

Inhibition of drug metabolism in human liver microsomes by nizatidine, cimetidine and omeprazole

S. FURUTA*, E. KAMADA, T. SUZUKI, T. SUGIMOTO,
Y. KAWABATA, Y. SHINOZAKI and H. SANO

Central Research Laboratories, Zeria Pharmaceutical Co., Ltd, 2512-1 Oshikiri,
Kohnan-machi, Ohsato-gun, Saitama 360-0111, Japan

Received 23 August 2000

1. The inhibitory effects of cimetidine, nizatidine and omeprazole on the metabolic activity of CYP2C9, 2C19, 2D6 and 3A were investigated in human liver microsomes. Both cimetidine and omeprazole inhibited each of the CYP subfamily enzymes; in particular, omeprazole extensively inhibited the hydroxylation of *S*-mephenytoin (CYP2C19, $K_i = 7.1 \mu\text{M}$). Nizatidine exhibited no inhibition of any of the CYP isoforms examined.

2. Cimetidine inhibited the hydroxylation of tolbutamide but not of diclofenac, whereas omeprazole inhibited the hydroxylation of diclofenac but not that of tolbutamide. The ability to inhibit CYP2C9 varied with incubation time, as measured by the metabolic rate constant for the substrates. Therefore, suitable substrates and incubation times must be selected in inhibition studies examining metabolic clearance and the mechanism of inhibition of these drugs.

3. Nizatidine did not inhibit the metabolism of cisapride, glibenclamide, benidipine and simvastatin. Omeprazole inhibited the metabolism of cisapride ($K_i = 0.4 \mu\text{M}$), glibenclamide ($11.7 \mu\text{M}$) and benidipine ($6.5 \mu\text{M}$), whereas cimetidine inhibited the metabolism of glibenclamide ($11.6 \mu\text{M}$). To avoid drug-drug interactions, care needs to be taken to select suitable medicines for co-administration with anti-ulcer drugs.

Introduction

Serious side-effects caused by drug-drug interactions attract much attention, and any significant information to help avoid such clinical incidents is valuable. It has been reported that the anti-ulcer drugs cimetidine and omeprazole inhibit oxidative metabolism by cytochrome P450 (CYP) in the liver (Pasanen *et al.* 1986, Humphries 1991, Ishizaki *et al.* 1995, Toon *et al.* 1995, Zech *et al.* 1996, Parker *et al.* 1997). Cimetidine, an H_2 -receptor antagonist, acts by binding to the haem moiety of CYP, which is the binding site of oxygen, resulting in non-specific inhibition of the enzymatic activity of CYP isoforms (Klotz 1987, Knodell *et al.* 1991). Therefore, caution is advised when drugs such as theophylline, warfarin, phenytoin, carbamazepine and lidocaine, which have a narrow range between their therapeutic and toxic levels, are co-administered with cimetidine (Knodell *et al.* 1991). Omeprazole, a proton pump inhibitor, extensively inhibits CYP2C19 (Karam *et al.* 1996, Ko *et al.* 1997). Plasma concentrations of diazepam and tolbutamide in particular are elevated when omeprazole is co-administered with these agents (Ishizaki *et al.* 1995, Toon *et al.* 1995). Furthermore, it has been reported that the clinical efficacy of omeprazole is changed by genetic polymorphism at the CYP2C19 locus (Humphries 1991, Andersson *et al.* 1998, Furuta *et al.* 1999). Another H_2 -receptor antagonist,

* Author for correspondence: e-mail: ken-taisya@zeria.co.jp

nizatidine, is known to inhibit the metabolism of CYP3A only very slightly (Klotz 1987, Wrighton and Ring 1994), and in clinical trials it was considered that no drug interaction occurred. However, the ability of nizatidine to inhibit other CYP isoforms is not clear, and there is little information available about the clinical interaction of nizatidine with other drugs. Moreover, there are no reports directly comparing the ability of nizatidine to inhibit the enzymatic activity of CYP isoforms or interfere with the metabolism of drugs such as cimetidine and omeprazole.

In the present study, the inhibition CYP isoforms by cimetidine, nizatidine and omeprazole was investigated using human liver microsomes, and the inhibitory abilities of these drugs were compared directly. Also investigated were the interactions between these drugs and the gastrokinetic agent cisapride, the calcium channel blocker benidipine, the diabetes treatment drug glibenclamide and the HMG-CoA reductase inhibitor simvastatin, which are all co-administered with anti-ulcer drugs in a clinical setting.

Materials and methods

Chemicals and reagents

Nizatidine was from Eli Lilly and Co. (Indianapolis, IN, USA). Cimetidine, glibenclamide, diclofenac, tolbutamide, nifedipine and chlorpropamide were from Sigma (St Louis, MO, USA). Omeprazole was from Union Quimico Farmaceutoca, SA (Barcelona, Spain). 4-Hydroxytolbutamide, 4'-hydroxydiclofenac, *S*-mephenytoin, 4'-hydroxymephenytoin, bufuralol, 1'-hydroxybufuralol and oxidized nifedipine were from Ultrafine Chemicals (Manchester, UK). Cisapride, benidipine and simvastatin were extracted and purified from Risamol granules (Yoshitomi Pharmaceutical Co. Ltd, Osaka, Japan), Coniel tablets (Kyowa Hakko Kogyo Co. Ltd, Tokyo, Japan) and Lipovas tablets (Banyu Pharmaceutical Co. Ltd, Tokyo, Japan), respectively by Zeria Pharmaceutical Co. (Tokyo, Japan), and their chemical purification was > 99%. Pooled microsomes from 10 male human livers (Lot no. HHM-0260) were from the International Institute for the Advancement of Medicine (Exton, PA, USA) with the approval of the local ethics committee and the coroner. Microsomes were prepared by differential centrifugation and contained 0.27 nmol P450 mg⁻¹ protein by the method of Omura and Sato (1964). All other reagents used were at least analytical grade.

In vitro metabolism using human liver microsomes

Enzyme assays were conducted according to the following methods with slight modification: hydroxylation of tolbutamide, *S*-mephenytoin and bufuralol (Ring *et al.* 1996), hydroxylation of diclofenac (Leemann *et al.* 1993) and oxidation of nifedipine (Guengerich *et al.* 1986). Incubation mixtures (total volume 0.5 or 1.0 ml) consisted of 0.1 M phosphate buffer (pH 7.4), 0.05 M EDTA.2 Na, the substrate, the inhibitor, an NADPH-generating system (consisting of 0.8 mM NADP⁺, 8.0 mM glucose 6-phosphate, 2 unit glucose 6-phosphate dehydrogenase, 10 mM MgCl₂) and human liver microsomes. Pre-incubation was performed at 37 °C for 5 min. The concentrations of microsomal proteins in the incubation mixtures were 0.2 mg ml⁻¹ for benidipine, 0.4 mg ml⁻¹ for simvastatin, 0.5 mg ml⁻¹ for tolbutamide, diclofenac and nifedipine, 1 mg ml⁻¹ for *S*-mephenytoin, bufuralol and glibenclamide, and 2.1 mg ml⁻¹ for cisapride. Enzyme activities were linear with respect to incubation time (data not shown). Incubation times employed were 5 min for diclofenac, nifedipine, cisapride and simvastatin, 10 min for benidipine, 30 min for bufuralol, and 60 min for tolbutamide, *S*-mephenytoin and glibenclamide. K_i were calculated from Dixon plots (1953) based on a competitive inhibition model.

Drug assays

Reactions were stopped by adding 20 µl 5 M HCl and 4 ml ethyl acetate for tolbutamide, 5 ml dichloromethane for *S*-mephenytoin, 50 µl perchloric acid for bufuralol, 2 ml acetonitrile for cisapride, and 0.5 ml acetonitrile for simvastatin. In the case of diclofenac, nifedipine, glibenclamide and benidipine, the reactants were added to 1 ml methanol after incubation to stop the reaction. The mixtures were vortex-mixed with each internal standard (chlorpropamide for tolbutamide, mefenamic acid for diclofenac, 5-hydroxy-1-tetralone for *S*-mephenytoin, 4-hydroxybenzoic acid isoamyl ester for nifedipine and benidipine, propranolol for cisapride, tolbutamide for glibenclamide, and 4-hydroxybenzoic

Table 1. Effect of varying concentrations of cimetidine, nizatidine and omeprazole on formation of 4-hydroxytolbutamide and 4'-hydroxydiclofenac by human liver microsomes.^a

Tolbutamide		25 μM	100 μM	250 μM
Control		13.6 $\pm$ 0.1 (100.0) ^b	34.4 $\pm$ 0.6 (100.0)	90.7 $\pm$ 2.1 (100.0)
Nizatidine	10 μM	14.2 $\pm$ 0.1 (104.5)	31.5 $\pm$ 0.1 (91.6)	N.T. ^c
	100 μM	13.6 $\pm$ 0.2 (99.7)	32.5 $\pm$ 1.9 (94.6)	N.T.
	1000 μM	13.6 $\pm$ 0.1 (100.2)	28.2 $\pm$ 1.8 (82.2)	N.T.
Cimetidine	10 μM	14.1 $\pm$ 0.6 (103.7)	37.0 $\pm$ 1.2 (107.8)	N.T.
	100 μM	10.7 $\pm$ 0.3 (78.3)	33.3 $\pm$ 1.8 (96.9)	N.T.
	1000 μM	5.0 $\pm$ 0.3 (36.4)	16.3 $\pm$ 0.8 (47.6)	N.T.
Omeprazole	100 μM	N.T.	N.T.	90.6 $\pm$ 0.3 (99.9)
	1000 μM	N.T.	N.T.	126.4 $\pm$ 13.3 (139.3)
Diclofenac		10 μM	50 μM	
Control		1010.8 $\pm$ 47.3 (100.0)	1658.4 $\pm$ 40.3 (100.0)	
Nizatidine	10 μM	1016.6 $\pm$ 40.9 (100.6)	1614.5 $\pm$ 43.7 (97.4)	
	100 μM	992.9 $\pm$ 45.8 (98.2)	1591.5 $\pm$ 58.2 (96.0)	
	1000 μM	942.1 $\pm$ 17.4 (93.2)	1560.5 $\pm$ 21.1 (94.1)	
Cimetidine	10 μM	933.5 $\pm$ 51.4 (92.3)	1548.9 $\pm$ 15.4 (93.4)	
	100 μM	939.8 $\pm$ 34.6 (93.0)	1602.9 $\pm$ 33.2 (96.7)	
	1000 μM	702.9 $\pm$ 48.8 (69.5)	1498.6 $\pm$ 107.2 (90.4)	
Omeprazole	10 μM	872.5 $\pm$ 20.6 (86.3)	1433.1 $\pm$ 27.5 (86.4)	
	100 μM	635.8 $\pm$ 28.1 (62.9)	853.3 $\pm$ 22.3 (51.5)	
	1000 μM	133.9 $\pm$ 2.9 (13.2)	312.5 $\pm$ 46.6 (18.8)	

^a Metabolic rates were calculated from the formation of 4-hydroxytolbutamide and 4'-hydroxydiclofenac (pmol mg⁻¹ protein min⁻¹, mean $\pm$ SE, n = 3).

^b Numbers in parentheses are percentages of the control.

^c N.T.; not tested.

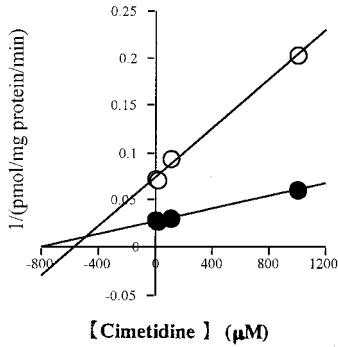
acid *n*-heptyl ester for simvastatin), and centrifuged for 10 min at 1000g. In the case of tolbutamide with omeprazole, metabolites were extracted with diethyl ether, and the organic layers washed with 0.5 M HCl to exclude interference from substances originating from omeprazole in the assay. Organic layers and supernatants were evaporated under nitrogen gas in a water bath at 50 °C, the residues dissolved in the mobile phase, and aliquots injected into an HPLC system (LC-6A, Shimadzu, Kyoto, Japan) connected to an analytical column (CAPCELL PAK C18 UG-120, 4.6 $\times$ 250 mm, SHISEIDO, Tokyo, Japan). The mobile phase consisted of acetonitrile-50 mM potassium phosphate buffer (30:70, pH 7.4) for 4'-hydroxydiclofenac, acetonitrile-25 mM perchloric acid (25:75, pH 2.5) for 1'-hydroxybufuralol, acetonitrile-0.1% trifluoroacetic acid (40:60) for oxidized nifedipine, acetonitrile-0.25% ammonium carbonate (45:55) for cisapride, acetonitrile-0.05% phosphoric acid (40:60) for glibenclamide, acetonitrile-20 mM ammonium acetate (50:50, pH 4.0) for benidipine, and acetonitrile-50 mM potassium phosphate buffer (60:40, pH 3.5) for simvastatin. In the case of 4-hydroxytolbutamide, the mobile phase was acetonitrile-0.05% phosphoric acid (25:75, pH 4.0) for cimetidine and nizatidine, acetonitrile-10 mM ammonium acetate buffer (20:80, pH 4.0) for omeprazole. In the case of 4'-hydroxymephenytoin, the mobile phase consisted of 50 mM potassium phosphate buffer (pH 4.0) and a linear gradient of 15-60% of acetonitrile. The UV absorbance was monitored at 224 nm for 4'-hydroxymephenytoin, 230 nm for 4-hydroxytolbutamide and glibenclamide, 237 nm for benidipine, 238 nm for simvastatin, 282 nm for 4'-hydroxydiclofenac, 270 nm for oxidized nifedipine, and 307 nm for cisapride. For 1'-hydroxybufuralol, the fluorescence was monitored at an excitation wavelength of 252 nm and an emission wavelength of 302 nm, and absolute concentrations determined against a standard calibration curve.

Results

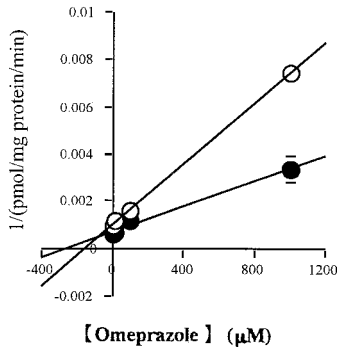
Inhibition study of CYP isoforms

The abilities of cimetidine, nizatidine and omeprazole to inhibit the metabolism of marker catalytic activities for CYP2C9, 2C19, 2D6 and 3A are shown in tables 1, 2, 3, and 4. The hydroxylation of tolbutamide and diclofenac (CYP2C9) was not inhibited by nizatidine (up to 1000 μM). Omeprazole also did not inhibit hy-

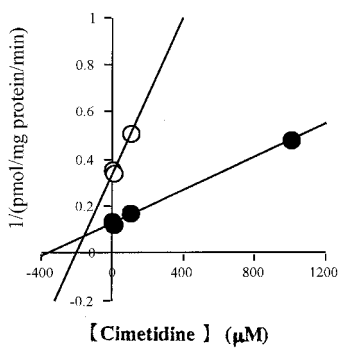
(a) Tolbutamide ($K_i = 495.0 \mu\text{M}$)



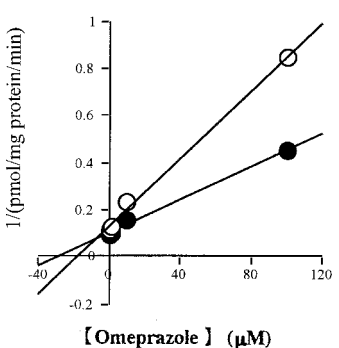
(b) Diclofenac ($K_i = 74.9 \mu\text{M}$)



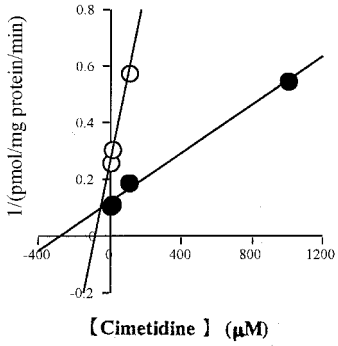
(c) S-Mephenytoin ($K_i = 157.8 \mu\text{M}$)



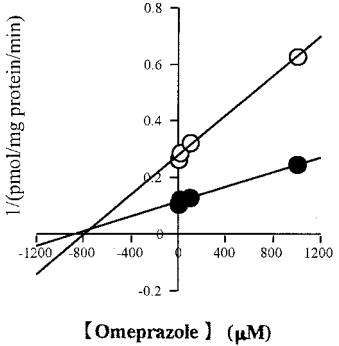
(d) S-Mephenytoin ($K_i = 7.1 \mu\text{M}$)



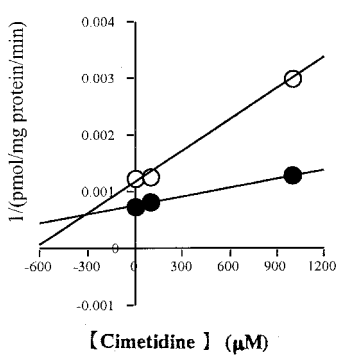
(e) Bufuralol ($K_i = 55.5 \mu\text{M}$)



(f) Bufuralol ($K_i = 745.1 \mu\text{M}$)



(g) Nifedipine ($K_i = 319.8 \mu\text{M}$)



(h) Nifedipine ($K_i = 367.5 \mu\text{M}$)

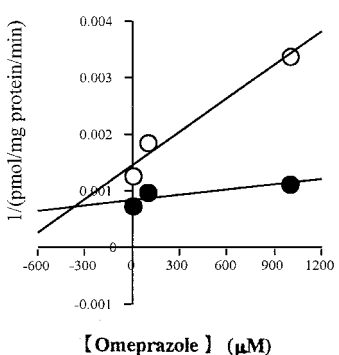


Figure 1. For legend see facing page.

Table 2. Effect of varying concentrations of nizatidine, cimetidine and omeprazole on formation of 4'-hydroxymephenytoin by human liver microsomes.^a

S-Mephenytoin		25 μM	100 μM
Control		2.8 $\pm$ 0.2 (100.0) ^b	7.5 $\pm$ 0.2 (100.0)
Nizatidine	10 μM	3.2 $\pm$ 0.2 (113.0)	6.8 $\pm$ 0.6 (91.1)
	100 μM	3.0 $\pm$ 0.2 (105.9)	8.0 $\pm$ 0.1 (106.4)
	1000 μM	2.4 $\pm$ 0.2 (86.5)	6.3 $\pm$ 0.3 (84.5)
Cimetidine	1000 μM	N.D. ^c	2.1 $\pm$ 0.2 (28.2)
Control		N.T. ^d	8.9 $\pm$ 0.2 (100.0)
Omeprazole		N.T.	1.2 $\pm$ 0.1 (13.3)

^a Metabolic rates were calculated from the formation of the 4'-hydroxymephenytoin (pmol mg⁻¹ protein min⁻¹, mean $\pm$ SE, n = 3).

^b Numbers in parentheses are percentages of the control.

^c N.D., not detectable.

^d N.T., not tested.

Table 3. Effect of varying concentrations of nizatidine, cimetidine and omeprazole on formation of 1'-hydroxybufuralol by human liver microsomes.^a

Bufuralol		5 μM	20 μM
Control		3.8 $\pm$ 0.1 (100.0) ^b	9.5 $\pm$ 0.5 (100.0)
Nizatidine	10 μM	4.3 $\pm$ 0.0 (111.4)	9.2 $\pm$ 0.3 (97.0)
	100 μM	3.7 $\pm$ 0.1 (96.9)	9.3 $\pm$ 0.6 (97.7)
	1000 μM	3.1 $\pm$ 0.0 (81.3)	8.3 $\pm$ 0.4 (86.9)
Cimetidine	1000 μM	N.D. ^c	1.8 $\pm$ 0.0 (19.2)
Omeprazole	1000 μM	1.6 $\pm$ 0.0 (41.6)	4.1 $\pm$ 0.0 (43.0)

^a Metabolic rates were calculated from the formation of 1'-hydroxybufuralol (pmol mg protein/min, mean $\pm$ SE, n = 3).

^b Numbers in parentheses are percentages of the control.

^c N.D., not detectable.

droxytolbutamide formation up to 1000 μM , but showed a tendency to inhibit the hydroxylation of diclofenac with 10 μM omeprazole, and the metabolic rate decreased to 18.8% of that of the control with 1000 μM omeprazole. The hydroxylation of diclofenac was not inhibited by cimetidine; however, inhibition of tolbutamide hydroxylation was confirmed with a high concentration of cimetidine, and the metabolic rate at 25 μM tolbutamide decreased to 36.4% that of the control with 1000 μM cimetidine. K_i calculated from Dixon plots (1953) using the data was 495.0 μM for cimetidine in the hydroxylation of tolbutamide and 74.9 μM for omeprazole in that of diclofenac (figure 1a and b). As shown in table 2, the hydroxylation of *S*-mephenytoin (CYP2C19) was not inhibited by nizatidine up to 1000 μM . However, *S*-mephenytoin metabolism was inhibited by cimetidine and omeprazole, with K_i = 157.8 and 7.1 μM respectively (figure 1c and d). As shown in table 3, the hydroxylation of bufuralol (CYP2D6) was not inhibited by nizatidine up to 1000 μM . On the other hand, the hydroxylation of bufuralol was decreased by

Figure 1. Dixon plots for the inhibition by cimetidine and omeprazole of tolbutamide 4-hydroxylation (a), diclofenac 4'-hydroxylation (b), *S*-mephenytoin 4'-hydroxylation (c, d), bufuralol 1'-hydroxylation (e, f) and nifedipine oxidation (g, h). Concentrations used were: tolbutamide 25 (○) and 100 (●) μM ; diclofenac 10 (○) and 50 (●) μM ; *S*-mephenytoin (c) 25 (○) and 100 (●) μM ; *S*-mephenytoin (d) 100 (○) and 200 (●) μM ; bufuralol 5 (○) and 20 (●) μM ; and nifedipine 20 (○) and 100 (●) μM .

Table 4. Effect of varying concentrations of nizatidine, cimetidine and omeprazole on formation of oxidized nifedipine by human liver microsomes.^a

Nifedipine		20 μM	100 μM
Control		800.8 $\pm$ 13.8 (100.0) ^b	1369.3 $\pm$ 41.0 (100.0)
Nizatidine	100 μM	778.4 $\pm$ 5.7 (97.2)	1353.3 $\pm$ 1.7 (98.8)
	1000 μM	564.1 $\pm$ 7.7 (70.4)	1258.8 $\pm$ 13.8 (91.9)
Cimetidine	1000 μM	332.1 $\pm$ 4.9 (41.5)	782.2 $\pm$ 23.9 (57.1)
Omeprazole	1000 μM	297.0 $\pm$ 20.1 (37.1)	891.0 $\pm$ 24.7 (65.1)

^a Metabolic rates were calculated from the formation of oxidized nifedipine (pmol mg⁻¹ protein min⁻¹, mean $\pm$ SE, n = 6).

^b Numbers in parentheses are percentages of the control.

Table 5. Effect of varying concentrations of nizatidine, cimetidine and omeprazole on metabolism of cisapride, glibenclamide, benidipine and simvastatin by human liver microsomes.^a

Cisapride		5 μM	50 μM
Control		194.0 $\pm$ 23.5 (100.0) ^b	1322.6 $\pm$ 252.3 (100.0)
Nizatidine	100 μM	195.2 $\pm$ 7.1 (100.6)	1267.1 $\pm$ 12.3 (95.8)
	1000 μM	130.5 $\pm$ 3.8 (67.3)	1262.2 $\pm$ 10.5 (95.4)
Cimetidine	100 μM	50.7 $\pm$ 15.1 (26.1)	302.8 $\pm$ 128.3 (22.9)
Omeprazole	100 μM	17.1 $\pm$ 5.9 (8.8)	189.3 $\pm$ 79.7 (14.3)

Glibenclamide		1 μM	5 μM
Control		4.3 $\pm$ 0.5 (100.0)	15.6 $\pm$ 0.8 (100.0)
Nizatidine	100 μM	4.5 $\pm$ 0.0 (105.8)	14.6 $\pm$ 0.6 (93.9)
	500 μM	4.7 $\pm$ 0.2 (109.6)	14.0 $\pm$ 0.5 (89.7)
Cimetidine	500 μM	0.3 $\pm$ 0.0 (8.0)	1.2 $\pm$ 0.0 (7.4)
Omeprazole	500 μM	0.5 $\pm$ 0.0 (12.1)	3.6 $\pm$ 1.4 (23.0)

Benidipine		2 μM	5 μM
Control		330.4 $\pm$ 31.6 (100.0)	1039.2 $\pm$ 19.4 (100.0)
Nizatidine	100 μM	315.7 $\pm$ 9.5 (95.6)	1059.7 $\pm$ 121.5 (102.0)
	1000 μM	226.4 $\pm$ 10.0 (68.5)	783.4 $\pm$ 35.8 (75.4)
Cimetidine	500 μM	61.2 $\pm$ 3.6 (18.5)	440.0 $\pm$ 136.0 (42.3)
Omeprazole	100 μM	40.7 $\pm$ 6.3 (12.3)	513.2 $\pm$ 67.8 (49.4)

Simvastatin		10 μM	25 μM
Control		2726.6 $\pm$ 250.3 (100.0)	2353.3 $\pm$ 191.3 (100.0)
Nizatidine	10 μM	2525.8 $\pm$ 232.5 (92.6)	2186.3 $\pm$ 99.8 (92.9)
	100 μM	2773.4 $\pm$ 230.2 (101.7)	2283.5 $\pm$ 774.7 (97.0)
	1000 μM	2279.7 $\pm$ 197.6 (83.6)	2134.8 $\pm$ 399.8 (90.7)
Control		1485.6 $\pm$ 74.8 (100.0)	1843.0 $\pm$ 200.3 (100.0)
Cimetidine	1000 μM	378.7 $\pm$ 78.2 (25.5)	437.9 $\pm$ 120.6 (23.8)
Omeprazole	1000 μM	260.6 $\pm$ 51.7 (17.5)	427.3 $\pm$ 170.6 (23.2)

^a Metabolic rates were calculated from the elimination of cisapride, glibenclamide, benidipine and simvastatin (pmol mg⁻¹ protein min⁻¹, mean $\pm$ SE, n = 3).

^b Numbers in parentheses are percentages of the control.

cimetidine and omeprazole, with K_i = 55.5 and 745.1 μM respectively (figure 1e and f). As shown in table 4, the formation of oxidized nifedipine (CYP3A) was not inhibited by nizatidine up to 1000 μM , although cimetidine and omeprazole decreased the oxidation of nifedipine. K_i for cimetidine and omeprazole in the oxidation of nifedipine was 319.8 and 367.5 μM respectively (figure 1g and h).

Drug interaction study

Drugs co-administered with anti-ulcer drugs in clinical use were selected to investigate the inhibition of their metabolism by cimetidine, nizatidine and omeprazole *in vitro* (table 5, figure 2). Nizatidine did not inhibit the metabolism of cisapride, benidipine, glibenclamide or simvastatin over the concentration range examined. However, the metabolism of cisapride was inhibited by cimetidine ($K_i = 79.7 \mu\text{M}$) and omeprazole ($0.4 \mu\text{M}$). Cimetidine inhibited the metabolism of glibenclamide ($K_i = 11.6 \mu\text{M}$), benidipine ($80.1 \mu\text{M}$) and simvastatin ($45.6 \mu\text{M}$), whereas omeprazole also inhibited the elimination of glibenclamide ($11.7 \mu\text{M}$), benidipine ($6.5 \mu\text{M}$) and simvastatin ($46.8 \mu\text{M}$).

Discussion

There have been many reports on CYP isoforms in human liver microsomes, and the specific substrates for these isoforms have been identified (Ono *et al.* 1996, Pelkonen *et al.* 1998). According to previous reports, inhibition studies of cimetidine, nizatidine and omeprazole were based on enzyme activity as indicated by the hydroxylation of tolbutamide (Ring *et al.* 1996) and diclofenac (Leemann *et al.* 1993) for CYP2C9, hydroxylation of *S*-mephenytoin for CYP2C19, hydroxylation of bufuralol for CYP2D6 (Ring *et al.* 1996), and oxidation of nifedipine for CYP3A (Guengerich *et al.* 1986). Omeprazole was metabolized by CYP2C19 (Ko *et al.* 1997); and the present results additionally showed that CYP2C9 (hydroxylation of diclofenac), 2D6 and 3A were also inhibited by omeprazole. This is thought to be because omeprazole was extensively metabolized via multiple metabolic pathways (Karam *et al.* 1996) or because secondary metabolites were transformed to further metabolites by other isoforms. The imidazole ring of cimetidine binds to the haem moiety of CYP, which is the binding site of oxygen, exhibiting an inhibitory effect on the hepatic elimination of this drug (Klotz 1987, Knodell *et al.* 1991). The results confirmed that cimetidine inhibited non-specifically the activity of each CYP isoform, whereas it inhibited the hydroxylation of tolbutamide, but not that of diclofenac, by CYP2C9. The incubation times were 60 min with tolbutamide and 5 min with diclofenac, based on their metabolic clearance; the inhibitory ability of cimetidine was dependent on incubation time. These results suggest that the inhibition of cimetidine might require a lag time for binding to CYP, as cimetidine does not inhibit the hydroxylation of diclofenac, which was eliminated rapidly during 5 min of incubation in *in vitro* metabolic studies. Therefore, the K_i of inhibitors that use the same mechanism as cimetidine vary with the substrates used and with incubation time, so that their inhibitory abilities have not been clarified. Omeprazole inhibits the hydroxylation of diclofenac with a short incubation time, but not tolbutamide with a long incubation time. Omeprazole was extensively metabolized within 60 min by human liver microsomes (data not shown) because of its large metabolic clearance (Katashima *et al.* 1998). The metabolism of tolbutamide in the healthy volunteer was inhibited by co-administration of cimetidine and omeprazole (Toon *et al.* 1995). It was suggested that the K_i of omeprazole in this inhibition study of the hydroxylation of tolbutamide was misleading. Therefore, a suitable substrate and incubation time must be selected, and the metabolic clearance and mechanism of inhibition considered. In contrast, nizatidine does not exhibit inhibition of CYP isoforms because the thiazole ring in the structure of nizatidine does not bind to the haem moiety of CYP (Klotz 1987), and this drug is eliminated

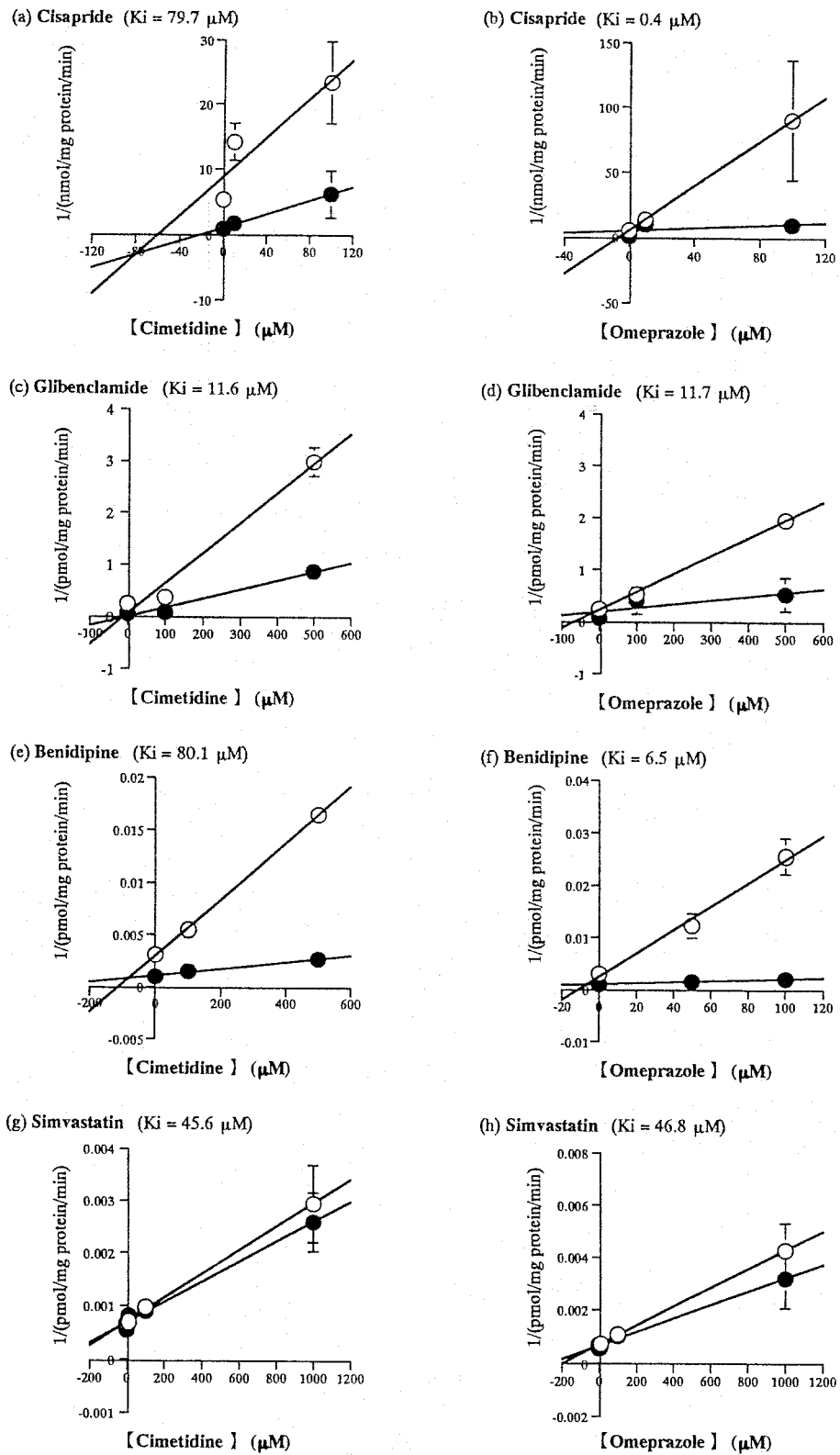


Figure 2. For legend see facing page.

mainly by renal excretion (Aronoff *et al.* 1988), whereas omeprazole is eliminated only by hepatic clearance (Sohn *et al.* 1992).

Inhibitors of acid secretion are frequently co-administered with other drugs in a clinical setting. Some are extensively metabolized and transformed into many secondary products, and their metabolites further transformed by multiple metabolic pathways. Since cisapride (Bedford and Rowbotham 1996), benidipine (Kobayashi and Kobayashi 1999, Katoh *et al.* 2000), glibenclamide (Mück *et al.* 1995, Rieutord *et al.* 1995) and simvastatin (Prueksaritanont *et al.* 1997) are co-administered with anti-ulcer drugs for clinical therapy, these drugs were selected in the present investigation of drug interactions. Because their metabolic pathways are complicated and drug interaction between cimetidine and omeprazole is predicted, the metabolic rate of each substrate was estimated using elimination of the parent drug and not metabolite formation. This is different from the usual approach for studying the inhibition of CYP isoforms. The results confirmed that cimetidine and omeprazole inhibited the metabolism of cisapride, glibenclamide, benidipine and simvastatin, whereas nizatidine did not inhibit any of these drugs. In particular, omeprazole inhibited the metabolism of cisapride ($K_i = 0.4 \mu\text{M}$), glibenclamide ($11.7 \mu\text{M}$) and benidipine ($6.5 \mu\text{M}$), whereas cimetidine inhibited of glibenclamide ($11.6 \mu\text{M}$). Considering the maximum plasma concentrations of omeprazole ($1.2 \mu\text{M}$, Sohn *et al.* 1992) and cimetidine ($6.3 \mu\text{M}$, Lin 1991) in humans, these results offer possible explanations for the interactions that occur when these drugs are co-administered with cimetidine and omeprazole. However, it was predicted that no drug interaction with nizatidine occurred because of its maximum plasma concentrations ($3.6 \mu\text{M}$, Aronoff *et al.* 1988). To ensure the safety of therapy, care needs to be taken to select suitable medicines for co-administration with inhibitors of acid secretion.

References

- ANDERSSON, T., HOLMBERG, J., RÖHSS, K. and WALAN, A., 1998, Pharmacokinetics and effect on caffeine metabolism of the proton pump inhibitors, omeprazole, lansoprazole, and pantoprazole. *British Journal of Clinical Pharmacology*, **45**, 369–375.
- ARONOFF, G. R., BERGSTROM, R. F., BOPP, R. J., SLOAN, R. S. and CALLAGHAN, J. T., 1988, Nizatidine disposition in subjects with normal and impaired renal function. *Clinical Pharmacology and Therapeutics*, **43**, 688–695.
- BEDFORD, T. A. and ROWBOTHAM, D. J., 1996, Cisapride drug interaction of clinical significance. *Drug Safety*, **15**, 167–175.
- DIXON, M., 1953, The determination of enzyme inhibitor constants. *Biochemical Journal*, **55**, 170–171.
- FURUTA, T., OHASHI, K., KOBAYASHI, K., IIDA, I., YOSHIDA, H., SHIRAI, N., TAKASHIMA, M., KOSUGE, K., HANAI, H., CHIBA, K., ISHIZAKI, T. and KANEKO, E., 1999, Effects of clarithromycin on the metabolism of omeprazole in relation to CYP2C19 genotype status in humans. *Clinical Pharmacology and Therapeutics*, **66**, 265–274.
- GUENGERICH, F. P., MARTIN, M. V., BEAUNE, P. H., KREMERS, P., WOLFF, T. and WAXMAN, D. J., 1986, Characterization of rat and human liver microsomal cytochrome P-450 forms involved in nifedipine oxidation, a prototype for genetic polymorphism in oxidative drug metabolism. *Journal of Biological Chemistry*, **261**, 5051–5060.
- HUMPHRIES, T. J., 1991, Clinical implications of drug interactions with the cytochrome P-450 enzyme system associated with omeprazole. *Digestive Diseases and Sciences*, **36**, 1665–1669.
- ISHIZAKI, T., CHIBA, K., MANABE, K., Koyama, E., HAYASHI, M., YASUDA, S., HORAI, Y., TOMONO, Y., YAMATO, C. and TOYOKI, T., 1995, Comparison of the interaction potential of a new proton pump inhibitor, E3810, versus omeprazole with diazepam in extensive and poor metabolizers of S-mephenytoin 4'-hydroxylation. *Clinical Pharmacology and Therapeutics*, **58**, 155–164.

Figure 2. Dixon plots for the inhibition by cimetidine and omeprazole of the metabolism of cisapride (a, b), glibenclamide (c, d), benidipine (e, f) and simvastatin (g, h). Concentrations used were: cisapride 5 (○) and 50 (●) μM ; glibenclamide 1 (○) and 5 (●) μM ; benidipine 2 (○) and 5 (●) μM ; and simvastatin 10 (○) and 25 (●) μM .

- KARAM, W. G., GOLDSTEIN, J. A., LASKER, J. M. and GHANAYEM, B. I., 1996, Human CYP2C19 is a major omeprazole 5-hydroxylase, as demonstrated with recombinant cytochrome P450 enzymes. *Drug Metabolism and Disposition*, **24**, 1081–1087.
- KATASHIMA, M., YAMAMOTO, K., TOKUMA, Y., HATA, T., SAWADA, Y. and IGA, T., 1998, Comparative pharmacokinetic/pharmacodynamic analysis of proton pump inhibitors omeprazole, lansoprazole and pantoprazole, in humans. *European Journal of Drug Metabolism and Pharmacokinetics*, **23**, 19–26.
- KATOH, M., NAKAJIMA, M., SHIMADA N., YAMAZAKI H. and YOKOI, T., 2000, Inhibition of human cytochrome P450 enzymes by 1,4-dihydropyridine calcium antagonists: prediction of *in vivo* drug-drug interactions. *European Journal of Clinical Pharmacology*, **55**, 843–852.
- KLOTZ, U., 1987, Lack of effect of nizatidine on drug metabolism. *Scandinavian Journal of Gastroenterology*, **22**, 18–23.
- KNODELL, R. G., BROWNE, D. G., GWOZDZ, G. P., BRIAN, W. R. and GUENGERICH, F. P., 1991, Differential inhibition of individual human liver cytochromes P-450 by cimetidine. *Gastroenterology*, **101**, 1680–1691.
- KO, J.-W., SUKHOVA, N., THACKER, D., CHEN, P. and FLOCKHART, D. A., 1997, Evaluation of omeprazole and lansoprazole as inhibitors of cytochrome P450 isoforms. *Drug Metabolism and Disposition*, **25**, 853–862.
- KOBAYASHI, H. and KOBAYASHI, S., 1999, Enantiomer-enantiomer interaction of a calcium channel antagonist, benidipine hydrochloride, during liver metabolism in the rat. *European Journal of Drug Metabolism and Pharmacokinetics*, **24**, 121–126.
- LEEMANN, T., TRANSON, C. and DAYER, P., 1993, Cytochrome P450(TB (CYP2C): a major monooxygenase catalyzing diclofenac 4'-hydroxylation in human liver. *Life Sciences*, **52**, 29–34.
- LIN, J. H., 1991, Pharmacokinetic and pharmacodynamic properties of histamine H₂-receptor antagonists: relationship between intrinsic potency and effective plasma concentrations. *Clinical Pharmacokinetics*, **20**, 218–236.
- MÜCK, W., HEINE, P. R., BREUEL, H. P., NIKLAUS, H., HORKULAK, J. and AHR, G., 1995, The effect of multiple oral dosing of nimodipine on glibenclamide pharmacodynamics and pharmacokinetics in elderly patients with type-2 diabetes mellitus. *International Journal of Clinical Pharmacology and Therapeutics*, **33**, 89–94.
- OMURA, T. and SATO, R., 1964, The carbon monoxide-binding pigment of liver microsomes. *Journal of Biological Chemistry*, **239**, 2370–2378.
- ONO, S., HATANAKA, T., HOTTA, H., SATOH, T., GONZALEZ, F. J. and TSUTSUI, M., 1996, Specificity of substrate and inhibitor probes for cytochrome P450s: evaluation of *in vitro* metabolism using cDNA-expressed human P450s and human liver microsomes. *Xenobiotica*, **26**, 681–693.
- PARKER, A. C., PRITCHARD, P., PRESTON, T., DALZELL, A. M. and CHOONARA, I., 1997, Lack of inhibitory effect of cimetidine on caffeine metabolism in children using the caffeine breath test. *British Journal of Clinical Pharmacology*, **43**, 467–470.
- PASANEN, M., ARVELA, P., PELKONEN, O., SOTANIEMI, E. and KLOTZ, U., 1986, Effect of five structurally diverse H₂-receptor antagonists on drug metabolism. *Biochemical Pharmacology*, **35**, 4457–4461.
- PELKONEN, O., MÄENPÄÄ, J., TAAVITSAINEN, P., RAUTIO, A. and RAUNIO, H., 1998, Inhibition and induction of human cytochrome P450 (CYP) enzymes. *Xenobiotica*, **28**, 1203–1253.
- PRUEKSARITANONT, T., GORHAM, L. M., MA, B., LIU, L., YU, X., ZHAO, J. J., SLAUGHTER, D. E., ARISON, B. H. and VYAS, K. P., 1997, *In vitro* metabolism of simvastatin in humans [SBT] identification of metabolizing enzymes and effect of the drug on hepatic P450s. *Drug Metabolism and Disposition*, **25**, 1191–1199.
- RIEUTORD, A., STUPANS, I., SHENFIELD, G. M. and GROSS, A. S., 1995, Gliclazide hydroxylation by rat liver microsomes. *Xenobiotica*, **25**, 1345–1354.
- RING, B. J., BINKLEY, S. N., VANDENBRANDEN, M. and WRIGHTON, S. A., 1996, *In vitro* interaction of the antipsychotic agent olanzapine with human cytochromes P450 CYP2C9, CYP2C19, CYP2D6 and CYP3A. *British Journal of Clinical Pharmacology*, **41**, 181–186.
- SOHN, D.-R., KOBAYASHI, K., CHIBA, K., LEE, K.-H., SHIN, S.-G. and ISHIZAKI, T., 1992, Disposition kinetics and metabolism of omeprazole in extensive and poor metabolizers of *S*-mephenytoin 4'-hydroxylation recruited from an oriental population. *Journal of Pharmacology and Experimental Therapeutics*, **262**, 1195–1202.
- TOON, S., HOLT, B. L., MULLINS, F. G. P. and KHAN, A., 1995, Effects of cimetidine, ranitidine and omeprazole on tolbutamide pharmacokinetics. *Journal of Pharmacy and Pharmacology*, **47**, 85–88.
- WRIGHTON, S. A. and RING, B. J., 1994, Inhibition of human CYP3A catalyzed 1'-hydroxy midazolam formation by ketoconazole, nifedipine, erythromycin, cimetidine, and nizatidine. *Pharmaceutical Research*, **11**, 921–924.
- ZECH, K., STEINIJANS, V. W., HUBER, R., KOLASSA, N. and RADTKE, H. W., 1996, Pharmacokinetics and drug interactions—relevant factors for the choice of a drug. *International Journal of Clinical Pharmacology and Therapeutics*, **34**, S3–6.

Non-enzymatic reduction of aliphatic tertiary amine *N*-oxides mediated by the haem moiety of cytochrome P450

K. TAKEKAWA†, S. KITAMURA†*, K. SUGIHARA† and S. OHTA†

† Safety Research Laboratory, Tanabe Seiyaku Co., Ltd, Yodogawa-ku, Osaka 532–8505, Japan

‡ Institute of Pharmaceutical Science, Hiroshima University School of Medicine, Kasumi 1–2–3, Minami-ku, Hiroshima 734–8551, Japan

1. The mechanism of reduction of aliphatic tertiary amine *N*-oxides to tertiary amines in liver microsomes was examined and a novel type of reduction by cytochrome P450 was found.

2. Rat liver microsomes exhibited a significant *N*-oxide reductase activity toward brucine *N*-oxide and imipramine *N*-oxide in the presence of both NAD(P)H and FAD under anaerobic conditions. These *N*-oxide reductase activities were inhibited by carbon monoxide or air. However, the activities were not abolished by boiling the microsomes; indeed, in the case of brucine *N*-oxide, the activity was enhanced.

3. The activity toward brucine *N*-oxide was also observed after the conversion of cytochrome P450 to cytochrome P420. Cytochrome P4502B1 alone exhibited the reductase activity in the presence of both NAD(P)H and FAD. After the removal of haem from cytochrome P4502B1, the activity was observed in the haem moiety, but not in the cytochrome P450 apoprotein.

4. Photochemically reduced FAD was effective in the reduction in place of NAD(P)H and FAD.

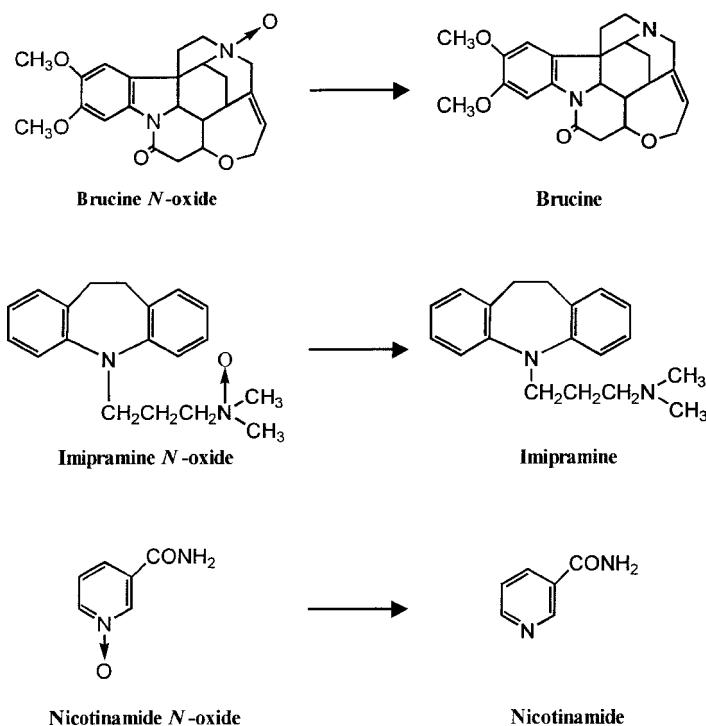
5. The *N*-oxide reduction appears to proceed non-enzymatically, catalysed by the haem group of cytochrome P450 in the presence of a reduced flavin.

Introduction

Aliphatic tertiary amines are generally oxidized at the nitrogen or the α -carbon atom of the tertiary amine moiety in the body, being transformed to *N*-oxides and secondary amines. The *N*-oxides thus formed are frequently reduced back to the parent tertiary amines. This interconversion between aliphatic tertiary amines and *N*-oxides is a well-known metabolic pathway (Bickel 1969). For example, imipramine *N*-oxide or imipramine was detected in rats dosed with imipramine or imipramine *N*-oxide, respectively. Such interconversion in the body might be effective to maintain the pharmacological effect of tertiary amines such as antidepressants. The enzyme systems involved in the oxidative metabolism have been well investigated, and the role of cytochrome P450 and flavin-containing monooxygenase have been established (Ziegler 1980). The enzyme system involved in the *N*-oxide reduction has been investigated in the livers of animals, and roles of the cytochrome P450 system in microsomal reduction and of aldehyde oxidase in cytosolic reduction have been demonstrated (Sugiura *et al.* 1974, 1976, Powis and Wincentsen 1980, Kitamura and Tatsumi 1984a, b, Sugihara *et al.* 1996).

Previous investigations have established that liver *N*-oxide reductase activity toward imipramine *N*-oxide, cyclobenzaprine *N*-oxide, tiaramide *N*-oxide or *N,N*-

* Author for correspondence: e-mail address: kitamura@pharm.hiroshima-u.ac.jp

Figure 1. Reduction of *N*-oxide compounds.

dimethylaniline *N*-oxide is located in the microsomal fraction, and have demonstrated a role of cytochrome P450 in these microsomal reduction (Sugiura *et al.* 1974, 1976, Kitamura and Tatsumi 1984b). Furthermore, reconstituted systems containing purified cytochrome P450, NADPH-cytochrome P450 reductase and NADPH reduce tiaramide *N*-oxide to tiaramide at rates comparable to those of the intact liver microsomes (Iwasaki *et al.* 1977). In contrast, Kato *et al.* (1976) reported that microsomal *N*-oxide reduction was markedly stimulated by the addition of a flavin.

Recently, the present authors investigated the reduction of *N*-oxide compounds, including aromatic type *N*-oxides and aliphatic tertiary amine *N*-oxides, to the corresponding amines by mammalian liver cytosol using nicotinamide *N*-oxide, imipramine *N*-oxide, cyclobenzaprine *N*-oxide and *S*-(-)-nicotine-1'-*N*-oxide as model substrates (Kitamura and Tatsumi 1984a, b, Sugihara *et al.* 1996). Mammalian liver cytosol exhibited significant *N*-oxide reductase activity toward these *N*-oxides when supplemented with an electron donor to aldehyde oxidase, such as 2-hydroxypyrimidine or *N*¹-methylnicotinamide, and the flavoenzyme functioned as an *N*-oxide reductase in liver cytosol (Kitamura and Tatsumi 1984a, b, Sugihara *et al.* 1996).

In the present study, rat liver microsomal *N*-oxide reduction was investigated using brucine *N*-oxide, imipramine *N*-oxide and nicotinamide *N*-oxide as model substrates of cyclic tertiary amine *N*-oxide, non-cyclic tertiary amine *N*-oxide and aromatic *N*-oxide (figure 1). We identified a novel mechanism of microsomal aliphatic tertiary amine *N*-oxide reduction, i.e. tertiary amine *N*-oxides are reduced by a non-enzymatic reaction involving cytochrome P450, catalysed by the haem in

the presence of both NAD(P)H and a flavin. In this system, tertiary amine N-oxides were reduced to tertiary amines by cytochrome P450B1 as if the reduction were an enzymatic reaction.

Materials and methods

Materials

Brucine N-oxide (99%), brucine (98%) and imipramine hydrochloride (> 99%) were from Aldrich Chemical Co., Inc. (Milwaukee, WI, USA). Nicotinamide (> 99%) and nicotinamide N-oxide (99%) were from Tokyo Chemical Co. Ltd (Tokyo, Japan). Imipramine N-oxide (> 99%) was prepared by the H_2O_2 -oxidation method (Dietrich 1967). Reduced flavins were prepared photochemically by the method of Yubisui *et al.* (1980).

Liver preparation

Male Sprague-Dawley rats (140–165 g) were used in this study. In some experiments, rats were given intraperitoneal sodium phenobarbital at 80 mg kg⁻¹ for 3 days, and killed 24 h after the last injection. The livers were immediately perfused with 1.15% KCl and homogenized in 4 vols of the latter KCl solution using a Potter-Elvehjem homogenizer. The homogenate was centrifuged for 20 min at 9000 g, and the supernatant fraction was centrifuged for 60 min at 105000g. The microsomal fraction was washed by resuspension in 1.15% KCl solution followed by resedimentation for 60 min at 105000g. The pellets were suspended in 2 vols of 1.15% KCl solution of the original liver weight. In some experiments, microsomes prepared as above were heated in a boiling water bath for 5 min to prepare boiled microsomes. By this treatment, pentoxoresorufin-*O*-dealkylase and ethoxoresorufin-*O*-dealkylase activities of cytochrome P450 system, and cytochrome *c*-reductase activity of NADPH-cytochrome P450 reductase in liver microsomes were completely abolished.

Treatment of liver microsomes with trypsin or sodium cholate

Liver microsomes (10 mg protein ml⁻¹) were treated with trypsin (1 mg ml⁻¹) overnight at 0 °C. The microsomes were separated by sedimentation at 105000g for 60 min, and the pellet was resuspended in the original volume of 1.15% KCl. The contents of cytochrome P450 in the supernatant and pellets were 0.3 and 0.9 nmol mg⁻¹ protein, respectively. Liver microsomes (3 mg protein ml⁻¹) were also treated with sodium cholate (a final concentration of 0.6%) for 60 min at 0 °C, then sedimented and resuspended as described above. The contents of cytochrome P450 in the supernatant and pellets were 0.9 and 0.3 nmol mg⁻¹ protein, respectively.

Preparation of cytochrome P450B1

Cytochrome P450B1 was prepared from rat liver microsomes by the method of Guengerich *et al.* (1982). Separation of the cytochrome P450 into the apoprotein and the haem moiety was performed by the method of Yu and Gunsalus (1974). Conversion of the cytochrome P450 to cytochrome P420 was performed by the method of Imai and Sato (1967).

Assay of N-oxide reduction

The incubation mixture consisted of 0.5 µmol imipramine N-oxide or brucine N-oxide, 2 µmol NADPH or NADH, 0.1 µmol FAD and a microsomal preparation (~0.5 mg protein) or 1 nmol haemoproteins in a final volume of 1 ml 0.1 M K,Na-phosphate buffer (pH 7.4). In some experiments, 0.2 µmol of a reduced flavin was used instead of both the pyridine nucleotide and FAD. The incubation was performed using a Thunberg tube under anaerobic conditions. The side arm contained NADPH or NADH, and the body contained all other components. The tube was gassed for 3 min with nitrogen, evacuated with an aspirator for 5 min and again gassed with nitrogen. The reaction was started by mixing the components of the side arm and the body together, continued for 5 min in the case of brucine N-oxide or for 10 min in the case of imipramine N-oxide at 37 °C and stopped by adding 0.1 ml 1 N NaOH. The mixture, after addition of 25 µg phenacetin or 12.5 µg acetanilide as an internal standard in the case of imipramine N-oxide or brucine N-oxide, respectively, was extracted once with 5 ml ethyl acetate and the extract was evaporated to dryness *in vacuo*. The residue was dissolved in 0.1 ml of methanol and then subjected to high-performance liquid chromatography (HPLC).

Some properties of the reaction were examined by using rat liver microsomes in the presence of NADPH and FAD. The reduction of imipramine N-oxide and brucine N-oxide proceeded linearly for at least 20 min and with increasing amount of protein of the microsomes up to 2 mg.

When nicotinamide N-oxide was used as a substrate in this experiment, the incubation was performed using 0.2 µmol of the N-oxide compound, in the same manner as described above. The assay for nicotinamide was conducted by the method described (Kitamura and Tatsumi 1984a).

HPLC

HPLC was performed in a Hitachi L-6000 high-performance liquid chromatograph (Tokyo, Japan) equipped with an ultraviolet absorption detector. The instrument was fitted with a 150×4.6 mm Inertsil ODS-3 (GL Sciences, Inc., Tokyo, Japan) column. In the case of brucine *N*-oxide, the mobile phase was acetonitrile-0.1 M KH_2PO_4 (1:4). The chromatograph was operated at a flow rate of 0.5 ml min^{-1} at ambient temperature and at a wavelength of 254 nm. Elution times of brucine *N*-oxide and brucine were 4.6 and 10.0 min, respectively. In the case of imipramine *N*-oxide, the mobile phase was acetonitrile-0.1 M KH_2PO_4 (3:7). The chromatograph was operated at 1.0 ml min^{-1} at ambient temperature, with detection at 254 nm. The elution times of imipramine *N*-oxide and its reduction product, imipramine, were 18.2 and 16.0 min, respectively. The amounts of reduction products were determined from the relative peak areas.

Measurement of cytochrome P450 contents

Cytochrome P450 concentration was determined by the method of Matsubara *et al.* (1976).

Determination of protein

Protein concentration was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as the standard.

Results

Unique aliphatic tertiary amine *N*-oxide reduction by rat liver microsomes

The liver microsomal *N*-oxide reducing activities of untreated rats towards imipramine *N*-oxide, brucine *N*-oxide and nicotinamide *N*-oxide were compared in the presence of NADH or NADPH, and FAD under anaerobic conditions. Rat liver microsomes exhibited *N*-oxide reductase activity towards brucine *N*-oxide and imipramine *N*-oxide in the presence of NADH or NADPH. The NADH- or NADPH-linked *N*-oxide reductase activity was markedly stimulated by addition of FAD. The highest activity was observed with brucine *N*-oxide, followed by imipramine *N*-oxide in both cases. However, nicotinamide *N*-oxide, an aromatic *N*-oxide, was not reduced to nicotinamide by liver microsomes even in the presence of NAD(P)H and FAD (figure 2).

The reductase activity toward brucine *N*-oxide in the presence of NADPH and FAD was enhanced by liver microsomes of phenobarbital-treated rats. When liver microsomes of untreated and phenobarbital-treated rats were boiled, cytochrome P450 and its reductase activities were abolished as described in the Materials and methods. However, the tertiary amine *N*-oxide reductase activity in the presence of NADPH and FAD was not abolished. Indeed, in the case of brucine *N*-oxide, it was enhanced. The reductase activity of native or boiled microsomes of untreated and phenobarbital-treated rats was markedly inhibited by carbon monoxide (figure 3). *N*-Oxide reducing activities towards brucine *N*-oxide and imipramine *N*-oxide of liver microsomes from phenobarbital-treated rats with NADPH or NADH were also stimulated by the addition of FMN and riboflavin instead of FAD. When the liver microsomes were boiled, the activity towards imipramine *N*-oxide was not abolished, as in the case of brucine *N*-oxide (table 1). These microsomal activities were also sensitive to inhibition by carbon monoxide (data not shown).

N-Oxide reduction by cytochrome P450 without the reductase

Aliphatic tertiary amine *N*-oxide reduction by trypsin-digested liver microsomes was examined. The pellets of trypsin-treated microsomes exhibited reductase activity toward brucine *N*-oxide in the presence of NAD(P)H and FAD, whereas

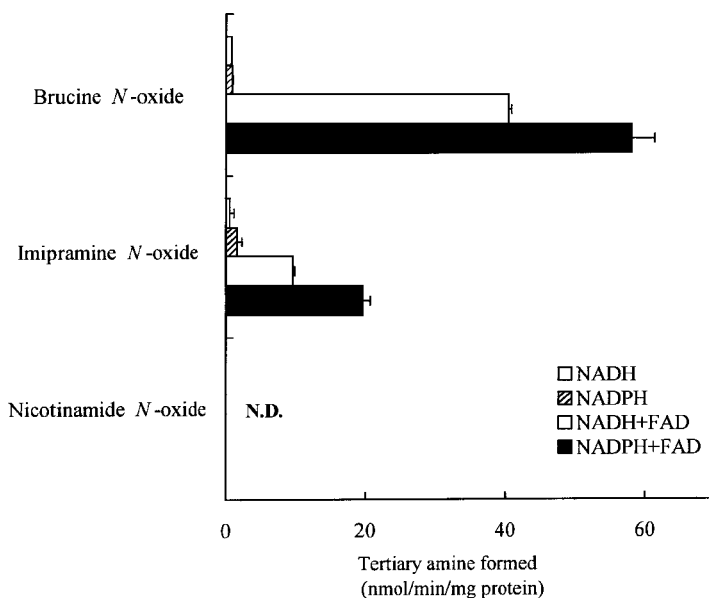


Figure 2. Reduction of *N*-oxide compounds by rat liver microsomes. Each value represents the mean $\pm$ SD of four experiments. Reactions were conducted with 50 μ l ($\sim$ 0.7 mg protein) of rat liver microsomes equivalent to 25 mg liver in the presence of cofactors at 37 $^{\circ}$ C for 5 min under anaerobic conditions. Reduction products were determined by HPLC. Other details are described in the Materials and methods. ND, not detected.

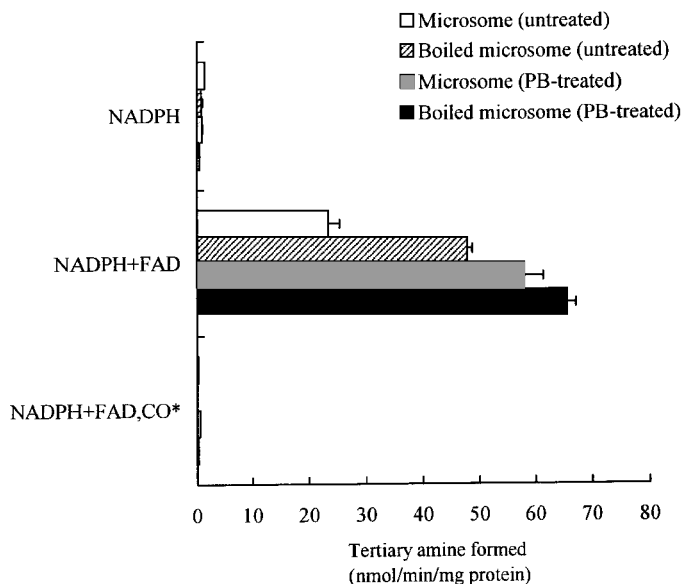


Figure 3. Reduction of brucine *N*-oxide by native or boiled liver microsomes of untreated or phenobarbital (PB)-treated rats. Each value represents the mean $\pm$ SD of four experiments. Reactions were conducted with 50 μ l ($\sim$ 0.7 mg protein) of rat liver microsomes equivalent to 25 mg liver in the presence of both NADPH and FAD at 37 $^{\circ}$ C for 5 min under anaerobic conditions. Brucine formed was determined by HPLC. Other details are described in the Materials and methods. *Reaction conducted under an atmosphere of carbon monoxide.

Table 1. Reduction of tertiary amine *N*-oxides by rat liver microsomes.

Addition	Brucine formed (nmol min ⁻¹ mg ⁻¹ protein)		Imipramine formed (nmol min ⁻¹ mg ⁻¹ protein)	
	Microsomes	Boiled microsomes	Microsomes	Boiled microsomes
NADH	0.75±0.07	0.29±0.04	0.54±0.61	nd
NADH + FAD	40.48±0.45	41.84±2.24	9.64±0.27	7.24±1.70
NADH + FMN	37.06±1.99	36.40±2.89	15.02±2.34	10.05±2.54
NADH + riboflavin	70.00±1.44	88.08±6.26	12.61±1.63	10.90±2.35
NADPH	0.91±0.09	0.50±0.02	1.57±0.66	0.15±0.12
NADPH + FAD	58.05±3.36	65.59±1.56	19.55±1.08	12.30±1.98
NADPH + FMN	59.29±3.19	58.24±4.63	18.57±1.29	9.65±1.04
NADPH + riboflavin	45.70±1.37	67.41±1.27	16.36±0.71	10.72±1.37

Each value represents the mean±SD of three animals.
ND, not detected.
Brucine *N*-oxide or imipramine *N*-oxide was incubated with liver microsomes of phenobarbital-treated rats in the presence of cofactors under anaerobic conditions.

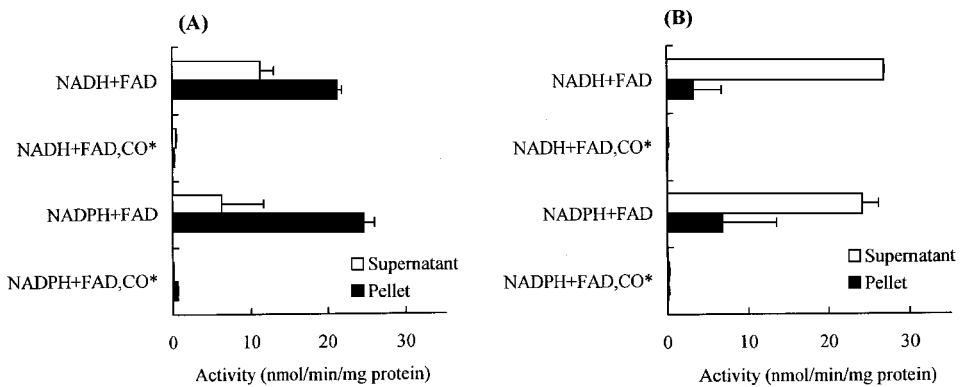


Figure 4. Effects of trypsin (A) or sodium cholate (B) treatment of rat liver microsomes on brucine *N*-oxide reductase activity. Each value represents the mean±SD of four experiments. Reactions were conducted with 50 μ l supernatant or a suspension of pellets of trypsin- or sodium cholate-treated microsomes in the presence of both NAD(P)H and FAD at 37 °C for 5 min under anaerobic conditions. Brucine formed was determined by HPLC. Other details are described in the Materials and methods. *Reaction conducted under an atmosphere of carbon monoxide.

the supernatant fraction showed low activity. In contrast, the activity was recovered in the supernatant fraction of sodium cholate-treated microsomes. In both cases, *N*-oxide reductase activities were markedly inhibited by carbon monoxide (figure 4). These facts suggest that the microsomal electron transfer system does not function in the NAD(P)H and flavin-dependent *N*-oxide reduction, and instead, a novel type of reduction system is involved in the *N*-oxide reduction by liver microsomes.

Next, the reduction of aliphatic tertiary amine *N*-oxide was examined with purified cytochrome P450. Rat liver cytochrome P4502B1 exhibited an *N*-oxide reducing activity in the presence of both NAD(P)H and a flavin without NADPH-cytochrome P450 reductase (figure 5). When the cytochrome P450 was boiled, the activity was again not abolished as was the case with the liver microsomal activity described above (data not shown). Cytochrome P420, an inactive form of cytochrome P450, prepared from cytochrome P4502B1 also exhibited an *N*-oxide

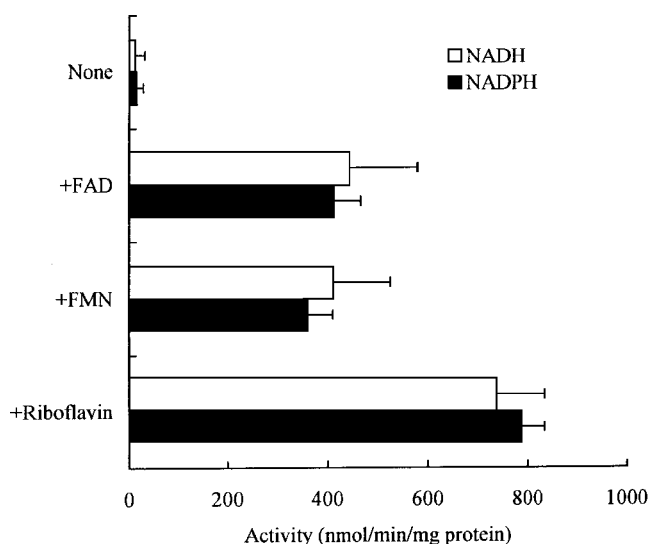


Figure 5. Reduction of brucine *N*-oxide by cytochrome P4502B1 in the presence of NAD(P)H and flavin. Each value represents the mean $\pm$ SD of four experiments. Reactions were conducted with 1 nmol cytochrome P4502B1 in the presence of 2 μ mol NADH or NADPH with or without 0.1 μ mol of a flavin at 37 $^{\circ}$ C for 5 min under anaerobic conditions. Other details are described in the Materials and methods.

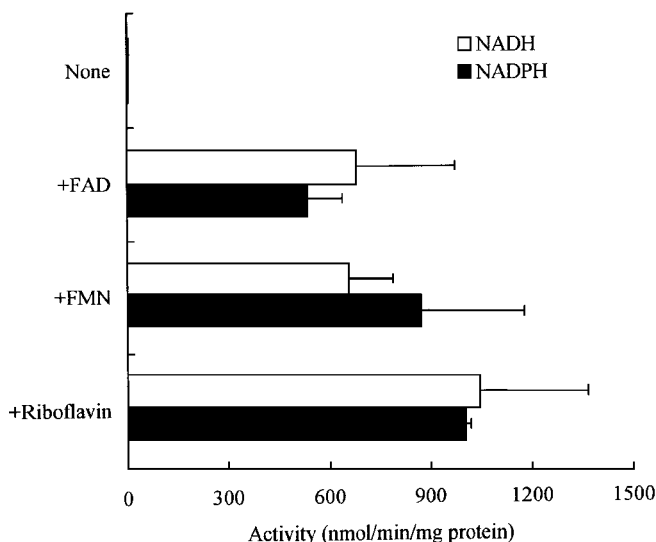


Figure 6. Reduction of brucine *N*-oxide by cytochrome P420 in the presence of NAD(P)H and flavin. Each value represents the mean $\pm$ SD of four experiments. Reactions were conducted with 1 nmol cytochrome P420 prepared from cytochrome P4502B1 in the presence of 2 μ mol NADH or NADPH with or without 0.1 μ mol of a flavin at 37 $^{\circ}$ C for 5 min under anaerobic conditions. Other details are described in the Materials and methods.

reducing activity in the presence of NAD(P)H and a flavin (figure 6). These facts suggest that the reduction in the presence of NAD(P)H and FAD proceeded with cytochrome P450 alone, without NADPH-cytochrome P450 reductase or NADH-cytochrome b_5 reductase.

Table 2. Reduction of brucine *N*-oxide by apo-cytochrome P4502B1 and haem.

Addition	Brucine formed (nmol min ⁻¹)	
	Apo-cytochrome P4502B1	Haem
None	0.02±0.01	0.34±0.07
NADH	0.05±0.01	0.44±0.12
NADPH	0.02±0.01	1.63±0.25
NADH+FAD	0.39±0.09	144.93±29.29
NADPH+FAD	0.20±0.04	118.23±21.80
NADH+FAD,CO*	0.03±0.01	13.47±5.11
NADPH+FAD,CO*	0.10±0.01	13.53±1.88

Each value represents the mean±SD of experiments.

* Assay was performed under an atmosphere of carbon monoxide. Reactions were conducted with dehaem protein or haem of cytochrome P4502B1 equivalent to 6 nmol cytochrome P450 in the presence of cofactors for 2 min under anaerobic conditions.

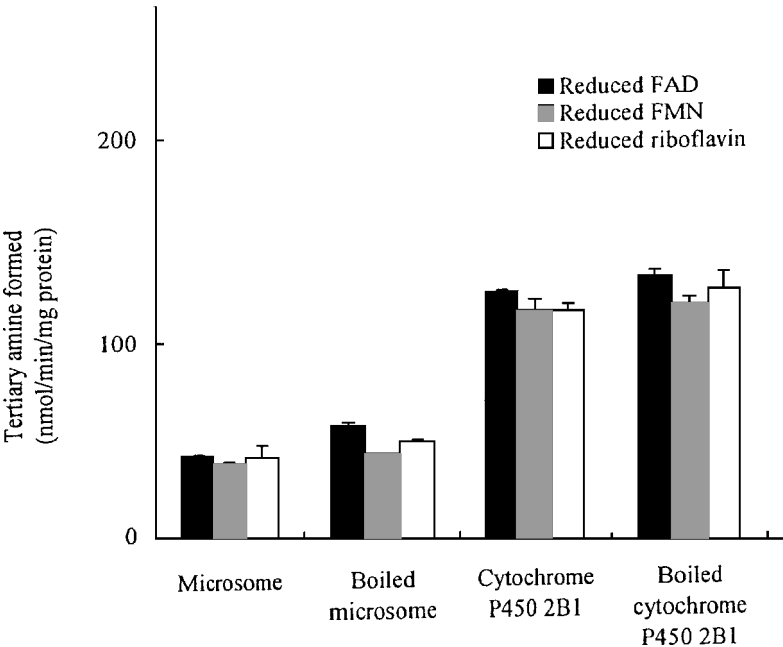


Figure 7. Reduction of brucine *N*-oxide by rat liver microsomes or cytochrome 2B1 in the presence of reduced flavins. Each value represents the mean+SD of four experiments. Reactions were conducted with 50 μ l of liver microsomes or 1 nmol cytochrome P4502B1 in the presence of 0.2 μ mol reduced FAD, FMN or riboflavin at 37 °C for 5 min under anaerobic conditions. Reduced flavin was produced spectrophotometrically. Other details are described in the Materials and methods.

Reduction of N-oxide by the haem moiety of cytochrome P450

The cytochrome P450 was separated into haem and apoprotein components in order to examine the involvement of these components in the *N*-oxide reduction. The haem isolated from cytochrome P4502B1 with both NADPH and FAD exhibited *N*-oxide reductase activity which was sensitive to carbon monoxide. However, the apoprotein moiety had little activity in the presence of both NADPH and FAD (table 2). Ferric chloride or ferrous chloride did not exhibit the reductase

activity even in the presence of NADPH and FAD. These results lead to the conclusion that aliphatic tertiary amine N-oxides are reduced by the non-enzymatic catalytic action of the haem group of cytochrome P450 in the presence of both FAD and a reduced pyridine nucleotide.

Reduced flavin-dependent N-oxide reduction by cytochrome P450

Liver microsomes also exhibited the reductase activity in the presence of photochemically reduced flavins in place of NAD(P)H and a flavin (figure 7). The activity with a reduced flavin was not abolished by the boiled microsomes. Cytochrome P450B1 also exhibited the reductase activity with a reduced flavin without the reductase. The boiled cytochrome P450 also exhibited the reductase activity with a reduced flavin (figure 7).

Discussion

Previously, it was reported that a flavin markedly enhanced the anaerobic reduction of aliphatic tertiary amine N-oxides by rat liver microsomes with NADPH or NADH (Kato *et al.* 1976), suggesting that a reduced flavin was enzymatically formed from a flavin by NADPH and NADPH-cytochrome P450 reductase, or NADH and NADH-cytochrome b_5 reductase, and then converted cytochrome P450 to its reduced form as an effective electron carrier, which in turn reduced the tertiary amine N-oxides to the corresponding amines. The overall reaction appeared to proceed enzymatically. However, experiments using boiled microsomes were not carried out at that time. The results of the present investigation have demonstrated cytochrome P450-mediated non-enzymatic reduction of aliphatic tertiary amine N-oxides by intact or boiled liver microsomes in the presence of both NAD(P)H and a flavin.

Based on the present results, the mechanism for the reduction of aliphatic tertiary amine N-oxides by rat liver microsomes is proposed as shown in figure 8. The first step involves the reduction of a flavin by a reduced pyridine nucleotide. In native microsomes, the flavin is mainly reduced by NADPH-cytochrome P450 reductase with NADPH or by NADH-cytochrome b_5 reductase with NADH as reported by Kato *et al.* (1976). In boiled microsomes, the flavin appears to be reduced non-enzymatically by NADPH or NADH as reported by Singer and Kearney (1950). However, the present authors cannot exclude the role of trace amounts of metal ions in catalysing this reduction as suggested by Crichton *et al.* (1975). The second step is the non-enzymatic reduction of the tertiary amine N-oxide to the corresponding amine by the reduced flavin as the electron donor, catalysed by the haem group of cytochrome P450. In this step, reduced flavins function as reducing agents of haem. Yubisui *et al.* (1980) reported that methemoglobin was stoichiometrically reduced by FADH_2 or FMNH_2 . They also showed that the reaction was very rapid, compared with the reduction of the flavin by flavin reductase of human erythrocytes. In the case of cytochrome P450, the haemoprotein was thought to be reduced by FADH_2 or FMNH_2 . Then, aliphatic tertiary amine N-oxides were reduced non-enzymatically by the reduced form of cytochrome P450. The reduction of flavins by flavin reductase in the first step is assumed to be the rate-limiting step in tertiary amine N-oxide reduction.

Kamm and Gillette (1963) reported that nitro- compounds were non-

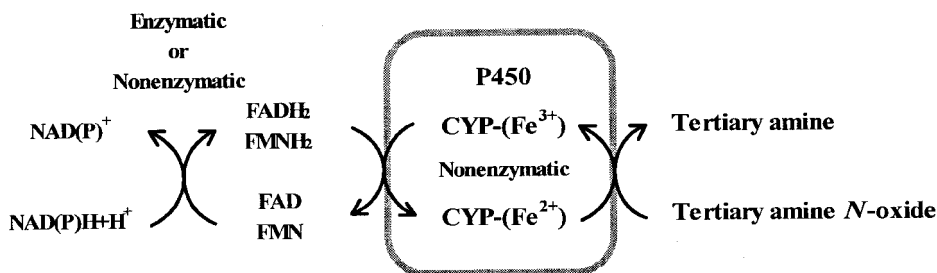


Figure 8. Proposed mechanism for tertiary amine *N*-oxide reduction by cytochrome P450 in the presence of NAD(P)H and flavins.

enzymatically reduced by reduced flavins under anaerobic conditions on the basis that the reduction of the nitro group to an amino group was accompanied by an increase of the absorbance at 446 nm due to oxidized flavins. *N*-oxide compounds used in this study were not directly reduced by reduced flavins without haem. There are a few reports of the involvement of haemoproteins in the reduction of tertiary amine *N*-oxides. They cover the aerobic reduction of trimethylamine *N*-oxide by haemoglobin in the presence of cysteine, the anaerobic reduction of indicine *N*-oxide by cytochrome *c* in the presence of ascorbic acid, and the aerobic reduction of 4-dimethylaminoazobenzene *N*-oxide by methaemoglobin (Vaisey 1956, Terayama 1963, Powis and DeGraw 1980). Patterson and Gorrod (1978) also reported that some 4-substituted *N*-ethyl-*N*-methylaniline *N*-oxides were reduced by boiled liver microsomes under aerobic conditions. Recently, the present authors demonstrated that imipramine *N*-oxide was anaerobically reduced by a quinone-dependent system catalysed by haemoproteins in liver cytosol of rats (Kitamura *et al.* 1999). Gillespie and Duffel (1989) reported that horseradish peroxidase catalysed *N*-oxide reduction of indicine *N*-oxide in the presence of high concentration of NADH (20 mM) and FMN (1 mM) under anaerobic conditions. In our preliminary study, haemoglobin and catalase also exhibited the *N*-oxide reducing ability in the presence of NAD(P)H and a flavin under anaerobic conditions. The reduced flavin-dependent *N*-oxide reduction may be usually observed in haemoproteins. However, the reduced flavin-dependent aliphatic tertiary amine *N*-oxide reduction by cytochrome P450 demonstrated in this study has not previously been reported except in our preliminary communication (Takekawa *et al.* 1997). Powis *et al.* (1982) proposed that the Fe(III)-EDTA complex stimulated anaerobic microsomal indicine *N*-oxide reduction. However, brucine *N*-oxide reducing activity estimated in the presence of NADH, FAD, ferric chloride and EDTA was much lower than that of cytochrome P450 demonstrated in this study. The non-enzymatic reduction catalysed by cytochrome P450 appears to function as a major *N*-oxide reducing system for various tertiary amine *N*-oxides.

Aliphatic tertiary amine *N*-oxides were reduced efficiently by the *N*-oxide reducing system of liver microsomes described in this study. In contrast, aromatic *N*-oxides such as nicotinamide *N*-oxide and purine *N*-oxides were anaerobically reduced by xanthine oxidase, a cytosolic flavoenzyme (Murray and Chaykin 1966, Stöhrer and Brown 1969). However, nicotinamide *N*-oxide was not reduced by this system. It has been reported that aliphatic tertiary amine *N*-oxides such as imipramine *N*-oxide and cyclobenzaprine *N*-oxide are also anaerobically reduced by the microsomal NADPH-dependent enzymatic system involving cytochrome P450,

though nicotinamide N-oxide is not enzymatically reduced by the cytochrome P450 system (Kitamura and Tatsumi 1984a, b). Nicotinamide N-oxide is not a substrate for enzymatic or non-enzymatic reductions involving cytochrome P450. The high water solubility of the N-oxide may be one reason for this. In contrast, aldehyde oxidase exhibited reductase activities toward both aliphatic tertiary amine N-oxides and aromatic type N-oxides, and in this case, extraordinarily high reductase activity toward nicotinamide N-oxide was observed (Kitamura and Tatsumi 1984a, b, Sugihara *et al.* 1996). Thus, there is a distinct difference between aliphatic tertiary amine N-oxides and aromatic type N-oxides from the viewpoint of biological reduction.

Kato and collaborators (Sugiura *et al.* 1974, 1976, Iwasaki *et al.* 1977) demonstrated NADPH-dependent tertiary amine N-oxide reduction in liver microsomes as an enzymatic reduction involving cytochrome P450. The reduced flavin-dependent N-oxide reducing activity was one order of magnitude greater than the microsomal NADPH-dependent N-oxide reductase activity. It is possible that the NADPH-dependent activity involved an intrinsic flavin-like electron carrier in part, in a similar manner to that shown in our study. In fact, about half of the NADPH-linked reducing activity remained even when the microsomes were boiled. Thus, the reduced flavin-dependent non-enzymatic reduction appears to function as a major system in the reduction of aliphatic tertiary amine N-oxides in liver microsomes.

Some N-oxide compounds are important as pharmacological or toxicological agents, e.g. in the groups of alkaloids, chemotherapeutics, antibiotics, psychotropic drugs, methemoglobin forming compounds and carcinogens. These activities are often decreased by the N-oxide reduction. Substrate specificity of N-oxide reductase(s) play an important role in the maintenance of the activity (Bickel 1969). In contrast, it is well known that tertiary amines are oxidized to the corresponding N-oxides in liver microsomes (Ziegler 1980). Thus, the reduction kinetics of tertiary amine N-oxides to the amines is complex because of the re-oxidation of tertiary amine to the parent N-oxide. It has been reported that amidines, amidinohydrazones and guanidines are oxidized to their N-hydroxyl metabolites, and retroreduction to the parent compounds occurs aerobically in liver microsomes (Clement *et al.* 1993, 1994, Clement and Jung 1994). The reduction system is composed of NADH, NADH-cytochrome b_5 reductase, cytochrome b_5 and a third protein component, which has many cytochrome P450-like characteristics (Clement *et al.* 1997). The reducing activity was much higher than the oxidative one in liver microsomes. This may be the reason why N-hydroxyl metabolites were not detected in the *in vivo* experiments. When imipramine was incubated with liver preparations in the presence of NADPH under aerobic conditions, it was oxidized to imipramine N-oxide. The oxidation rate in microsomes of rat liver was $10.3 \text{ nmol}/30 \text{ min mg}^{-1}$ protein (Kitamura *et al.* 1999). The activity is very low compared with that of the reduction of imipramine N-oxide to the amine in liver microsomes reported in the current study, as in the case of interconversion between amidine derivatives and their N-hydroxyl metabolites. High reductase activity is of major relevance for the maintenance of effective concentrations of active tertiary amines. When imipramine was similarly incubated under anaerobic conditions, the formation of imipramine N-oxide was not observed in liver microsomes, even in the presence of NADPH. The velocity of the aerobic N-oxide reduction in liver preparations decreased to $\sim 1/10$ of that under anaerobic conditions. The reason for this may be the

competition of the substrate with oxygen at the active site. However, the re-oxidation of imipramine to the *N*-oxide may contribute to the decreased formation of the reduction product under aerobic conditions.

The microsomal, reduced flavin-dependent aliphatic tertiary amine *N*-oxide reduction was observed widely among species, i.e. rats, mice, guinea pigs, hamsters and rabbits (data not shown). The reduced flavin-dependent *N*-oxide reducing activity was one order of magnitude greater than the *N*-oxide reductase activity of the aldehyde oxidase or enzymatic NADPH-dependent cytochrome P450 system described in the literature (Kitamura and Tatsumi 1984b). The present authors have also reported a quinone-dependent *N*-oxide reducing system, composed of NAD(P)H, quinone reductase and haemoproteins. This system also exhibits high reducing activity (Kitamura *et al.* 1999). Thus, the reduced flavin-dependent reduction appears to function as a major system in the reduction of aliphatic tertiary amine *N*-oxides in liver microsomes, similar to the quinone-dependent *N*-oxide reduction system in liver cytosol. It is noteworthy that the *N*-oxidation of aliphatic tertiary amines was suggested to be a route of detoxification (Bickel 1969). If this is so, the system demonstrated in this study would result in the pharmacological reactivation of aliphatic tertiary amines from the *N*-oxides.

References

- BICKEL, M. H., 1969, The pharmacology and biochemistry of *N*-oxides. *Pharmacological Review*, **21**, 325–355.
- CLEMENT, B. and JUNG, F., 1994, *N*-Hydroxylation of the antiprotozoal drug pentamidine catalysed by rabbit liver cytochrome P-450 2C3 or human liver microsomes, microsomal retroreduction, and further oxidative transformation of the formed amidoximes. *Drug Metabolism and Disposition*, **22**, 486–497.
- CLEMENT, B., LOMB, R. and MÖLLER, W., 1997, Isolation and characterization of the protein components of the liver microsomal O₂-insensitive NADH-benzamidoxime reductase. *Journal of Biological Chemistry*, **272**, 19615–19620.
- CLEMENT, B., SCHULTZE-MOSGAU, M. H., RICHTER, P. H. and BESCH, A., 1994, Cytochrome P450-dependent *N*-hydroxylation of an aminoguanidine (amidinohydrazone) and microsomal retro-reduction of the *N*-hydroxylated product. *Xenobiotica*, **24**, 671–688.
- CLEMENT, B., SCHULTZE-MOSGAU, M. H. and WOHLERS, H., 1993, Cytochrome P450 dependent *N*-hydroxylation of a guanidine (debrisoquine), microsomal catalysed reduction and further oxidation of the *N*-hydroxy-guanidine metabolite to the urea derivative. *Biochemical Pharmacology*, **46**, 2249–2267.
- CRICHTON, R. R., ROMAN, F. and WAUTERS, M., 1975, Reductive mobilization of ferritin iron by reduced nicotinamide-adenine dinucleotide flavin mononucleotide. *Biochemical Society Transactions*, **3**, 946–948.
- DIETRICH, H., 1967, 5-Dialkylaminoalkyl-5H-dibenz[b,f]azepine *N*-oxides. *Chemical Abstract*, **66**, 10857n.
- GILLESPIE, S. G. and DUFFEL, M. W., 1989, Peroxidase as a model for reduction of tertiary amine oxides catalysed by rat hepatic supernatant and microsomal fractions. *Biochemical Pharmacology*, **38**, 573–579.
- GUENGERICH, F. P., DANNAN, G. A., WRIGHT, S. T., MARTIN, M. V. and KAMINSKY, L. S., 1982, Purification and characterization of liver microsomal cytochrome P-450. *Biochemistry*, **21**, 6019–6030.
- IMAI, Y. and SATO, R., 1967, Conversion of cytochrome P-450 to P-420 by neutral salts and some other reagents. *European Journal of Biochemistry*, **1**, 419–426.
- IWASAKI, K., NOGUCHI, H., KATO, R., IMAI, Y. and SATO, R., 1977, Reduction of tertiary amine *N*-oxide by purified cytochrome P-450. *Biochemical and Biophysical Research Communication*, **77**, 1143–1149.
- KAMM, J. J. and GILLETTE, J. R., 1963, Mechanism of stimulation of mammalian nitro reductase by flavins. *Life Sciences*, **4**, 254–260.
- KATO, R., IWASAKI, K. and NOGUCHI, H., 1976, Stimulatory effect of FMN and methyl viologen on cytochrome P-450 dependent reduction of tertiary amine *N*-oxide. *Biochemical and Biophysical Research Communication*, **72**, 267–274.

- KITAMURA, S., SUGIHARA, K. and TATSUMI, K., 1999, A unique tertiary amine N-oxide reduction system composed of quinone reductase and haem in rat liver preparations. *Drug Metabolism and Disposition*, **27**, 92–97.
- KITAMURA, S. and TATSUMI, K., 1984a, Involvement of liver aldehyde oxidase in the reduction of nicotinamide N-oxide. *Biochemical and Biophysical Research Communication*, **120**, 602–606.
- KITAMURA, S. and TATSUMI, K., 1984b, Reduction of tertiary amine N-oxides by liver preparations: function of aldehyde oxidase as a major N-oxide reductase. *Biochemical and Biophysical Research Communication*, **121**, 749–754.
- LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. and RANDALL, R. J., 1951, Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry*, **193**, 265–275.
- MATSUBARA, T., KOIKE, M., TOCHINO, A. and SUGENO, K., 1976, Quantitative determination of cytochrome P-450 in rat liver homogenate. *Analytical Biochemistry*, **2**, 264–289.
- MURRAY, K. N. and CHAYKIN, S., 1966, The reduction of nicotinamide N-oxide by xanthine oxidase. *Journal of Biological Chemistry*, **241**, 3468–3473.
- PATTERSON, L. H. and GORROD, J. W., 1978, The metabolism of some 4-substituted N-ethyl-N-methylaniline N-oxides. In J. W. Gorrod (ed.), *Biological Oxidation of Nitrogen* (Amsterdam: Elsevier), pp. 471–478.
- POWIS, G. and DEGRAW, C., 1980, N-Oxide reduction by haemoglobin, cytochrome c and ferrous ions. *Research Communications Chemical Pathology and Pharmacology*, **30**, 143–150.
- POWIS, G., SVINGEN, B. A. and DEGRAW, C., 1982, Iron-EDTA stimulated reduction of indicine N-oxide by hepatic microsomal fraction, isolated hepatocytes, and the intact rat. *Biochemical Pharmacology*, **31**, 293–299.
- POWIS, G. and WINCENTSEN, L., 1980, Pyridine nucleotide cofactor requirements of indicine N-oxide reduction by hepatic microsomal cytochrome P-450. *Biochemical Pharmacology*, **29**, 347–351.
- SINGER, T. P. and KEARNEY, F. B., 1950, The non-enzymatic reduction of cytochrome c by pyridine nucleotides and its catalysis by various flavins. *Journal of Biological Chemistry*, **183**, 409–429.
- STÖHRER, G. and BROWN, G. B., 1969, Purine N-oxides XXVIII. The reduction of purine N-oxides by xanthine oxidase. *Journal of Biological Chemistry*, **244**, 2498–2502.
- SUGIHARA, K., KITAMURA, S. and TATSUMI, K., 1996, S(-)-Nicotine-1'-N-oxide reductase activity of rat liver aldehyde oxidase. *Biochemistry and Molecular Biology International*, **40**, 535–541.
- SUGIURA, M., IWASAKI, K. and KATO, R., 1976, Reduction of tertiary amine N-oxides by liver microsomal cytochrome P-450. *Molecular Pharmacology*, **12**, 322–334.
- SUGIURA, M., WASAKI, K., NOGUCHI, H. and KATO, R., 1974, Evidence for the involvement of cytochrome P-450 in tiaramide N-oxide reduction. *Life Sciences*, **15**, 1433–1442.
- TAKEKAWA, K., SUGIHARA, K., KITAMURA, S. and TATSUMI, K., 1997, Non-enzymatic reduction of brucine N-oxide by the haem group of cytochrome P450. *Biochemistry and Molecular Biology International*, **42**, 977–981.
- TERAYAMA, H., 1963, Aminoazo dye amine-N-oxide as a possible intermediate metabolite preceding N-demethylation and ortho-hydroxylation, as well as azo dye-protein binding. *Gann*, **55**, 195–204.
- VAISEY, E. B., 1956, The non-enzymic reduction of trimethylamine oxide to trimethylamine, dimethylamine, and formaldehyde. *Canadian Journal of Biochemical Physiology*, **34**, 1085–1090.
- YU, C.-A. and GUNSALUS, I. C., 1974, Cytochrome P-450cam III. Removal and replacement of ferriprotoporphyrin IX. *Journal of Biological Chemistry*, **249**, 107–110.
- YUBISUI, T., MATSUKAWA, S. and YONEYAMA, Y., 1980, Stopped flow studies on the non-enzymatic reduction of methemoglobin by reduced flavin mononucleotide. *Journal of Biological Chemistry*, **255**, 11694–11697.
- ZIEGLER, D. M., 1980, Microsomal flavin-containing monooxygenase. In W. B. Jakoby (ed.), *Enzymatic Basis of Detoxication*, vol. 1 (New York: Academic Press), pp. 201–227.

Lack of interaction between broprimine and 5-fluorouracil on human dihydropyrimidine dehydrogenase

S. YAMAZAKI†‡*, M. HAYASHI†, L. N. TOTH† and N. OZAWA†

† Toxicology and Efficacy Research, Tsukuba Research Laboratories, Pharmacia & Upjohn Ltd, Tsukuba, Ibaraki 300-4247, Japan

‡ Pharmacokinetics and Bioanalytical Research, Pharmacia Corporation, Kalamazoo, MI 49007, USA

Received 11 October 2000

1. Broprimine (2-amino-5-bromo-6-phenyl-4-pyrimidinone) is a member of a class of antineoplastic agents that are administered concomitantly or sequentially with anticancer 5-fluorouracil (5-FU) prodrugs in clinical patients. Interactions between broprimine and 5-fluorouracil (5-FU) were investigated on dihydropyrimidine dehydrogenase (DPD) activity, the rate-limiting enzyme of 5-FU metabolism, in human liver cytosol. Apparent DPD activity was determined by measuring the recovery of [^{14}C]5-FU by HPLC.

2. The apparent activity of 5-FU metabolism (2.1–100 μM) showed a linear relationship in the Eadie-Hofstee plot in the pooled cytosol, suggesting that a single enzyme is responsible for apparent 5-FU metabolism. K_m and V_{max} were estimated to be 23 μM and 0.32 $\text{nmol min}^{-1} \text{mg}^{-1}$ protein, respectively. Apparent DPD activity for 5-FU (25 μM) in the cytosol from 12 individual donors ranged from 0.017 to 0.39 (0.16 ± 0.12) $\text{nmol min}^{-1} \text{mg}^{-1}$ protein, indicating a large intersubject variance.

3. The suicidal inactivators of the DPD enzyme, (*E*)-5-(2-bromovinyl)uracil and 5-bromouracil (6.3–50 μM), illustrated concentration-dependent inhibition on DPD activity. Isocytosine (6.3–100 μM), used as a negative control, did not affect DPD activity. Broprimine (6.3–100 μM) also did not show any inhibition of DPD activity. Therefore, broprimine is unlikely to cause increases in 5-FU levels in clinical patients after co-administration of broprimine with 5-FU prodrugs.

Introduction

In 1993, 15 deaths in Japanese patients occurred in association with the co-administration of the new antiviral drug for herpes, sorivudine (1- β -D-arabinofuranosyl-(*E*)-5-(2-bromovinyl)uracil), with one of the anticancer oral 5-fluorouracil (5-FU) prodrugs (Pharmaceutical Affairs Bureau 1994, Swinbanks 1994). This serious accident was possibly caused by significant increases in 5-FU levels due to interactions between 5-FU and the antiviral drug sorivudine. An active metabolite of sorivudine, (*E*)-5-(2-bromovinyl)uracil (BVU), irreversibly inactivates dihydropyrimidine dehydrogenase (DPD), which is the initial rate-limiting enzyme in 5-FU metabolism and dehydrogenates at the C5, 6-double bond of 5-FU (Desgranges *et al.* 1986, Ogura *et al.* 1998). Hepatic DPD, a homodimeric 210 KDa flavoprotein with Fe-S clusters, has been recognized as a key enzyme-regulating plasma and tissue concentrations of 5-FU administered to humans (Lu *et al.* 1992). In addition to BVU, 5-bromouracil (5-BU) (Naguib *et al.* 1989, Porter *et al.* 1992), 5-iodouracil (Porter *et al.* 1991) and 5-ethynyluracil (Porter *et al.* 1992) have also been reported to be irreversible inactivators of the DPD enzyme. These reports

* Author for correspondence at Pharmacia Corporation; e-mail: shinji.yamazaki@am.pnu.com

suggest that some pyrimidine analogues, which have an electron-withdrawing group such as a halogen or conjugated double or triple bonds at the C5 position on the pyrimidine ring, could inactivate DPD activity as suicide substrates.

Bropirimine (2-amino-5-bromo-6-phenyl-4-pyrimidinone) is an aryl pyrimidinone that induces interferon production and modulates a number of other aspects of the immune system. It has activity against a number of viral illnesses, as well as activity against several different tumours (Wierenga 1985). Anticancer 5-FU prodrugs are likely to be administered concomitantly or sequentially with bropirimine in clinical patients. Bropirimine has bromine at the C5 position of the pyrimidine ring, so it had been predicted that bropirimine could irreversibly inhibit DPD activity. Therefore, it was necessary for the drug development to estimate *in vitro* interactions between bropirimine and 5-FU to extrapolate the interaction *in vivo*. In the present study, the possible *in vitro* interactions between bropirimine and 5-FU have been investigated in the human liver cytosol.

Materials and methods

Chemicals

Bropirimine (chemical purity 98%) was from Pharmacia & Upjohn (Kalamazoo, MI, USA) (Skulnick *et al.* 1985). Radiolabelled 5-FU (radiochemical purity > 99%) with ^{14}C at the C2 position was from American Radiolabelled Chemicals, Inc. (St Louis, MO, USA). 5-BU, cytosine, dithiothreitol, NADPH and tetrabutylammonium hydrogen sulphate were from Wako Pure Chemical Industries Ltd (Osaka, Japan). BVU, human serum albumin and isocytosine were from Sigma Chemical Co. (St Louis, MO, USA). INSTA-GEL® (Packard Instrument Co., Meriden, CT, USA) was used for the measurement of radioactivity as the scintillation cocktail. Other reagents were of reagent grade.

Human liver cytosol

Pooled liver cytosol (five male) and individual liver cytosol from seven male and five female donors were from the International Institutes for the Advancement of Medicine (IIAM, Exton, PA, USA). All donors were Caucasians (5–63 and 21–67 years old for males and females, respectively). The Ethical Committee of Pharmacia & Upjohn approved the use of the human liver cytosol for the study.

5-FU metabolism in human liver cytosol

The enzyme kinetics of 5-FU metabolism were investigated in the pooled liver cytosol. The reaction mixture contained [^{14}C]5-FU (2.1–100 μM), NADPH (0.2 mM), dithiothreitol (2 mM) and liver cytosol (1.0 mg protein) in 0.1 ml (final volume) of potassium phosphate buffer (50 mM, pH 7.4). The incubation mixture was pre-incubated for 2 min at 37 °C after the addition of NADPH, and the reaction was initiated by the addition of [^{14}C]5-FU. The incubation reaction proceeded for 5 min and then was terminated by the addition of acetonitrile (0.4 ml) containing non-labelled 5-FU as a carrier and cytosine as an internal standard for the HPLC analysis. Sample work-up and chromatographic conditions are described below. Michaelis-Menten parameters (apparent K_m and $V_{\max}$) for 5-FU metabolism were estimated by linear least-square regression analysis between 5-FU concentration divided by apparent rates of DPD activity and 5-FU concentration. Apparent rates of 5-FU metabolism were also determined in the cytosol of seven male and five female donors. The reaction mixture and incubation conditions were the same as the above experiments using the pooled cytosol with the exception of a concentration of [^{14}C]5-FU of 25 μM .

Effect of pyrimidine analogues on 5-FU metabolism in human liver cytosol

The effect of pyrimidine analogues on 5-FU metabolism was investigated in human liver cytosol. The reaction mixture contained [^{14}C]5-FU (13, 25 and 50 μM), pyrimidine analogues (6.3–100 μM), NADPH (0.2 mM), dithiothreitol (2 mM) and liver cytosol (1.0 mg protein) in 0.1 ml (final volume) of potassium phosphate buffer (50 mM, pH 7.4). The pyrimidine analogue was added to the incubation mixture just before incubation and incubation conditions were the same as the experiment described above. Apparent K_i for the pyrimidine analogues in the pooled cytosol was estimated by linear least-square regression analysis between the reciprocal of the apparent rate of DPD activity and the inactivator concentration. The apparent IC_{50} for the pyrimidine analogues in individual cytosols was determined by linear least-square regression analysis of three points in the plot of apparent activity (% of control) versus the inactivator concentration.

HPLC analysis of 5-FU metabolism in human liver cytosol

The apparent rate of 5-FU metabolism was determined by measuring the recovery of [^{14}C]5-FU from the incubation mixture. After the incubation, acetonitrile (0.4 ml) containing non-labelled 5-FU as a carrier and cytosine as an internal standard were added to the incubation mixture to terminate the reaction. After centrifugation (1500g) for 10 min, the supernatant was evaporated and the residue was reconstituted with 0.1 ml HPLC mobile phase. The solution (0.05 ml) was injected into the HPLC, which consisted of two ODS columns (Hypersil ODS-5, 4.6 $\times$ 250 mm, GL Science, Inc., Tokyo, Japan; Spherisorb ODS-1-5, 4.6 $\times$ 250 mm, GL Science) used in tandem, and a fraction of [^{14}C]5-FU was collected for measurement of radioactivity. The HPLC mobile phase was 1.5 mM potassium phosphate buffer (pH 8.0) containing 5 mM tetrabutylammonium hydrogen sulphate and was delivered at 1.0 ml min $^{-1}$. Under these conditions, 5-FU and cytosine eluted at $\sim$ 4 and 8 min, respectively, at a UV absorbance of 320 nm. Radioactivity was determined by a liquid scintillation counter (LSC-3500, Aloka Co., Ltd, Tokyo, Japan). Counting efficiency was determined by an external standard technique with a suitably constructed standard curve. The lower limit of quantitation for the recovery of 5-FU was set at radioactivity level that was three times higher than the background level. Accordingly, the lower limit of quantitation corresponded to $\sim$ 2% of 5-FU concentration. Intra- and inter-recoveries of quality control samples ranged from 85 to 115% with coefficient variances of $<$ 10%.

Measurement of protein concentration in human liver cytosol

Protein concentration in liver cytosol was measured by the Bradford method (1976) using a protein assay kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Human serum albumin was used as standard protein.

Results

The effects of cytosol protein concentration and incubation time on the apparent rate of 5-FU metabolism were evaluated in the pooled cytosol. Mixtures containing 5-FU at 25 μM and cytosol protein at 0.4–2.0 mg protein were incubated for 2–10 min at 37 $^{\circ}\text{C}$ following 2-min pre-incubation. The apparent rate of 5-FU metabolism was linear for cytosol protein content (0.4–1.0 mg; $r^2 = 0.98$) and for incubation time (2–5 min; $r^2 = 0.98$) (data not shown). Based on these results, experiments were therefore performed under linear conditions to determine the kinetic parameters.

The apparent rate of 5-FU metabolism (2.1–100 μM) was determined in the pooled human liver cytosol. A linear relationship between the apparent rates of DPD activity divided by 5-FU concentration and the apparent rates of DPD activity was observed by Eadie-Hofstee graphical analysis (figure 1), suggesting that a single enzyme is responsible for apparent 5-FU metabolism in the liver cytosol. K_m and $V_{\max}$ were estimated as being 23 μM and 0.32 nmol min $^{-1}$ mg $^{-1}$ protein, respectively. Furthermore, the apparent rate of 5-FU metabolism (25 μM) was determined in liver cytosol of seven male and five female donors and the mean apparent activity for 5-FU was 0.16 ± 0.12 nmol min $^{-1}$ mg $^{-1}$ protein (0.017–0.39 nmol min $^{-1}$ mg $^{-1}$ protein), indicating a large intersubject variance.

The effects of BVU (6.3–50 μM) on the metabolism of 5-FU (13, 25 and 50 μM) were determined in the pooled cytosol. Inhibition of 5-FU metabolism by BVU was concentration-dependent at each 5-FU concentration and the apparent K_i was estimated to be 7.5 μM (figure 2). Apparent IC_{50} values of BVU (6.3–50 μM) were also investigated in individual cytosols with the exception of three donors which showed low DPD activity ($<$ 0.06 nmol min $^{-1}$ mg $^{-1}$ protein). BVU illustrated concentration-dependent inhibition of 5-FU metabolism and the apparent IC_{50} for BVU ranged from 18 to 37 (26 ± 7) μM . Another suicidal inactivator, 5-BU (6.3–50 μM), also showed concentration-dependent inhibition for 5-FU metabolism in the pooled cytosol, whereas isocytosine (6.3–100 μM) did not show any effects

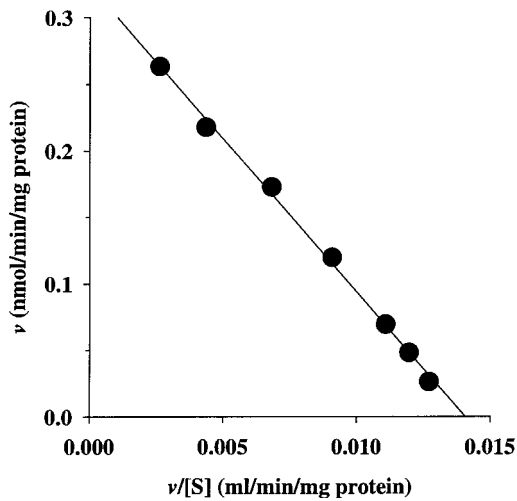


Figure 1. Eadie-Hofstee plot of apparent DPD activity in pooled human liver cytosol. The incubation mixture containing [¹⁴C]5-FU (2.1–100 μ M), NADPH (0.2 mM), dithiothreitol (2 mM) and liver cytosol (1.0 mg protein) in 0.1 ml (final volume) of potassium phosphate buffer (50 mM, pH 7.4) was incubated for 5 min at 37 °C following a 2-min pre-incubation with NADPH. The reaction was initiated by the addition of [¹⁴C]5-FU. The apparent rate of 5-FU metabolism was determined by measurement of the recovery of [¹⁴C]5-FU by HPLC. Data are the mean of duplicate determinations.

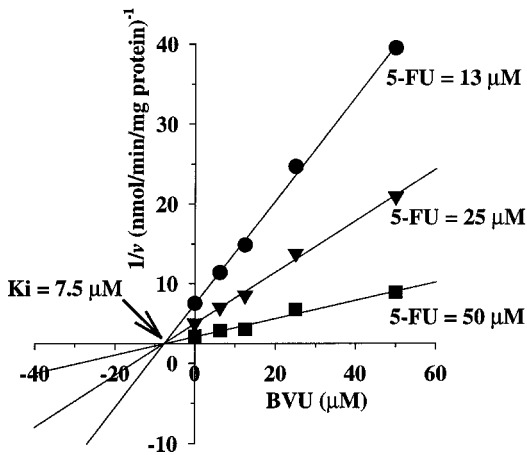


Figure 2. Dixon plot of apparent DPD activity in pooled human liver cytosol in the presence of BVU. The incubation mixture contained [¹⁴C]5-FU (13, 25 or 50 μ M), BVU (6.3–50 μ M), NADPH (0.2 mM), dithiothreitol (2 mM) and liver cytosol (1.0 mg protein) in 0.1 ml (final volume) of potassium phosphate buffer (50 mM, pH 7.4). The incubation conditions and the measurement of 5-FU metabolism were the same as described in figure 1. BVU was added to the incubation mixture just before incubation. Data are the mean of duplicate determinations.

(figure 3). These results indicate that the present assay system is suitable to estimate the effects of pyrimidine analogues on 5-FU metabolism in humans. The effects of bropirimine (6.3–100 μ M) on 5-FU metabolism were also investigated in the present study. In the pooled cytosol, the apparent rate of 5-FU metabolism with bropirimine at a maximum concentration of 100 μ M was 106% compared with tests without bropirimine (figure 3). The apparent rates of 5-FU metabolism with bropirimine

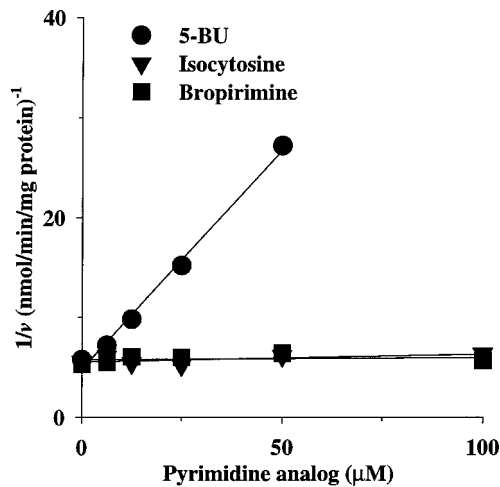


Figure 3. Dixon plot of apparent DPD activity in pooled human liver cytosol with 5-BU, isocytosine or bropirime. The incubation mixture contained [^{14}C]5-FU ($25\text{ }\mu\text{M}$), pyrimidine analogue (5-BU $6.3\text{--}50\text{ }\mu\text{M}$ or isocytosine and bropirime $6.3\text{--}100\text{ }\mu\text{M}$), NADPH (0.2 mM), dithiothreitol (2 mM) and liver cytosol (1.0 mg protein) in 0.1 ml (final volume) of potassium phosphate buffer (50 mM , $\text{pH } 7.4$). The incubation conditions and the measurement of 5-FU metabolism were the same as described in figures 1 and 2. Data are the mean of duplicate determinations.

($100\text{ }\mu\text{M}$) in individual donors ranged from 91 to 124 % ($108\pm 11\%$) compared with tests without bropirime. These results indicate that bropirime does not effect 5-FU metabolism in human liver cytosol.

Discussion

In the present study, apparent DPD activity was determined by measuring the recovery of 5-FU. A single enzyme seemed to be responsible for the apparent 5-FU metabolism on the basis of Eadie-Hofstee graphical analysis. Therefore, the predominant factor influencing 5-FU metabolism appears to be DPD activity. A large intersubject variance was observed for the apparent DPD activity in the cytosol of seven male and five female donors. Lu *et al.* (1995) reported a large intersubject variance on apparent activities of 5-FU metabolism *in vitro* in human liver (range $0.049\text{--}0.89$, mean $\pm\text{SE}$ $0.37\pm 0.02\text{ nmol min}^{-1}\text{ mg}^{-1}\text{ protein}$, $n = 138$). DPD activity in human liver was also reported to be highly variable with a 20-fold range in the general population (McMurrough and McLeod 1996). Clearance of 5-FU *in vivo* is known to show a large intersubject variance (Fleming *et al.* 1992, Milano *et al.* 1992, Etienne *et al.* 1994). Thus, DPD activity *in vivo* seems to have a large intersubject variance. The tests for DPD inhibition with known inhibitors, BVU and 5-BU, showed concentration-dependent inhibition in the present study. Isocytosine, known not to inhibit DPD enzyme, did not show any effects. These results indicate that the present assay system using human liver cytosol is useful to estimate the effects of various substrates of DPD in humans.

The rate-limiting enzyme of 5-FU metabolism, DPD, is irreversibly inactivated by some pyrimidine analogues such as BVU, 5-BU, 5-iodouracil and 5-ethynyluracil, which have an electron-withdrawing group at the C5 position of pyrimidine ring (Desgranges *et al.* 1986, Naguib *et al.* 1989, Porter *et al.* 1991, 1992, Ogura *et al.* 1998). Although bropirime is also a pyrimidine derivative and has

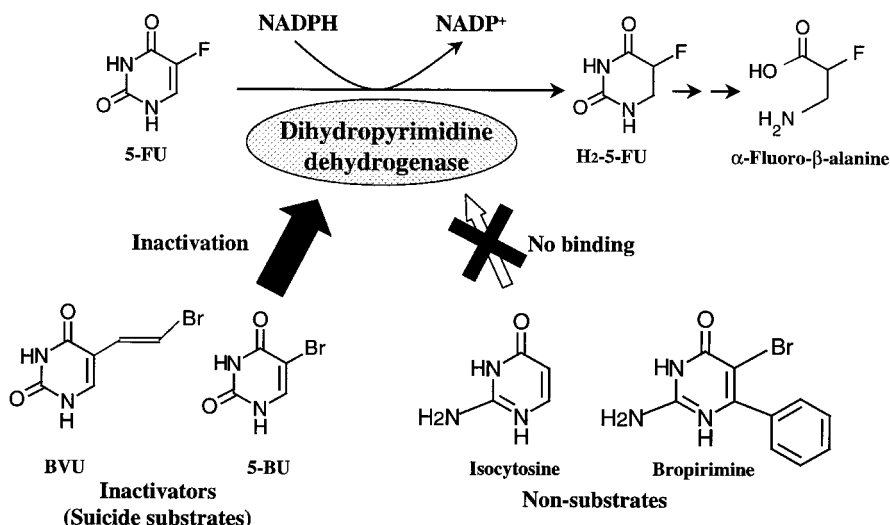


Figure 4. Inactivators and non-substrates for dihydropyrimidine dehydrogenase in human liver cytosol. Suicidal inactivators such as BVU and 5-BU cause significant increases in levels of 5-FU *in vivo* by irreversible inactivation of DPD in humans, whereas the pyrimidine analogues having an amino group at the C2 or C4 position such as bropirimine and isocytosine are not reduced by dihydropyrimidine dehydrogenase.

bromine at the C5 position, bropirimine did not inhibit DPD activity *in vitro*. Therefore, the probability of an increase in 5-FU levels *in vivo* after co-administration of bropirimine with 5-FU prodrugs is unlikely. It is estimated that DPD degrades > 85% of an administered dose of 5-FU in humans (Woodcock *et al.* 1979). For bropirimine, the main metabolites in humans are the 4-*O*-glucuronide, 2-*N*-glucuronide, cysteine conjugate and oxidized metabolites at the phenyl ring after oral administration of bropirimine (Hauer *et al.* 1997, Wynalda *et al.* 1998). This metabolite profile, indicating non-reduction at the C5, 6-double bond, is also consistent with the present results. It suggests that two intact oxo groups at the C2 and C4 positions of pyrimidine are necessary to bind to hepatic DPD enzyme in the mouse (Naguib *et al.* 1989). Because bropirimine has an amino group at the C2 position, it may not bind to the DPD enzyme in human liver (figure 4). In conclusion, bropirimine did not show any inhibition of the apparent DPD activity in the present assay system using human liver cytosol. Thus, bropirimine is unlikely to cause increases in levels of 5-FU in clinical patients after co-administration of bropirimine with 5-FU prodrugs.

References

- BRADFORD, M., 1976, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, **72**, 248–254.
- DESRANGES, C., RAZAKA, G., CLERCO, E. D., HARDEWIJN, P., BALZARINI, J., DROUILLET, F. and BRICAUD, H., 1986, Effect of (*E*)-5-(2-bromovinyl)uracil on the catabolism and antitumor activity of 5-fluorouracil in rats and leukemic mice. *Cancer Research*, **46**, 1094–1104.
- ETIENNE, M. C., LAGRANGE, J. L., DASSONVILLE, O., FLEMING, R., THYSS, A., RENNE, N., SCHNEIDER, M., DEMARD, F. and MILANO, G., 1994, Population study of dihydropyrimidine dehydrogenase in cancer patients. *Journal of Clinical Oncology*, **12**, 2248–2253.
- FLEMING, R. A., MILANO, G., THYSS, A., ETIENNE, M. C., RENEE, N., SCHNEIDER, M. and DEMARD, F., 1992, Correlation between dihydropyrimidine dehydrogenase activity in peripheral mononuclear cells and systemic clearance of fluorouracil in cancer patients. *Cancer Research*, **52**, 2899–2902.

- HAUER, M. J., THORNBURGH, B. A., FAGERNESS, P. E., WIENKERS, L. C., VRBANAC, J. J. and WICKREMA SINHA, A. J., 1997, Identification of the major urinary metabolites of bropirimine following oral administration of [^{14}C]bropirimine to healthy human volunteers. Presented at the 8th North American ISSX Meeting (Abstract 219), October, South Carolina.
- LU, Z.-H., ZHANG, R. and DIASIO, R. B., 1992, Purification and characterization of dihydropyrimidine dehydrogenase from human liver. *Journal of Biological Chemistry*, **267**, 17102–17109.
- LU, Z., ZHANG, R. and DIASIO, R. B., 1995, Population characterization of hepatic dihydropyrimidine dehydrogenase activity, a key metabolic enzyme in 5-fluorouracil chemotherapy. *Clinical Pharmacology and Therapeutics*, **58**, 512–522.
- McMURROUGH, J. and McLEOD, H. L., 1996, Analysis of the dihydropyrimidine dehydrogenase polymorphism in a British population. *British Journal of Clinical Pharmacology*, **41**, 425–427.
- MILANO, G., ETIENNE, M. C., CASSUTO-VIGUIER, E., THYSS, A., SANTINI, J., FRENAY, M., RENEE, N., SCHNEIDER, M. and DEMARD, F., 1992, Influence of sex and age on fluorouracil clearance. *Journal of Clinical Oncology*, **10**, 1171–1175.
- NAGUIB, F. N. M., KOUNI, M. H. and CHA, S., 1989, Structure-activity relationship of ligands of dihydropyrimidine dehydrogenase from mouse liver. *Biochemical Pharmacology*, **38**, 1471–1480.
- OGURA, K., NISHIYAMA, T., TAKUBO, H., KATO, A., OKUDA, H., ARAKAWA, K., FUKUSHIMA, M., NAGAYAMA, S., KAWAGUCHI, Y. and WATABE, T., 1998, Suicidal inactivation of human dihydropyrimidine dehydrogenase by (E)-5-(2-bromovinyl)uracil derived from the antiviral, sorivudine. *Cancer Letters*, **122**, 107–113.
- PHARMACEUTICAL AFFAIRS BUREAU, JAPANESE MINISTRY OF HEALTH AND WELFARE, 1994, *A Report on Investigation of Side Effects of Sorivudine: Deaths Caused by Interaction Between Sorivudine and 5-FU Prodrugs* [in Japanese].
- PORTER, D. J. T., CHESTNUT, W. G., TAYLOR, L. C. E., MERRIL, B. M. and SPECTOR, T., 1991, Inactivation of dihydropyrimidine dehydrogenase by 5-iodouracil. *Journal of Biological Chemistry*, **266**, 19988–19994.
- PORTER, D. J. T., CHESTNUT, W. G., MERRIL, B. M. and SPECTOR, T., 1992, Mechanism-based on inactivation of dihydropyrimidine dehydrogenase by 5-ethynyluracil. *Journal of Biological Chemistry*, **267**, 5236–5242.
- SKULNICK, H. I., WEED, S. D., EIDSON, E. E., RENIS, H. E., WIERENGA, W. and STRINGFIELD, D. A., 1985, Pyrimidinones. 1: 2-Amino-5-halo-6-aryl-4(3H)-pyrimidinones. Interferon-inducing antiviral agents. *Journal of Medicinal Chemistry*, **28**, 1864–1869.
- SWINBANKS, D., 1994, Death bring clinical trials under scrutiny in Japan. *Nature*, **369**, 697.
- WIERENGA, W., 1985, Antiviral and other bioactivities of pyrimidinones. *Pharmacology and Therapeutics*, **30**, 67–89.
- WOODCOCK, T. M., MARTIN, D. S., DAMIN, L. E. M., KEMENY, N. E. and YOUNG, C. W., 1979, Combination clinical trials with thymidine and fluorouracil: A phase I and clinical pharmacological evaluation. *Cancer*, **45**, 1135–1143.
- WYNALDA, M. A., HAUER, M. J. and WIENKERS, L. C., 1998, Human biotransformation of bropirimine: Characterization of the major bropirimine oxidative metabolites formed *in vitro*. *Drug Metabolism and Disposition*, **26**, 1048–1051.

Metabolism of 2-nitrofluorene, 2-aminofluorene and 2-acetylaminofluorenes in rat and dog and the role of intestinal bacteria

O. UEDA, S. KITAMURA*, R. KUBO, Y. YANO, Y. KANZAKI, T. FUJIMOTO, K. TATSUMI and S. OHTA

Institute of Pharmaceutical Science, Hiroshima University School of Medicine, Kasumi 1-2-3, Minami-ku, Hiroshima 734-8551, Japan

Received 11 October 2000

1. The *in vivo* metabolism of 2-nitrofluorene (NF), an environmental pollutant, and 2-aminofluorene (AF) and its acylated derivatives, 2-formylaminofluorene (FAF) and 2-acetylaminofluorene (AAF), was examined in rat and dog.

2. 7-Hydroxy-2-nitrofluorene, 5-hydroxy-2-nitrofluorene, AF, AAF, FAF, 7-hydroxy-2-aminofluorene, 5-hydroxy-2-aminofluorene, 7-hydroxy-2-acetylaminofluorene, 5-hydroxy-2-acetylaminofluorene, 7-hydroxy-2-formylaminofluorene and 5-hydroxy-2-formylaminofluorene were identified as urinary and faecal metabolites of NF in rat and dog.

3. AAF and its hydroxylated derivatives were detected as major metabolites of NF in rat, but FAF and its hydroxylated metabolites were mainly excreted in dog.

4. AF, AAF, FAF and their hydroxylated metabolites were also identified as urinary and faecal metabolites of AF, AAF or FAF in rat, suggesting that AAF and FAF are interconverted via AF.

5. Treatment of rat and dog with antibiotics significantly decreased the urinary and faecal excretion of AF and its derivatives after oral administration of NF, and partly decreased the excretion of acylated metabolites after an oral dose of AF.

6. The caecal contents of untreated rats and some species of intestinal bacteria exhibited nitro-reductase activity toward NF, and acylating activity toward AF, affording AAF and FAF.

Introduction

Nitropolycyclic aromatic hydrocarbons, which are carcinogenic, enter the environment from diesel engine exhaust, urban pollution sources and cigarette smoking among others (Wang *et al.* 1980, Rosenkranz and Mermelstein 1983, Hayakawa *et al.* 1995). To assess the possible risks associated with human exposure to these pollutants, it is essential to understand their metabolism. Several reports have indicated that nitropolycyclic aromatic hydrocarbons are mainly metabolized by nitro-reduction, ring hydroxylation, acylation and conjugation in mammalian species (Fu 1990). 2-Nitrofluorene (NF) is a typical carcinogenic nitropolycyclic aromatic hydrocarbon (Miller *et al.* 1955, 1962, Möller *et al.* 1989). NF has been detected in diesel exhaust particles as a major component, together with nitropyrenes (Campbell and Milton 1984). NF is a potent mutagen and forms DNA adducts in animals (Möller *et al.* 1994).

The present authors previously examined the *in vitro* metabolism of NF in mammals, focusing on nitro-reduction (Kitamura *et al.* 1983, Tatsumi *et al.* 1986). When the nitro compound was incubated with liver cytosol of the rabbit, its reduction product 2-aminofluorene (AF) was isolated from the mixture. Evidence

* Author for correspondence; e-mail: kitamura@pharm.hiroshima-u.ac.jp

was obtained that liver aldehyde oxidase catalyzed this nitro-reduction in the cytosolic fraction, and cytochrome P450 catalyzed the reduction in liver microsomal fraction of rabbit. In contrast, Törnquist *et al.* (1990) reported that rat lung microsomes exhibited oxidase activity toward NF, and that this activity was induced by β -naphthoflavone. The latter authors suggested that constitutive lung microsomal cytochrome P4502B1 catalyzed the 9-oxidation of NF, and inducible cytochrome P4501A1 catalyzed oxidation at other positions of NF.

In mammalian liver, nitro-reduction of nitro-PAH is followed by *N*-acetylation and *N*-formylation of the arylamines formed (Tatsumi *et al.* 1989). The acetylation with acetyl-CoA is catalyzed by arylamine acetyltransferase in liver cytosol. In contrast, the formylation is catalyzed by cytosolic formamidase in the presence of *N*-formyl-L-kynurenine. It was demonstrated that 1-nitropyrene is metabolized to the reduced and acylated metabolites, 1-aminopyrene, 1-acetylaminopyrene and 1-formylaminopyrene, in fish *in vivo* and *in vitro* (Kitamura and Tatsumi 1996, 1997). Also demonstrated was that NF is metabolized to AF, 2-acetylaminofluorene (AAF) and 2-formylaminofluorene (FAF) *in vivo* in rabbit (Tatsumi and Amano 1987, Tatsumi *et al.* 1989). Möller *et al.* (1987) examined the *in vivo* metabolism of NF in rat. They identified two metabolites: 7-hydroxy-2-acetylaminofluorene (7-OH-AAF) and 5-hydroxy-2-acetylaminofluorene (5-OH-AAF), and speculated that NF was reduced to AF, AF thus formed was acetylated to AAF, and AAF was further metabolized via the known AAF metabolic pathway. However, they did not detect the formylamino derivatives as metabolites of NF.

In the current study, a detailed examination of NF metabolism was conducted, and FAF and its hydroxylated derivatives were isolated and identified as novel major *in vivo* metabolites of NF, and also as metabolites of AF and AAF in rat and dog. As described above, nitro-reductase activity toward NF and acylating activity toward AF have been detected in mammalian liver preparations. However, it is not clear whether these activities in liver can exclusively account for the metabolism of the chemical in the whole animal. The metabolism of certain xenobiotics is also mediated by intestinal bacteria (Scheline 1973). In fact, 1-nitropyrene is reduced to 1-aminopyrene by pure intestinal bacteria or caecum contents from animals (Kinouchi *et al.* 1982, Howard *et al.* 1983, Narai *et al.* 1984). Therefore, also examined was the role of the intestinal bacteria in nitro-reduction and acylation of arylamines *in vivo* using the conventional and antibiotic-treated rat and dog.

Materials and methods

Chemicals

NF (98% pure), AF (>98%) and AAF (>98%) were from Tokyo Chemical Industry Co., Ltd (Tokyo, Japan). Diethylstilbestrol (DES), streptomycin sulphate and bacitracin were from Wako Pure Chemical Industries, Ltd (Osaka, Japan). Neomycin sulphate was from Sigma Chemical Co. (St Louis, MO, USA). β -Glucuronidase (100000 Fishman units ml⁻¹)/arylsulphatase (800000 Roy units ml⁻¹) from *Helix pomatia* was from Loche Diagnostics (Mannheim, Germany). FAF was prepared as described (Tatsumi and Amano 1987). 7-Hydroxy-2-nitrofluorene (7-OH-NF, 97%), 7-hydroxy-2-aminofluorene (7-OH-AF, >99%) and 7-OH-AAF (98%) were synthesized according to Bielschowsky (1945). 5-Hydroxy-2-nitrofluorene (5-OH-NF, 96%), 5-hydroxy-2-aminofluorene (5-OH-AF, 97%) and 5-OH-AAF (97%) were synthesized according to Weisburger and Weisburger (1955). 7-Hydroxy-2-formylaminofluorene (7-OH-FAF, 98%) and 5-hydroxy-2-formylaminofluorene (5-OH-FAF, 96%) were prepared from 7-OH-AF and 5-OH-AF, respectively, by formylation according to Ueda *et al.* (1996).

Animals

Male Wistar (Slc: Wistar/ST) rats, 120–150 g, and male beagle and mongrel dogs (3.6–5.7 kg) were from Japan SLC, Inc. (Shizuoka, Japan). Rats and dogs were fed standard pellet MM-3 (Funabashi Farm, Shizuoka, Japan) and commercial dog meal, Vita-One (Japanese Pet Food Co., Tokyo, Japan), respectively.

Caecal contents and bacterial strains

Caecal contents rapidly prepared from the exsanguinated conventional and antibacterial-treated rat were suspended in 2 vols GAM broth 'Nissui' (Gifu Anaerobic Medium Broth, Nissui Pharmaceutical Co., Ltd, Tokyo, Japan). After gentle centrifugation (500g for 5 min) to exclude food materials, the supernatant was used as a suspension of caecal contents. Pure strains of intestinal bacteria, *Clostridium sporogenes*, *Bifidobacterium bifidum*, *Escherichia coli*, *Salmonella typhimurium* and *Bacteroides vulgatus*, were from the Institute for Fermentation (Osaka, Japan). Facultative anaerobic bacteria were grown in *l*-broth at 37 °C and harvested by centrifugation at 10000 g for 10 min. Cells were washed twice with sterilized 0.1 M K₂Na-phosphate buffer (pH 7.4), and resuspended in the buffer. Anaerobic bacteria were grown in GAM broth using a BBL Gas Pack anaerobic jar (Becton-Dickinson, Cockeysville, MD, USA). Cells harvested by centrifugation were suspended in sterilized GAM broth. The concentration of the cell suspensions was adjusted on the basis of the absorbance at 600 nm, namely when the absorbance was 0.1, the cell concentration was estimated to be 2×10^8 cells ml⁻¹.

Treatment of rat and dog with antibiotics

Treatment of rat and dog with antibiotics was performed according to Kinouchi *et al.* (1993). A mixture of neomycin sulphate, streptomycin sulphate and bacitracin dissolved in sterilized water was given orally to rat twice daily for 3 days at a dose of 200 mg kg⁻¹ each. These antibiotics in capsules were also administered similarly to dog. In the experiments *in vivo*, the antibiotic treatment was continued for 2 further days after administration of NF or AF. The antibiotic-treated rat and dog were maintained in sterilized metabolic cages that permitted separation of urine and faeces, and allowed free access to steam-sterilized diet and drinking water. The control rat and dog were kept in standard cages with free access to diet and water.

Administration of chemicals to rat and dog

NF, AF, AAF or FAF was suspended in 0.5% carboxymethyl cellulose and given orally to antibiotic-treated or control rat at a single dose of 100 mg kg⁻¹. These chemicals induced vomiting at high dose in dog, and were given as capsules (p.o.) at a single dose of 10 mg kg⁻¹.

Liver preparations

The control and antibiotic-treated rat were exsanguinated. The livers were immediately perfused with 1.15% KCl and homogenized in 4 vols KCl solution with a Potter-Elvehjem homogenizer. Microsomal and cytosolic fractions were from the homogenate by successive centrifugation at 9000g for 20 min and 105000 g for 60 min. The microsomal fraction was resuspended in the KCl solution and recentrifuged at 105000 g.

Isolation of the metabolites of NF, AF, AAF and FAF

NF, AF, AAF and FAF were each given orally to five rats and three dogs at 100 or 10 mg kg⁻¹, respectively. The urine and faeces were collected separately in a light-shielded vessel for 2 days following administration of these compounds. The urine and faeces from the rat and dog before administration were used as controls. One hundred millilitres of the urine of rat (total 230 ml) or dog (total 550 ml) were incubated with β -glucuronidase 30 000 units/arylsulphatase 240000 units in 100 ml 0.1 M citrate-phosphate buffer (pH 6.0) at 37 °C for 16 h. The incubation mixtures were extracted twice with three volumes of ethyl acetate and the combined extracts evaporated to dryness *in vacuo*. TLC and HPLC showed that the extract contained eleven metabolites with *R_f* and retention times corresponding to those of authentic samples. These metabolites were purified by preparative TLC and HPLC after silica gel column chromatography. Silica gel column chromatography was performed as described below. The urine sample was applied to a silica gel column (Kieselgel 60, 70–230 mesh ASTM, Merck, 5 × 40 cm) equilibrated with benzene, and eluted stepwise with 100 ml each of benzene, benzene-acetone (9:1), benzene-acetone (8:2), benzene-acetone (6:4), benzene-acetone (4:6) and benzene-acetone (2:8). Each fraction was evaporated to dryness, and the residue was further purified by preparative TLC and HPLC.

The faeces of rat (40 g) and dog (210 g) were dried, pulverized in a mortar and extracted twice with 10 vols methanol by sonication and shaking for 20 min each. The combined extracts were passed through a Florisil (Wako Pure Chemical Industries Ltd, Osaka, Japan, 5 × 40 mm) column, and the metabolites were purified by preparative TLC and HPLC after silica gel column chromatography in the same manner as the metabolites in the urine of rat and dog.

Quantitative determination of the metabolites of NF, AF, AAF and FAF

After administration of NF, AF, AAF or FAF, the urine and faeces were collected separately for 48 h. Urine (1 ml) was incubated with β -glucuronidase 3000 units/arylsulphatase 24000 units in 2 ml 0.1 M citrate-phosphate buffer (pH 6.0) at 37 °C for 16 h. The faeces were dried over P_2O_5 *in vacuo* and pulverized in a mortar. An aliquot (0.5 g) of the pulverized faeces was extracted twice with 20 vols each of methanol by shaking. After centrifugation, the supernatants were combined and evaporated to dryness *in vacuo*. The precipitates were further extracted once with 3 ml citrate-phosphate buffer by shaking. The buffer extract together with β -glucuronidase 3000 units/arylsulphatase 24000 units was added to the residues from the supernatants, and incubated as described above. Each incubation mixture, after addition of 10 μ g DES, was extracted once with 5 vols benzene and the benzene extract was washed with 6 ml water by shaking. The washed benzene extract was evaporated to dryness *in vacuo*, and the residue was dissolved in a small amount of methanol and subjected to HPLC.

Metabolism of NF and AF by intestinal bacteria and liver preparations

The incubation was performed at 37 °C for 30 min under an atmosphere of nitrogen in a Thunberg tube unless otherwise stated. A typical incubation mixture consisted of 0.1 μ mol of NF or AF in 10 μ l methanol, and caecal contents (0.1 g) or bacteria (10^{10} cells) in a final volume of 2 ml 0.1 M K, Na-phosphate buffer (pH 7.4). Each incubation mixture, after addition of 10 μ g DES as an internal standard, was extracted with 5 ml ethyl acetate and the extract was evaporated to dryness *in vacuo*. The residue was dissolved in 0.1 ml methanol and analysed by HPLC. When liver microsomes or cytosol were used, 1 μ mol of a cofactor mixture was used. To detect the reducing activity of liver microsomes and cytosol, NF was incubated with liver microsomes (~2 mg protein) and NADPH, or liver cytosol (~3 mg protein) and 2-hydroxypyrimidine under anaerobic conditions. To detect the acylating activity of liver cytosol, AF was incubated with liver cytosol (~3 mg protein) and acetyl-CoA or N-formyl-L-kynurenine under aerobic conditions. After incubation, each mixture was extracted with ethyl acetate as above and analysed by HPLC. The limits of detection were 0.002, 0.004 and 0.004 nmol for AF, AAF and FAF, respectively.

Thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC)

Silica gel plates (Kieselgel 60 GF₂₅₄, 0.25 mm thick; E. Merck, Darmstadt, Germany) were developed in benzene-acetone (7:3 v/v) saturated with ammonia water. Spots were visualized under UV light (254 nm). The R_f of authentic samples were: 7-OH-AAF 0.17, 7-OH-FAF 0.20, 5-OH-AAF 0.23, 5-OH-FAF 0.26, 3-OH-AAF 0.32, AAF 0.45, FAF 0.48, 7-OH-NF 0.53, 5-OH-NF 0.56, AF 0.64 and NF 0.78.

HPLC was performed in a Hitachi L-6000 chromatograph (Tokyo, Japan) fitted with a 125 × 4 mm LiChrosorb RP-18 column (E. Merck). The mobile phase was acetonitrile- H_2O (3:7 v/v) for 25 min, acetonitrile- H_2O (linear gradient from 3:7 to 7:3 v/v) for 25 min and acetonitrile- H_2O (7:3 v/v) for 20 min. The chromatograph was operated at a flow rate of 0.6 ml min⁻¹ at a wavelength of 280 nm. The elution times of NF and its metabolites were: 7-OH-FAF 12.7, 7-OH-AAF 14.1, 7-OH-AF 15.2, 5-OH-FAF 21.2, 5-OH-AAF 22.8, 5-OH-AF 23.5, 3-OH-AAF 38.1, FAF 48.4, AAF 50.6, AF 52.9, 7-OH-NF 54.7, DES (an internal standard) 56.2, 5-OH-NF 57.2 and NF 62.3 min. The amounts of the metabolites were determined from these peak areas.

Analytical procedures

Electron impact mass spectra were taken with a Japan Electron Optics Laboratory (JEOL) JMS-SX102GCG mass spectrometer. UV spectra were recorded with a Hitachi 320 spectrophotometer.

Results

Identification of the metabolites of NF in rat

NF was orally administered to rat, the urine was extracted with ethyl acetate after treatment with β -glucuronidase/aryl-sulphatase, and the extract was subjected

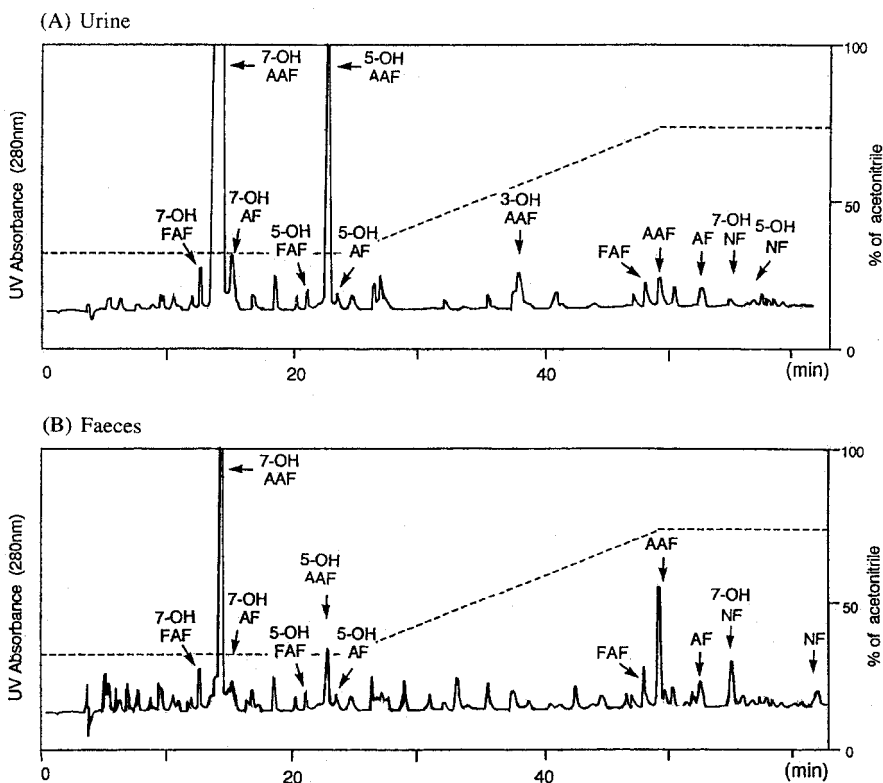


Figure 1. HPLC of the *in vivo* metabolites of 2-nitrofluorene (NF) in rat. (A) Extract of urine of rat dosed with NF and (B) extract of faeces. The absorbance (full scale = 0.016 AU) was measured at 280 nm. 7-OH-FAF, 7-hydroxy-2-formylaminofluorene; 7-OH-AAF, 7-hydroxy-2-acetylaminofluorene; 7-OH-AF, 7-hydroxy-2-aminofluorene; 5-OH-FAF, 5-hydroxy-2-formylaminofluorene; 5-OH-AAF, 5-hydroxy-2-acetylaminofluorene; 5-OH-AF, 5-hydroxy-2-aminofluorene; 3-OH-AAF, 3-hydroxy-2-acetylaminofluorene; FAF, 2-formylaminofluorene; AAF, 2-acetylaminofluorene; AF, 2-aminofluorene; 7-OH-NF, 7-hydroxy-2-nitrofluorene; 5-OH-NF, 5-hydroxy-2-nitrofluorene.

to HPLC as described in the Materials and methods. Several peaks due to NF metabolites were detected together with a peak due to unchanged NF (figure 1A). These peaks were not detected in the HPLC chromatogram of the urine extract of the control rat (data not shown). Retention times of the 12 metabolites at 12.7, 14.1, 15.2, 21.2, 22.8, 23.5, 48.4, 50.6, 52.9, 54.7, 57.2 and 62.3 corresponded to those of authentic 7-OH-FAF, 7-OH-AAF, 7-OH-AF, 5-OH-FAF, 5-OH-AAF, 5-OH-AF, FAF, AAF, AF, 7-OH-NF, 5-OH-NF and NF, respectively. These metabolites were isolated from the extract by silica gel column chromatography, TLC and HPLC as described in the Materials and methods. Mass and UV spectral data of these metabolites are shown in table 1. The mass spectra of the metabolites detected at 12.7 and 21.2 min showed the same molecular ions at m/z 225, and the same fragment ion at m/z 196 ($M^+ - \text{CHO}$), suggesting that these metabolites are hydroxylated 2-formylaminofluorenes. From the UV spectra, the metabolites detected at 12.7 and 21.2 min were identified as 7-OH-FAF and 5-OH-FAF, respectively. The mass spectra of other metabolites showed different molecular ions with characteristic fragment ions (table 1). The mass and UV spectra, the R_f in TLC

Table 1. Mass and UV spectra of the metabolites of 2-nitrofluorene in rat.

Metabolite	Mass spectral peak (<i>m/z</i>)	UV spectral peak (nm)
AF	181(M ⁺),165,152	288,314(S)
AAF	223(M ⁺),181,165	276,286,300(S),313(S)
FAF	209(M ⁺),181,165	278,288,300(S),313(S)
7-OH-AF	197(M ⁺),180	286,326(S)
5-OH-AF	197(M ⁺),180	268(S),276,302
7-OH-AAF	239(M ⁺),197,181	276(S),291,320(S)
5-OH-AAF	239(M ⁺),197,181	272(S),283,299,309(S)
3-OH-AAF	239(M ⁺),197,181	274,280,318
7-OH-FAF	225(M ⁺),196	278(S),290,315(S)
5-OH-FAF	225(M ⁺),196	278(S),283,300,309(S)
7-OH-NF	227(M ⁺),211,197,181	360
5-OH-NF	227(M ⁺),211,197,181	350

S, shoulder; AF, 2-aminofluorene; AAF, 2-acetylaminofluorene; FAF, 2-formylaminofluorene; 7-OH-AF, 7-hydroxy-2-aminofluorene; 5-OH-AF, 5-hydroxy-2-aminofluorene; 7-OH-AAF; 7-hydroxy-2-acetylaminofluorene; 5-OH-AAF, 5-hydroxy-2-acetylaminofluorene; 7-OH-FAF, 7-hydroxy-2-formylaminofluorene; 5-OH-FAF, 5-hydroxy-2-formylaminofluorene; 7-OH-NF, 7-hydroxy-2-nitrofluorene; 5-OH-NF, 5-hydroxy-2-nitrofluorene.

and the elution times in HPLC of the metabolites were identical with those of authentic samples. Also detected was another metabolite, of which the retention time in HPLC was 38.1 min, from the urine. This metabolite was suggested to be monohydroxylated AAF from the mass spectrum. The UV spectrum corresponded to that of 3-hydroxy-2-acetylaminofluorene (3-OH-AAF) (Weisburger and Weisburger 1954). Based on these facts, it is concluded that NF was metabolized to the hydroxylated metabolites of NF, 7-OH-NF and 5-OH-NF, and the reduced and acylated metabolites, AF, AAF and FAF, and their hydroxylated metabolites, 7-OH-AF, 5-OH-AF, 7-OH-AAF, 5-OH-AAF, 3-OH-AAF, 7-OH-FAF and 5-OH-FAF.

The faeces of rat dosed with NF also contained these metabolites except 3-OH-AAF (HPLC, figure 1B), which were identified by comparison with authentic samples as in the case of the urine.

Metabolism of AF, AAF and FAF in rat

The *in vivo* secondary metabolism of AF, AAF and FAF detected as metabolites of NF was examined in rat. AF, AAF, FAF and their hydroxylated metabolites were all detected in HPLC and TLC of the extracts of the urine and faeces of rat dosed with AF, AAF or FAF. These metabolites isolated by silica gel column chromatography, TLC and HPLC were identified by comparison with authentic samples, as in the case of NF metabolites in rat.

Quantitative determination of the metabolites of NF, AF, AAF and FAF in rat

The amounts of the metabolites excreted in the urine and faeces of rat dosed with NF, AF, AAF or FAF were compared. From the urine and faeces of rat dosed with NF, 7-OH-AAF was detected as a major metabolite, together with 5-OH-AAF, 3-OH-AAF and AAF, and small amounts of 7-OH-NF, 7-OH-AF and FAF (figure

2A). In the case of AF or AAF administration, 7-OH-AAF, 5-OH-AAF and AAF were detected as major metabolites, and FAF and its hydroxylated metabolites were also excreted. In the case of AF, some unchanged AF was detected. In contrast, 7-OH-FAF was excreted as a major metabolite from the urine and faeces of rat dosed FAF, together with 7-OH-AAF, 5-OH-AAF, 5-OH-FAF and FAF (figure 2C-E). These facts suggest that NF was readily reduced to AF, and the AF formed was also readily converted to its acylated metabolites, AAF and FAF, which were inter-converted, perhaps via AF, in rat *in vivo*.

Effect of antibiotic treatment of rat on NF metabolism

The relative contribution of intestinal bacteria to the reductive metabolism of NF to AF and its derivatives *in vivo* was examined in rat. Treatment with antibiotics caused a significant decrease in the urinary and faecal excretion of AF and its acylated derivatives, AAF and FAF, and their hydroxylated metabolites. In contrast, the amounts of 7-OH-NF, 5-OH-NF and NF excreted were increased by the treatment (figure 2B). These facts suggest that intestinal bacteria function mainly in the reductive metabolism of NF *in vivo*.

Metabolism of NF, AF and AAF in dog

When NF was dosed to dog, AF, AAF, FAF and their hydroxylated metabolites, except 3-OH-AAF, were also detected in the urine and faeces. In this case, 7-OH-FAF and 5-OH-FAF were major metabolites, together with 5-OH-AF, 7-OH-NF and 5-OH-NF, but 7-OH-AAF and 5-OH-AAF were excreted in only small amounts. In contrast, when NF was dosed to dog treated with antibiotics, the amounts of these metabolites excreted in the urine and faeces were markedly decreased, but 7-OH-NF was markedly increased (figure 3A,B). Next, the metabolism of AAF and AF in dog was examined. When AAF was dosed to dog, 7-OH-AAF was isolated as a major metabolite. In contrast, FAF and its hydroxylated derivatives were excreted as major metabolites of AF, as in the case of NF metabolism in dog. In this case, AF and 7-OH-AF were also detected as major metabolites (figure 3C,D). These facts suggest that NF was also mainly reduced by intestinal bacteria, and the AF formed was mainly acylated to FAF in dog.

Effect of antibiotic treatment of rat and dog on AF metabolism

Furthermore, the relative contribution of intestinal bacteria to the acylation of AF to AAF and FAF *in vivo* was examined in rat and dog. Treatment of rat with antibiotics caused a decrease in the urinary excretion of AAF and FAF, and these hydroxylated metabolites to some extents. The urinary excretion of 7-OH-AAF and 5-OH-AAF was notably decreased after the antibiotic treatment. However, total amounts of these metabolites excreted in urine and faeces changed little, with the exception of an decrease in excretion of 7-OH-AAF from 18 to 13% by antibiotic treatment (figure 4).

When AF was orally administered to antibiotic-treated dog, the amounts of AAF

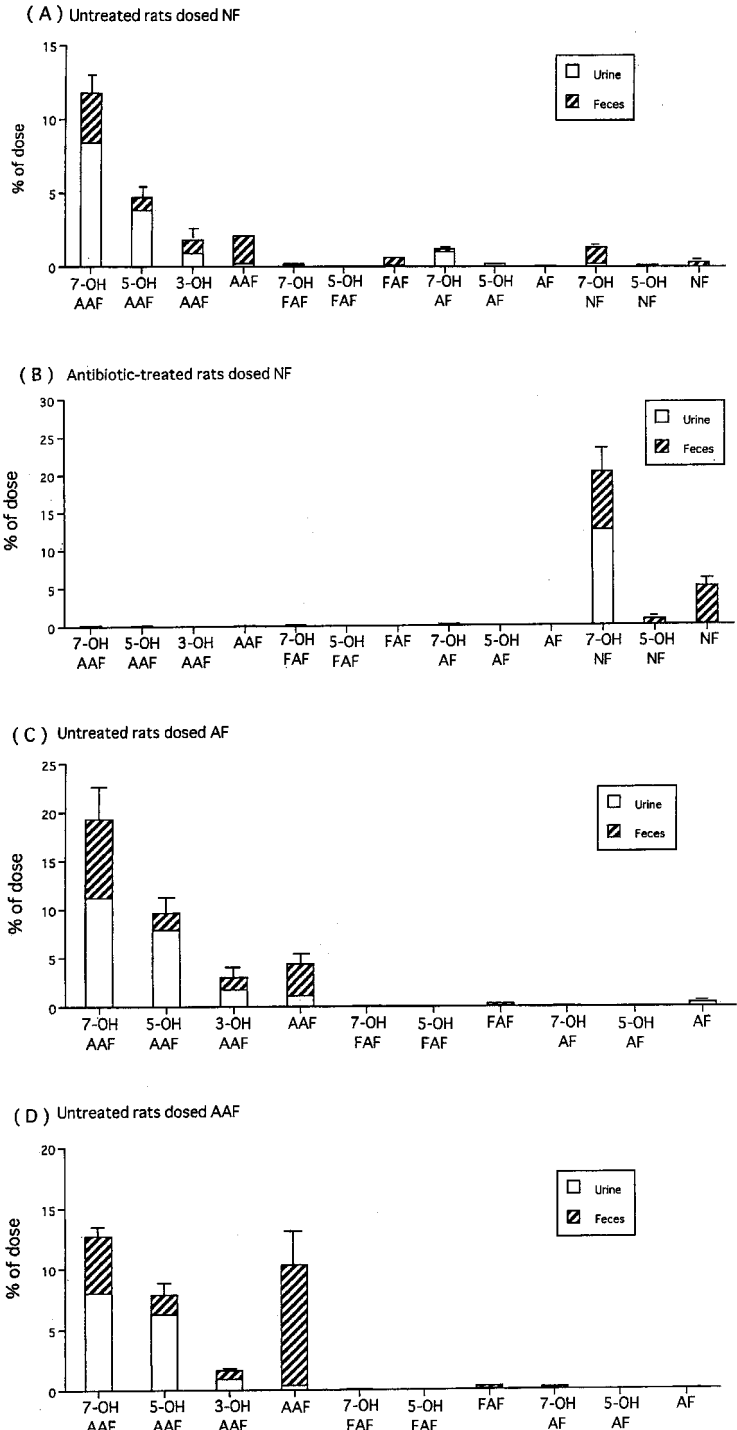


Figure 2. For legend see opposite.

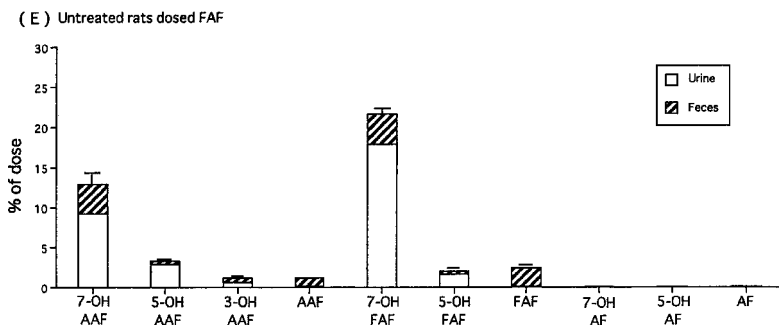


Figure 2. Urinary and faecal excretion of 2-nitrofluorene (NF), 2-aminofluorene (AF), 2-acetylaminofluorene (AAF), 2-formylaminofluorene (FAF) and their hydroxylated derivatives by untreated rats given NF, AF, AAF or FAF, and by antibiotic-treated rats given NF. (A) Excretion (% of dose) of metabolites of NF in untreated rats, (B) excretion (% of dose) of metabolites of NF in antibiotic-treated rats, (C) excretion (% of dose) of metabolites of AF in untreated rats, (D) excretion (% of dose) of metabolites of AAF in untreated rats and (E) excretion (% of dose) of metabolites of FAF in untreated rats. Each bar represents the mean $\pm$ SD of three experiments. The metabolites were extracted from the urine and faeces of rats dosed with NF, AF, AAF or FAF as described in the Materials and methods. The amounts of metabolites excreted were determined by HPLC. 7-OH-AAF, 7-hydroxy-2-acetylaminofluorene; 5-OH-AAF, 5-hydroxy-2-acetylaminofluorene; 3-OH-AAF, 3-hydroxy-2-acetylaminofluorene; 7-OH-FAF, 7-hydroxy-2-formylaminofluorene; 5-OH-FAF, 5-hydroxy-2-formylaminofluorene; 7-OH-AF, 7-hydroxy-2-aminofluorene; 5-OH-AF, 5-hydroxy-2-aminofluorene; 7-OH-NF, 7-hydroxy-2-nitrofluorene; 5-OH-NF, 5-hydroxy-2-nitrofluorene.

and FAF excreted decreased from 1.7 to 0.1% and from 2.6 to 1.4%, respectively, compared with those in the untreated dog. These facts suggest that intestinal bacteria also catalyse the acetylation and formylation of AF in rat and dog.

Metabolism of NF and AF by rat caecal contents and liver preparations

When NF was incubated with the caecal contents of untreated rat under anaerobic conditions, AF and its acylated metabolites, FAF and AAF, were detected by HPLC in the extract of the incubation mixture. Furthermore, FAF and AAF were also detected in the mixture after AF was incubated with rat caecum contents (figure 5). However, little or no nitro-reducing or acylating activity was observed with the caecal contents of antibiotic-treated rat. Furthermore, the influence of antibiotic treatment on hepatic nitro-reductase activity toward NF and acylating activity toward AF was examined. Little difference between the control and antibiotic-treated rat was detected in the liver microsomal NF reductase activity with NADPH or cytosolic activity with 2-hydroxypyrimidine, an electron donor for aldehyde oxidase. No significant difference between untreated and antibiotic-treated rat in formylating or acetylating activity of liver cytosol toward AF in the presence of *N*-formyl-L-kynurenine or acetyl-CoA was observed (table 2).

Metabolism of NF and AF by pure strains of intestinal bacteria

Five pure strains of intestinal bacteria were also tested for reducing ability toward NF and acylating ability toward AF under anaerobic conditions. Very high reducing activity toward NF was observed in *Clostridium sporogenes*, followed by

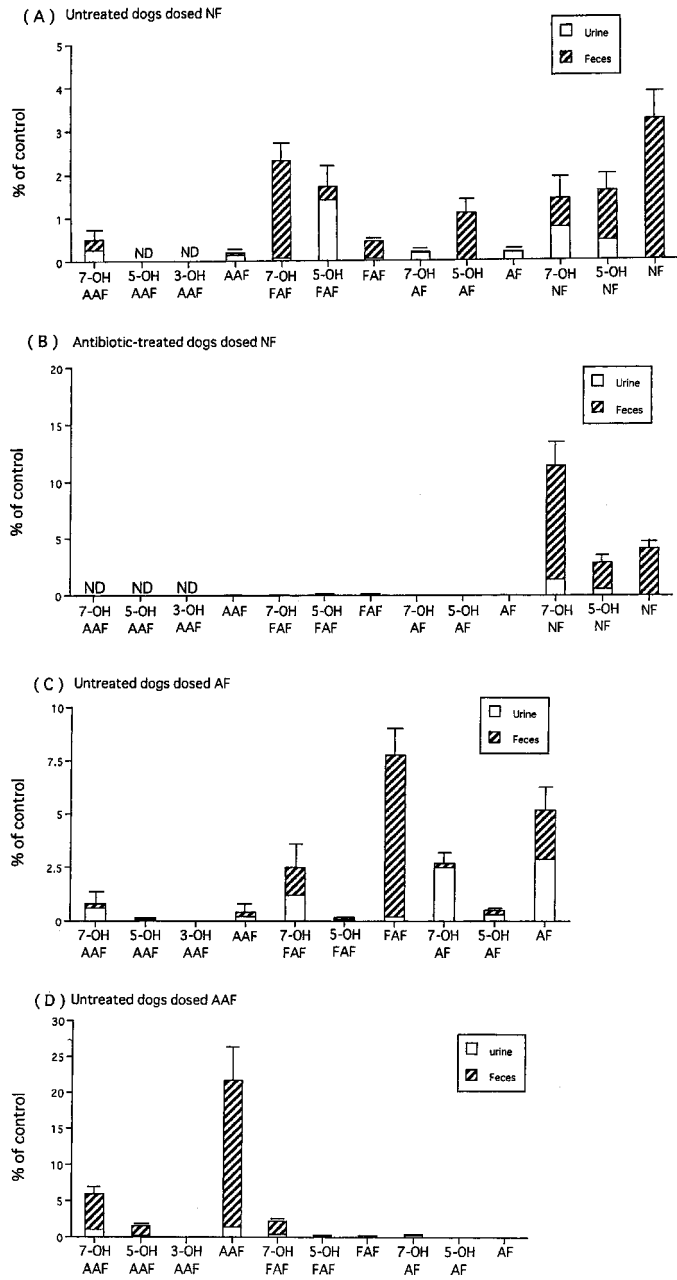
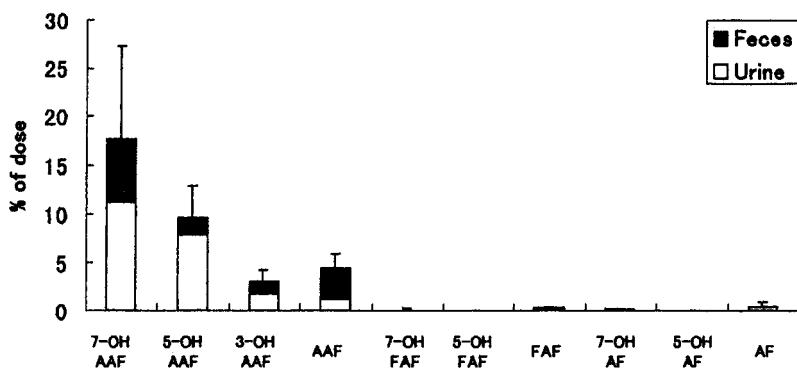


Figure 3. Urinary and faecal excretion of 2-nitrofluorene (NF), 2-aminofluorene (AF), 2-acetylaminofluorene (AAF), 2-formylaminofluorene (FAF) and their hydroxylated derivatives by untreated dogs given NF, AF or AAF, and antibiotic-treated dogs given NF. (A) Excretion (% of dose) of metabolites of NF in untreated dogs, (B) excretion (% of dose) of metabolites of NF in antibiotic-treated dogs, (C) excretion (% of dose) of metabolites of AF in untreated dogs and (D) excretion (% of dose) of metabolites of AAF in untreated dogs. Each bar represents the mean $\pm$ SD of three experiments. SD was calculated from the total amounts of the metabolites excreted in the urine and faeces. The metabolites were extracted from the urine and faeces of dogs dosed with NF, AF or AAF as described in the Materials and methods. The amounts of metabolites excreted were determined by HPLC. 7-OH-AAF, 7-hydroxy-2-acetylaminofluorene; 5-OH-AAF, 5-hydroxy-

(A) Untreated



(B) Antibiotic-treated

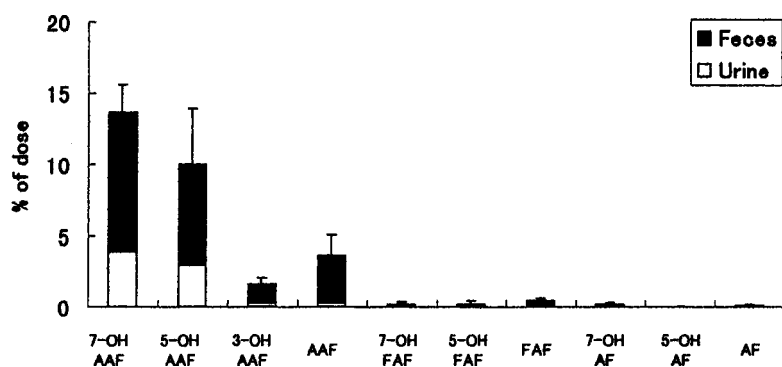


Figure 4. Urinary and faecal excretion of 2-aminofluorene (AF), 2-acetylaminofluorene (AAF), 2-formylaminofluorene (FAF) and their hydroxylated derivatives by untreated and antibiotic-treated rats given AF. (A) Excretion (% of dose) of metabolites of AF in untreated rats and (B) excretion (% of dose) of metabolites of AF in antibiotic-treated rats. Each bar represents the mean $\pm$ SD of three experiments. The metabolites were extracted from the urine and faeces of rats dosed with AF as described in the Materials and methods. The amounts of metabolites excreted were determined by HPLC. 7-OH-AAF, 7-hydroxy-2-acetylaminofluorene; 5-OH-AAF, 5-hydroxy-2-acetylaminofluorene; 3-OH-AAF, 3-hydroxy-2-acetylaminofluorene; 7-OH-FAF, 7-hydroxy-2-formylaminofluorene; 5-OH-FAF, 5-hydroxy-2-formylaminofluorene; 7-OH-AF, 7-hydroxy-2-aminofluorene; 5-OH-AF, 5-hydroxy-2-aminofluorene.

Bacteroides vulgatus. Some bacterial strains also exhibited acylating activity toward AF. Relatively high activity was observed in *Salmonella typhimurium* and *Clostridium sporogenes*, followed by *Bacteroides vulgatus* and *Escherichia coli*. In these strains, the acetylating activity was somewhat higher than the formylating activity (table 3).

Based on these results, we suggest that intestinal bacteria play a major role in the reductive metabolism of NF to AF, and also partly in the acylation of AF to AAF and FAF in animals.

2-acetylaminofluorene; 7-OH-FAF, 7-hydroxy-2-formylaminofluorene; 5-OH-FAF, 5-hydroxy-2-formylaminofluorene; 7-OH-AF, 7-hydroxy-2-aminofluorene; 5-OH-AF, 5-hydroxy-2-amino-fluorene; 7-OH-NF, 7-hydroxy-2-nitrofluorene; 5-OH-NF, 5-hydroxy-2-nitrofluorene.

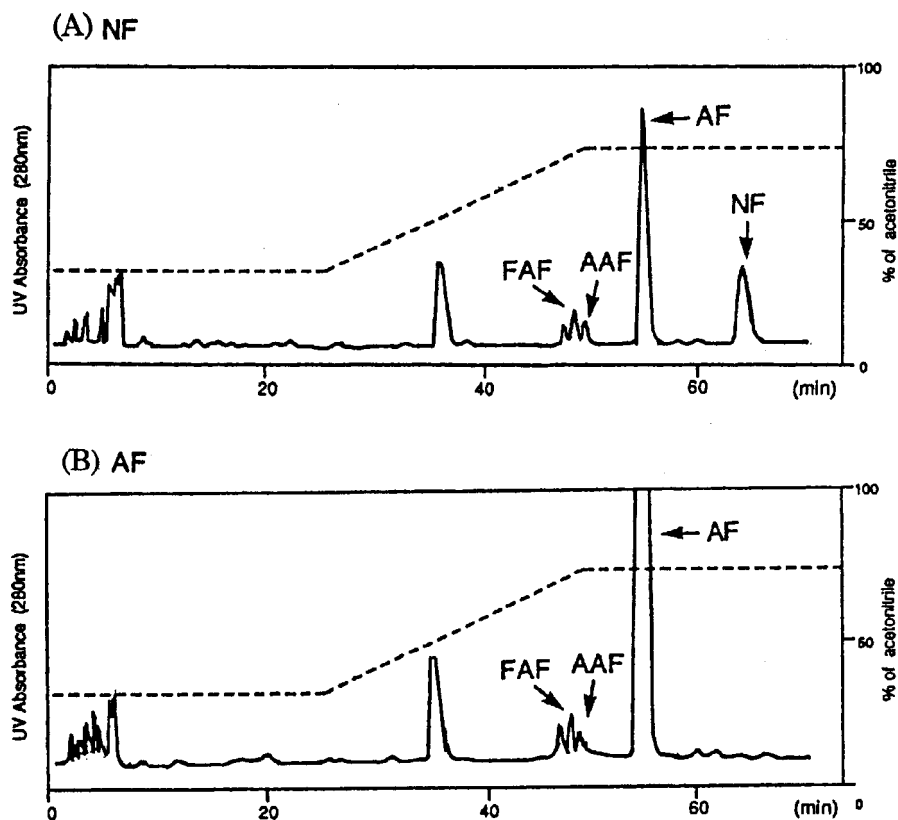


Figure 5. HPLC of the metabolites of 2-nitrofluorene (NF) or 2-aminofluorene (AF) produced by rat caecum contents. (A) Extract of the incubation mixture of NF with rat caecum contents and (B) extract of the incubation mixture of AF with caecum contents. The absorbance (full scale = 0.016 AU) was measured at 280 nm. AAF, 2-acetylaminofluorene; FAF, 2-formylaminofluorene.

Discussion

In the present study, it has been demonstrated that NF is predominantly metabolized to AF and its acylated metabolites in rat and dog. It was proposed that NF and 1-nitropyrene are metabolized reductively by aldehyde oxidase and cytochrome P450 to the corresponding amino compounds *in vitro* (Saito *et al.* 1984, Tatsumi *et al.* 1986). Recently, it was reported that zonisamide, an anticonvulsant agent, was reduced to 2-sulphamylacetylphenol, via the ring-fused intermediate, by aldehyde oxidase *in vitro* (Sugihara *et al.* 1996). However, when zonisamide was administered to antibiotic-treated rat, the amount of the reduced metabolite excreted was markedly decreased compared with that in the case of untreated rat (Kitamura *et al.* 1997). It is therefore suggested that the reductive metabolism of zonisamide mainly involved intestinal bacteria, but not aldehyde oxidase or the cytochrome P450 system. The present findings also suggest that intestinal bacteria play a predominant role in the reduction of NF *in vivo*. Möller *et al.* (1988) also suggested the role of intestinal bacteria in the NF metabolism from the experiments using germ-free rat. Intestinal bacteria are known to participate in the *in vivo* reduction of the azo linkage of prontosil and salicylazosulphapyridine (Gingell *et al.* 1971, Peppercorn and Goldman 1972), *N*-hydroxy-2-acetylaminofluorene (Weisburger *et*

Table 2. Reduction of 2-nitrofluorene and acylation of 2-aminofluorene by the caecum contents and liver preparations of the untreated and antibiotic-treated rat.

Preparation	Untreated		Antibiotic-treated			
	NF reducing activity ¹		AF acylating activity ²		NF reducing activity ¹	
	AF formed	(nmol/h/g contents)	AAF formed	FAF formed	AF formed	AAF formed
Caecum contents	1569±245	(pmol/h/g contents)	506±160	519±118	(nmol/h/g contents)	(pmol/h/g contents)
Liver cytosol	180±22	(pmol/min/mg protein)	21±5	81±11	187±14	25±17
Liver microsomes	242±38		nd	nd	234±26	19±7
						85±17
						nd

Each value represents the mean ± SD of three experiments. ND, not detected.
¹ 2-Nitrofluorene (NF) was incubated with rat caecum contents or liver preparations under anaerobic conditions. 2-Aminofluorene (AF) formed was determined by HPLC.
² AF was incubated with rat caecum contents or liver preparations. 2-Acetylaminofluorene (AAF) and 2-formylaminofluorene (FAF) formed were determined by HPLC. Other details were described in the Materials and methods.

The difference is thought to be due to the fact that arylamine acetyltransferase is deficient in dog tissues. In the current study, 3-OH-AAF was tentatively identified from the spectral data of the metabolite isolated from the urine of rat dosed with NF. Möller *et al.* (1987) also isolated 3-OH-AAF from the urine of rat dosed with NF, and proposed a formation pathway via *O*-acetyl *N*-hydroxyl-2-acetylaminofluorene. In the present preliminary study using ^{14}C -AAF in rat, the main excretion route of the radioactivity was urine. After the treatment of the urine with β -glucuronidase/arylsulphatase, the majority of the radioactivity was extracted with ethyl acetate, as compared with only 8% of the radioactivity before the treatment. This fact suggests that the hydroxylated metabolites of AF, AAF or FAF are excreted largely as glucuronic or sulphuric acid conjugates.

The present authors previously reported that various type of aryl amines were acylated to the acetyl amino and formyl amino derivatives in rabbit and fish by arylamine acetyltransferase and formamidase, respectively (Tatsumi *et al.* 1989, Kitamura and Tatsumi 1996). Furthermore, formyl amino derivatives were hydrolyzed *in vivo* (Ueda *et al.* 1996, Kitamura and Tatsumi 1997). In the current study, it was demonstrated that AF is acylated to acetyl amino and formyl amino derivatives by intestinal bacteria. The amounts of AAF and its hydroxylated metabolites in control rat and dog were decreased when AF was administered to antibiotic-treated rat and dog. Intestinal bacteria may therefore play a role in the acylation of AF to AAF and FAF *in vivo* in animals. It is remarkable that *N*-acylation of arylamines is mediated by intestinal bacteria as there are a few reports concerning the acylation of arylamines by intestinal bacteria. Dull *et al.* (1987) reported that when sulphasalazine was incubated with guinea pig caecal contents, the *N*-acetyl derivative was detected as an acylated metabolite. In our preliminary study, acylated metabolites of AF were excreted in germ-free rat dosed with AF. However, the amounts of the metabolites were about half of those in conventional rat. It was also previously demonstrated that *N*-acetylated metabolites were excreted as metabolites of arylamines in dog in spite of the absence of arylamine acetyltransferase in dog (Okumura *et al.* 1995). It is suggested that intestinal bacteria in dog were responsible for the acylation. In this study, AAF and its hydroxylated metabolites were detected as metabolites of NF and AF in dog. The effect of antibiotic treatment on the excretion of the acylated metabolites in dog dosed with AF, supported the idea that in dog the acetylation of arylamines is mainly catalyzed by intestinal bacteria.

It was demonstrated that bacterial drug-reducing enzyme systems can reduce 1-nitropyrene and nitrofurazone. The cell-free extract from *Escherichia coli*, containing NAD(P)H and FMN-dependent reductase, exhibited nitro-reductase activity (Narai *et al.* 1984). Recently, we also showed that zonisamide, (α -bromoisovaleryl)urea, α -, β -unsaturated ketones, styrene oxide and stilbene oxide are reduced by an NAD(P)H and flavins-dependent enzyme system in the cell-free extract of *Clostridium sporogenes* (Ishida *et al.* 1996, Kitamura *et al.* 1997, 1999a, 2000). The reducing enzyme system in bacteria responsible for the reduction of NF is assumed to be the same as the 1-nitropyrene reducing enzyme system (Narai *et al.* 1984). However, the acylating enzyme system has not been examined. In a preliminary experiment on the acylating enzyme in bacteria, we found that the acetylating activity of the cell-free extract of intestinal bacteria toward AF was independent of acetyl CoA, which is the acetyl donor of mammalian arylamine acetyltransferase. Formylation of AF was not enhanced by the addition of *N*-formyl-L-kynurenine, a formyl donor of mammalian formamidase. Further studies

on the bacterial enzyme system responsible for the acylation of arylamines are underway in this laboratory.

References

- BIELSCHOWSKY, F., 1945, A metabolite of 2-acetamidofluorene. *Biochemical Journal*, **39**, 287–289.
- CAMPBELL, R. M. and MILTON, L. L., 1984, Capillary column gas chromatographic determination of nitro polycyclic aromatic compounds in particulate extracts. *Analytical Chemistry*, **56**, 1026–1030.
- DULL, B. J., SALATA, K. and GOLDMAN, P., 1987, Role of the intestinal flora in the acetylation of sulphasalazine metabolites. *Biochemical Pharmacology*, **36**, 3772–3774.
- FU, P. P., 1990, Metabolism of nitro-polycyclic aromatic hydrocarbons. *Drug Metabolism Reviews*, **22**, 209–268.
- GARDNER, D. M. and RENWICK, A. G., 1978, The reduction of nitrobenzoic acids in the rat. *Xenobiotica*, **8**, 679–690.
- GINGELL, R., BRIDGES, J. W. and WILLIAMS, R. T., 1971, The role of the gut flora in the metabolism of prontosil and neoprontosil in the rat. *Xenobiotica*, **1**, 143–156.
- GOLDIN, B. R., 1990, Intestinal microflora: Metabolism of drugs and carcinogens. *Annals of Medicine*, **22**, 43–48.
- HAYAKAWA, K., MURAHASHI, T., BUTOH M., MIYAZAKI, M., 1995, Determination of 1,3-, 1,6-, and 1,8-dinitropyrenes and 1-nitropyrene in urban air by high-performance liquid chromatography using chemiluminescence detection. *Environmental Science and Technology*, **29**, 928–932.
- HOWARD, P. C., BELAND, F. A. and CERNIGLIA, C. E., 1983, Reduction of the carcinogen 1-nitropyrene to 1-aminopyrene by rat intestinal bacteria. *Carcinogenesis*, **4**, 985–990.
- ISHIDA, M., KITAMURA, S. and TATSUMI, K., 1996, Purification of an α,β -ketoalkene double bond reductase from *Salmonella typhimurium*. *Biochemistry and Molecular Biology International*, **39**, 1157–1165.
- KINOCHI, T., KATAOKA, K., MIYANISHI, K., AKIMOTO, S. and OHNISHI, Y., 1993, Biological activities of the intestinal microflora in mice treated with antibiotics or untreated and the effects of the microflora on absorption and metabolic activation of orally administered glutathione conjugates of K-region epoxides of 1-nitropyrene. *Carcinogenesis*, **14**, 869–874.
- KINOCHI, T., MANABE, Y., WAKISAKA, K. and OHNISHI, Y., 1982, Biotransformation of 1-nitropyrene in intestinal anaerobic bacteria. *Microbiology and Immunology*, **26**, 993–1005.
- KITAMURA, S., KUWASAKO, M., OHTA, S. and TATSUMI, K., 1999a, Reductive debromination of α -(bromoisovaleryl)urea by intestinal bacteria. *Journal of Pharmacy and Pharmacology*, **51**, 79–84.
- KITAMURA, S., MITA, M., MATSUDA, K., OHTA, S. and TATSUMI, K., 2000, Reduction of stilbene oxide and styrene oxide to the corresponding alkenes by intestinal bacteria. *Xenobiotica*, **30**, 359–369.
- KITAMURA, S., MITA, M., SHIMIZU, Y., SUGIHARA, K. and OHTA, S., 1999b, Conversion of dieldrin to aldrin by intestinal bacteria in rats. *Biological and Pharmaceutical Bulletin*, **22**, 880–882.
- KITAMURA, S., NARAI, N., HAYASHI, M. and TATSUMI, K., 1983, Rabbit liver enzymes responsible for reduction of nitropolycyclic aromatic hydrocarbons. *Chemical and Pharmaceutical Bulletin*, **31**, 776–779.
- KITAMURA, S., SUGIHARA, K., KUWASAKO, M. and TATSUMI, K., 1997, The role of mammalian intestinal bacteria in the reductive metabolism of zonisamide. *Journal of Pharmacy and Pharmacology*, **49**, 253–256.
- KITAMURA, S. and TATSUMI, K., 1996, Metabolism *in vivo* of 1-nitropyrene, an environmental pollutant, in fish. *Biological and Pharmaceutical Bulletin*, **19**, 1524–1526.
- KITAMURA, S. and TATSUMI, K., 1997, 1-Nitropyrene-metabolizing activities of fish liver preparations. *Bulletin of Environmental Contamination and Toxicology*, **58**, 448–455.
- LEVIN, A. A. and DENT, J. G., 1982, Comparison of the metabolism of nitrobenzene by hepatic microsomes and cecal microflora from Fischer-344 rats *in vitro* and the relative importance of each *in vivo*. *Drug Metabolism and Disposition*, **10**, 450–454.
- MILLER, E. C., FLETCHER, T. L., MARGRETH, A. and MILLER, J. A., 1962, The carcinogenicity of derivatives of fluorene and biphenyl: fluoro derivatives as probe for active sites in 2-acetylaminofluorene. *Cancer Research*, **22**, 1002–1014.
- MILLER, J. A., SANDIN, R. B., MILLER, E. C. and RUSCH, H. P., 1955, The carcinogenicity of compounds related to 2-acetylaminofluorene. II. Variations in the bridges and the 2-substituent. *Cancer Research*, **15**, 188–199.
- MÖLLER, L., CORRIE, M., MIDTVEDT, T., RAFTER, J. and GUSTAFSSON, J.-Å., 1988, The role of the intestinal microflora in the formation of mutagenic metabolites from the carcinogenic air pollutant 2-nitrofluorene. *Carcinogenesis*, **9**, 823–830.
- MÖLLER, L., RAFTER, J. and GUSTAFSSON, J.-Å., 1987, Metabolism of the carcinogenic air pollutant 2-nitrofluorene in the rat. *Carcinogenesis*, **8**, 637–645.
- MÖLLER, L., TORNDAL, U.-B., ERIKSSON, L. C. and GUSTAFSSON, J.-Å., 1989, The air pollutant 2-

- nitrofluorene as initiator and promoter in a liver model for chemical carcinogenesis. *Carcinogenesis*, **10**, 435–440.
- MÖLLER, L., ZEISIG, M., MIDTVEDT, T. and GUSTAFSSON, J.-Å., 1994, Intestinal microflora enhances formation of DNA adducts following administration of 2-NF and 2-AAF. *Carcinogenesis*, **15**, 857–861.
- NARAI, N., KITAMURA, S. and TATSUMI, K., 1984, A comparative study on 1-nitropyrene and nitrofurazone reductases in *Escherichia coli*. *Journal of Pharmacobio-Dynamics*, **7**, 407–413.
- OKUMURA, F., UEDA, O., KITAMURA, S. and TATSUMI, K., 1995, *N*-acetylation and *N*-formylation of carcinogenic arylamines and related compounds in dogs. *Carcinogenesis*, **16**, 71–76.
- PEPPERCORN, M. A. and GOLDMAN, P., 1972, The role of intestinal bacteria in the metabolism of salicylazosulfapyridine. *Journal of Pharmacology and Experimental Therapeutics*, **181**, 555–562.
- RENWICK, A. G., EVANS, S. P., SWEATMAN, T. W., CUMBERLAND, J. and GEORGE, C. F., 1982, The role of the gut flora in the reduction of sulphinpyrazone in the rat. *Biochemical Pharmacology*, **31**, 2649–2656.
- ROSENKRANZ, H. S. and MERMELSTEIN, R., 1983, Mutagenicity and genotoxicity of nitroarenes, All nitro-containing chemicals were not created equal. *Mutation Research*, **114**, 217–267.
- SAITO, K., KAMATAKI, T. and KATO, R., 1984, Participation of cytochrome P-450 in reductive metabolism of 1-nitropyrene by rat liver microsomes. *Cancer Research*, **44**, 3169–3173.
- SCHELINE, R. R., 1973, Metabolism of foreign compounds by gastrointestinal microorganisms. *Pharmacological Reviews*, **25**, 451–523.
- SUGIHARA, K., KITAMURA, S. and TATSUMI, K., 1996, Involvement of mammalian liver cytosols and aldehyde oxidase in reductive metabolism of zonisamide. *Drug Metabolism and Disposition*, **24**, 199–202.
- TATSUMI, K. and AMANO, H., 1987, Biotransformation of 1-nitropyrene and 2-nitrofluorene to novel metabolites, the corresponding formylamino compounds, in animal bodies. *Biochemical and Biophysical Research Communications*, **142**, 376–382.
- TATSUMI, K., KITAMURA, S. and NARAI, N., 1986, Reductive metabolism of aromatic nitro compounds including carcinogens by rabbit liver preparations. *Cancer Research*, **46**, 1089–1093.
- TATSUMI, K., KITAMURA, S., AMANO, H. and UEDA, K., 1989, Comparative study on metabolic formation of *N*-arylformamides and *N*-arylacetamides from carcinogenic arylamines in mammalian species. *Cancer Research*, **49**, 2059–2064.
- TÖRNQUIST, S., MÖLLER, L., GABRIELSSON, J., GUSTAFSSON, J.-Å. and TOFTGÅRD, R., 1990, 2-Nitrofluorene metabolism in the rat lung. Pharmacokinetic and metabolic effects of b-naphthoflavone treatment. *Carcinogenesis*, **11**, 1249–1254.
- UEDA, O., KITAMURA, S. and TATSUMI, K., 1996, Further metabolism of 2-formylaminofluorene, a metabolite of 2-aminofluorene, in rats. *Biological and Pharmaceutical Bulletin*, **19**, 330–333.
- WANG, C. Y., LEE, M. S., KING, C. M., WARNER, P. O., 1980, Evidence for nitroaromatics as direct-acting mutagens of airborne particulates. *Chemosphere*, **9**, 83–87.
- WEISBURGER, J. H., GRANTHAM, P. H., HORTON, R. E. and WEISBURGER, E. K., 1970, Metabolism of the carcinogen *N*-hydroxy-*N*-2-fluorenylacetamide in germ-free rats. *Biochemical Pharmacology*, **19**, 151–162.
- WEISBURGER, E. K. and WEISBURGER, J. H., 1954, *ortho*-Hydroxy derivatives of the carcinogen 2-acetylaminofluorene. *Journal of Organic Chemistry*, **19**, 964–972.
- WEISBURGER, E. K. and WEISBURGER, J. H., 1955, 2-Acetylamino-5-hydroxyfluorene, a metabolite of the carcinogen 2-acetylaminofluorene. *Journal of Organic Chemistry*, **20**, 1396–1401.
- ZACHARIAH, P. K. and JUCHAU, M. R., 1974, The role of gut flora in the reduction of aromatic nitro-groups. *Drug Metabolism and Disposition*, **2**, 74–78.

Pharmacokinetics and system linearity of tea catechins in rat

M. ZHU†, Y. CHEN† and R. C. LI†‡*

†Department of Pharmacy, The Chinese University of Hong Kong, Shatin, N.T., Hong Kong

‡Pharmacokinetics/Pharmacodynamics, Bioanalytical & Drug Disposition, Wyeth-Ayerst Research, Pearl River, 10965 NY, USA

Received 4 September 2000

1. The pharmacokinetics and dose proportionality of three tea catechins were assessed.
2. Male Sprague-Dawley rats (210–230 g) received intravenous (i.v.) doses (50, 100, 200 and 300 mg kg⁻¹) of a decaffeinated tea fraction containing (-)-epicatechin (EC: 5%), (-)-epigallocatechin gallate (EGCG: 50%) and (-)-epicatechin gallate (ECG: 13%).
3. Catechins in plasma, urine and faeces were quantitated by HPLC.
4. A two-compartment model was utilized to describe the bi-exponential disposition exhibited by the three catechins.
5. Over this dose range, the central distribution volume (V_c) for these catechins increased significantly ($p < 0.05$) from 0.17–0.34 to 0.42–0.66 l kg⁻¹.
6. A concentration-dependent increase in the plasma free fraction of catechins that mirrored the increases in V_c was observed.
7. The estimates of steady-state volume of distribution (V_{ss}) were between 0.68 and 2.08 l kg⁻¹ at the lowest dose, and tended to increase with dose.
8. Appreciable amount of catechins partitioned into red blood cells (range 9–43%) and was apparently independent of concentration.
9. The mean elimination half-life ($t_{1/2,\beta}$) for EC, EGCG and ECG across doses were 43, 124, and 222 min respectively, and were invariant with dose.
10. Parallel to V_c , a 2–3-fold dose-dependent increase ($p < 0.05$) in systemic clearance (CL) was observed for the three catechins.
11. Urinary recovery was highest (21–31%) for EC, while those for EGCG and ECG was only ~3–5%.
12. Faecal recoveries of the catechins were between 0.5 and 5%.
13. In conclusion, the pharmacokinetics of the catechins appeared to be non-linear; dose-dependent changes in xenobiotic distribution might contribute to this observation.

Introduction

Many studies have been conducted world-wide on tea, a widely consumed beverage, to evaluate its nutritional or pharmaceutical values. Data collected in these studies suggest that green tea may have preventive effect against liver injury, dental caries, various cancers, arteriosclerosis and inflammations induced by lipid peroxidation (Weisburger 1999). The underlying mechanisms possibly involve its powerful anti-oxidative properties, its ability to inhibit cell replication and growth of neoplasm, and its modulating effects on intestinal microflora (Haslam 1996, Zhang *et al.* 1997, Steele *et al.* 1999, Weisburger 1999).

Chemical investigations on the composition of the leaves of *Camellia sinensis* revealed the presence of polyphenols (36%), carbohydrates (25%), proteins (15%),

* Author for correspondence; e-mail: rcli@war.wyeth.com

lignin (6.5%), amino acids (4%), methyl xanthines (3.5%), lipids (2%), organic acids (1.5%), chlorophyll (0.5), carotenoids (< 0.1%) and volatile oils (< 0.1%), etc. (Yamamoto 1997). It is generally believed that catechin derivatives including (-)-epicatechin (EC), (-)-epigallocatechin (EGC), (-)-epicatechin gallate (ECG) and (-)-epigallocatechin gallate (EGCG) in the polyphenol fraction are bioactive (Yamamoto 1997). As with the content of individual tea catechins, EGCG in various tea preparations is the highest (Zhang *et al.* 1991, 1997).

To date, however, most pharmacological studies carried out on these tea catechins have been *in vitro* in nature, and *in vivo* data pertaining to the pharmacokinetics (PK) of tea components are not extensive (Chen *et al.* 1997, van het Hof *et al.* 1998, Yang *et al.* 1998, 1999). To assess fully the nutritional and pharmacological values of tea, it is informative to define the pharmacokinetics of these catechins. As the amount and frequency of tea intake varies substantially among individuals, proportionality of the pharmacokinetic system (dose-dependency) is an important determinant, not only for the total systemic exposure of the catechins as a function of dose, but also its relationship to the intended pharmacological effects. The primary objectives of this study are to characterize the pharmacokinetics of three major tea catechins and linearity of the pharmacokinetic systems when they were administered intravenously (*i.v.*) in the form of a catechin fraction over a 6-fold dose range. Additional studies on plasma protein binding and red blood cell partitioning of the catechins were also conducted to maximize data interpretation.

Materials and methods

Preparation of tea catechins

Lung-Jing, a variety of tea cultivated in southern China, was purchased commercially in Shanghai. To prepare a decaffeinated catechin fraction for the pharmacokinetic studies, green tea leaves (250 g) were extracted with 2 litres of boiling de-ionized and distilled water for 30 min. The resultant solution was then partitioned with ethyl acetate for three times (0.7 litres each) to separate the catechins from other water-soluble components. The combined ethyl acetate fraction was concentrated under vacuum at 50 °C and the resultant extract (30 g) was subjected to the removal of caffeine, xanthine derivatives and water-soluble chlorophyll using silica-gel column chromatography. The column was initially eluted with a mixture of chloroform-methanol (20:1), decaffeinated catechins were obtained by eluting with 100% methanol. The yield of a tea-catechin fraction (18 g) was accounted for 60% of the ethyl acetate fraction and 7.2% of the dry tea-leaves.

Separation and structural identification of EGCG, ECG and EC, the major ingredients of the catechin-fraction, followed the approach described by Seto *et al.*, and authentic compounds purchased from Sigma (St Louis, MO, USA) were employed as references (purity > 95–98%). Retention times and UV spectra of these compounds were also examined and compared to the reference standards. The respective content of EC, ECG and EGCG in this preparation was quantitatively determined to be 5, 13 and 50% of the fraction, respectively, using HPLC. Aliquots of this standardized catechin-fraction from a single batch were employed for the PK study which were kept at –80 °C to minimize oxidation. The content of each catechin was verified each time before use, and no chemical decomposition was observed for at least 1 year. Chemical structures of the three catechins are shown in figure 1.

HPLC analysis of tea catechins

An HPLC system (Hewlett Packard series 1050), consisting of an UV-detector, an autosampler, a reversed-phase ODS column (4.6 mm i.d. X 250 mm; particle size: 5 µm, Beckman) and a guard column (Novapak C₁₈, Waters), was employed for the simultaneous quantitation of the three catechins in biofluids. Pure EC, ECG and EGCG standards were utilized as references in the analysis. Acetonitrile (HPLC grade, Mallinckrodt-Baker, Inc., USA) and other chemical reagents were acquired commercially.

The HPLC assay developed by Lee *et al.* (1995) was employed with slight modifications. A gradient system (pH 2.4) consisting of two eluting solutions of A (5% acetonitrile in 0.4 M NaH₂PO₄) and B (25% acetonitrile in 0.4 M NaH₂PO₄) was used. Ratio of the solutions A and B in the mobile phase was changed from 9:1 to 2:8 after the first 45 min, then was changed back to 9:1 for another 5 min. The flow rate of

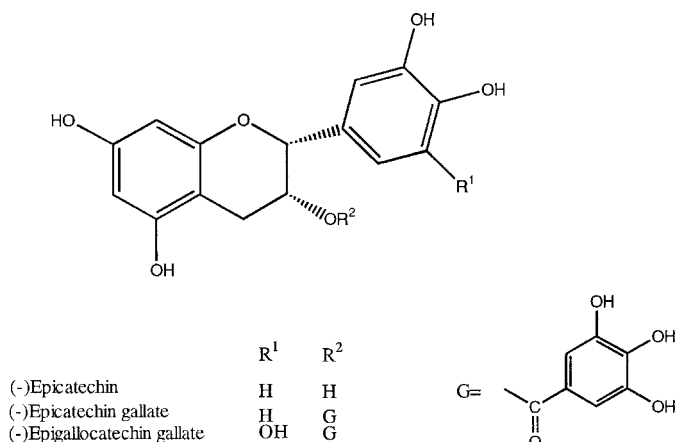


Figure 1. Chemical structures of EC, EGCG and ECG.

the mobile phase was set at 1.0 ml min^{-1} and detection of catechins was preformed at 278 nm. Complete separation of EC, EGCG and ECG was achieved at the retention times of 25.0, 26.5 and 37.7 min, respectively. The linear ranges of the calibration curve for EC, EGCG and ECG were 0.2–50, 0.7–300 and 0.5–100 $\mu\text{g ml}^{-1}$, respectively, and the correlation coefficients were 0.999 for all three catechins. Coefficients of variation for the inter- and intraday assay variability for a representative quality control sample in mid calibration range and a concentration ratio of EC:EGCG:ECG = 1:10:2 were assessed to be 7.4% (EC), 4.4% (EGCG), 8.2% (ECG) and 3.7% (EC), 3.5% (EGCG), 5.5% (ECG), respectively. The interday extraction recoveries ($n = 5$) of EC, EGCG and ECG were 89.3 ± 5.3 , 89.7 ± 3.7 , and $87.3 \pm 5.5\%$, respectively.

Pharmacokinetic study of tea catechins

Male Sprague-Dawley rats (210–230 g) were housed under controlled conditions (23–25 °C, 55% relative humidity, and 12 h light/dark cycle) and were allowed to free access to food (Purina rat chow, St Louis, MO, USA) and water before the experiments. Five animals were randomly assigned to four dose groups. A single intravenous dose of the catechin-fraction was administered to the study animals at the 50, 100, 200 and 300 mg kg^{-1} dose groups. For each animal, the right jugular vein was cannulated 24 h before dosing. The rats were then fasted overnight with free access to water. Preparation of the test doses required accurate weighing of the stock catechin fraction before dissolving it in saline containing 5% DMSO to make up to the designed concentrations. Blood (0.3 ml) was withdrawn before i.v. dosing ($t = 0$) and at 5, 10, 20, 40, 60, 90, 120, 180 and 300 min post-dosing. The samples were immediately centrifuged at 6000g for 5 min to separate the plasma. To prevent oxidation of the catechins, 50 μl 20% ascorbic acid buffer containing 0.4 M NaH_2PO_4 and 0.1% Na_2EDTA (pH 3.6) was added to 200 μl of each plasma sample. The mixture was then sonicated for 1 min followed by the adding ethyl acetate for extraction (two times; 4 and 2 ml). The pooled ethyl acetate fraction was centrifuged at 4000g for 5 min and was dried at 35 °C under nitrogen. The resultant residue was kept at -80°C until assay. At the time of assay, the frozen sample was reconstituted with 400 μl 10% acetonitrile aqueous solution (pH 2.4) and a 100- μl aliquot was injected onto the HPLC system. No decomposition of the catechins was observed in storage for > 1 month.

Urine and faeces samples were collected for 24 h post-dosing and the total urine volume and faecal weight were recorded for each animal. To prevent oxidation, 2 ml ascorbic acid buffer (20%, pH 3.6) was added to the urine samples before the study. Similarly, ethyl acetate was used for the extraction of catechins (three times) from the urine samples. The faeces collected were homogenized with 0.4 M phosphate buffer (pH 2.4) to yield a 10% suspension. The solution was sonicated for 10 min, and the supernatant of faeces (2 ml) was extracted twice with ethyl acetate (4 ml $\times$ 2). The extracted urine and faeces samples were dried with nitrogen and reconstituted with 1 ml 10% acetonitrile aqueous solution (pH 2.4), 100- μl aliquot was injected on to the HPLC.

Determination of plasma protein binding of tea catechins

Binding of the three catechins to plasma proteins was examined using ultrafiltration. The study was performed using a micropartition system (4104, Amicon, Epernon, France) and a YMT membrane with a molecular weight cut-off of 30000 Daltons. Blood was collected from rats and pooled to harvest the

plasma by centrifugation. The stock catechin fraction were diluted with saline phosphate buffer (pH 7.4, 0.0011 M). The final concentrations used in the protein binding studies were similar to those achieved *in vivo* after i.v. dosing. After spiking fresh rat plasma with the catechin solution, the sample was incubated for 20 min at 37 °C and was centrifuged at 2000g for 20 min (Eppendorf Centrifuge, 5810R). The ultrafiltrate collected was submitted to the HPLC assay. Each experiment was performed in triplicate. Non-specific binding was corrected by replacing the plasma with the same volume of saline phosphate buffer in the solution containing catechins at the test concentrations.

The percentage of protein binding was calculated as:

$$\% \text{ Protein bound} = 100\% (1 - C_{\text{filtrate}}/C_{\text{plasma}}),$$

where, C_{filtrate} is the concentration of free catechin in the ultrafiltrate and C_{plasma} is the concentration of catechin in plasma.

Determination of partitioning of tea catechins into red blood cells

The degree of partitioning of individual catechins into red blood cells was examined. Fresh blood (10 ml with 10 units heparin per ml) was collected and mixed with 1 ml of the diluted catechin extract. Following incubation at 37 °C for 10 min, the plasma and red blood cells were separated by centrifugation at 6000g for 20 min. To 100 μ l plasma and red blood cells collected, 50 μ l phosphate buffer containing 20% ascorbic acid (pH 3.6) was added and the catechins were extracted with ethyl acetate for the HPLC assay. Owing to the low recovery of catechins from red blood cells (< 30%), and the presence of interfering peaks from these cells, the partitioning of catechins was calculated as:

$$\text{Fraction of catechin partitioned into red blood cells} = 1 - 0.55 \times (C_{\text{plasma}}/C_{\text{added}}),$$

where C_{plasma} is the concentration of catechin in plasma and C_{added} is the known concentration of catechin added to whole blood. A nominal value of 0.55 was used to correct for the plasma volume in blood.

Pharmacokinetic analysis of tea catechins

Pharmacokinetic analysis of the concentration versus time data for the three catechins from individual animals was carried out using WinNonlin software (Pharsight, Cary, NC, USA). To describe best the biphasic disposition profile of the three compounds, a two compartment model was utilized along with a weighing function of $1/y^2$ for data fitting and parameter estimation. The use of weights improved the model description of the terminal elimination phase. A three compartment model was also tested. However, the reduction in the precision of parameter estimation along with the lack of substantial improvement in various statistical parameters including the AIC or SC criterion and the sums of squared residuals did not justify adding additional parameters to the two-compartment model. Differences in the pharmacokinetic parameter estimates among dosing groups were assessed by ANOVA and the level of statistical significance (α) was set at 0.05.

Results

Pharmacokinetics of catechins dosed as catechin fraction

Following i.v. administration of the tea catechin-fraction to the study rats at doses ranging from 50 to 300 mg kg⁻¹, plasma concentrations of the three compounds declined in a bi-exponential manner showing a rapid distributive phase and a relatively slower elimination phase (figure 2). Pertinent pharmacokinetic parameter estimates depicting the profiles for these catechins are given in tables 1–3.

Across the doses studied, EC, EGCG and ECG showed similar central volumes of distribution (V_c) ranging from 0.2 to 0.7 l kg⁻¹, which were in the same order of magnitude of total body water (0.6 l kg⁻¹). Tissue distribution of the three compounds was rapid as indicated by the short distribution half-life ($t_{1/2,\alpha}$) of 3–15 min. As with the estimates of steady-state volume of distribution (V_{ss}), respective means for the doses tested were 0.68–1.32, 1.29–1.73 and 2.08–3.19 l kg⁻¹ for EC, EGCG and ECG, and were 3–6-fold higher than the V_c estimates at the corresponding dose level. This observation suggests that these catechins exhibited a relatively high degree of tissue distribution that was in agreement with the short $t_{1/2,\alpha}$ estimates.

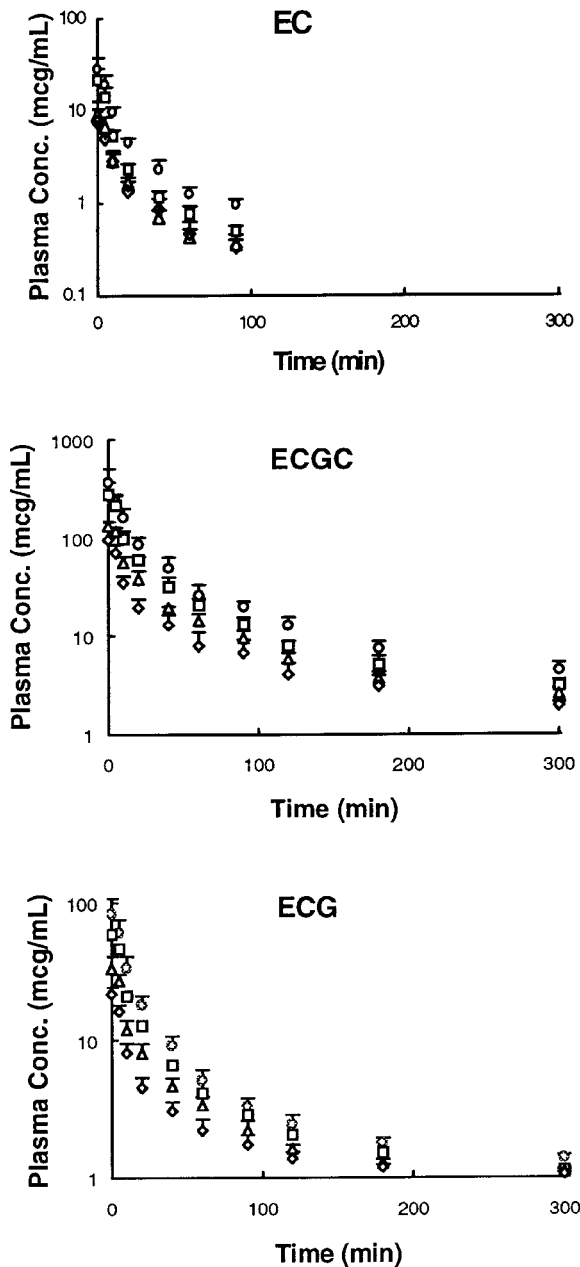


Figure 2. Mean (SD) plasma concentration versus time profiles of tea catechins following intravenous dosing ($n = 5$). (Top) EC at actual doses of 15 ($\circ$), 10 ($\square$), 5 ($\triangle$) and 2.5 ($\diamond$) mg kg^{-1} ; (middle) ECGC at actual doses of 150 ($\circ$), 100 ($\square$), 50 ($\triangle$) and 25 ($\diamond$) mg kg^{-1} ; (bottom) ECG at actual doses of 39 ($\circ$), 26 ($\square$), 13 ($\triangle$) and 6.5 ($\diamond$) mg kg^{-1} . Note: all three catechins exhibited biphasic disposition profiles and parallel terminal elimination was observed at the various doses tested.

Elimination of catechins varied among EC, ECGC and ECG, as the respective terminal elimination half-lives ($t_{1/2,\beta}$) were assessed to be 43, 124 and 222 min and were apparently dose-independent (figure 2). Total clearance (CL) of the three catechins were estimated to be 0.01–0.04 $\text{l min}^{-1} \text{kg}^{-1}$ among the doses tested and a

Table 1. Pharmacokinetic parameter estimates of EC following i.v. dosing ($n = 5$).

Parameter estimates	EC in tea catechin-fraction				p
Dose (mg kg ⁻¹)	2.5	5.0	10	15	–
$C_{\max}$ (mg l ⁻¹)	16.7±5.7	17.5±5.9	35.3±12.6	40.1±15.8	–
V_e (l kg ⁻¹)	0.17±0.07	0.26±0.11	0.34±0.20	0.42±0.15	0.06
V_{ss} (l kg ⁻¹)	0.68±0.19	1.03±0.19	1.21±0.43	1.32±0.71	> 0.05
K_{10} (min ⁻¹)	0.11±0.03	0.13±0.05	0.12±0.03	0.09±0.03	> 0.05
K_{12} (min ⁻¹)	0.12±0.06	0.10±0.05	0.08±0.02	0.07±0.02	> 0.05
K_{21} (min ⁻¹)	0.036±0.012	0.030±0.009	0.030±0.007	0.040±0.019	> 0.05
$t_{1/2,\alpha}$ (min)	3.08±1.11	3.70±1.14	3.60±1.23	4.35±1.40	> 0.05
$t_{1/2,\beta}$ (min)	46.3±11.8	42.1±8.8	46.5±8.4	38.3±9.3	> 0.05
CL (l min ⁻¹ kg ⁻¹)	0.016±0.002	0.031±0.003	0.040±0.008	0.033±0.007	0.0002
AUC _{0→∞} (mg l ⁻¹ min)	150±16	161±18	294±61	463±85	–
MRT (min)	40.8±12.4	32.9±4.1	33.9±6.4	40.8±25.6	> 0.05
Urinary recovery (%)	31.4±8.9	28.0±4.2	20.7±1.4	21.3±2.7	< 0.05
CL _r (l min ⁻¹ kg ⁻¹)×10 ⁻⁴	78±22	82±12	72±4	70±8	> 0.05
Faeces recovery (%)	3.52±1.10	5.09±1.82	3.47±0.99	2.69±0.54	< 0.05

Table 2. Pharmacokinetic parameter estimates of EGCG following i.v. dosing ($n = 5$).

Parameter estimates	EGCG in tea catechin-fraction				p
Dose (mg kg ⁻¹)	25	50	100	150	–
$C_{\max}$ (mg l ⁻¹)	95±38	143±60	214±71	236±45	–
V_e (l kg ⁻¹)	0.30±0.11	0.39±0.13	0.58±0.14	0.66±0.15	0.002
V_{ss} (l kg ⁻¹)	1.29±0.48	1.42±0.44	1.57±0.48	1.73±0.43	> 0.05
K_{10} (min ⁻¹)	0.04±0.01	0.036±0.014	0.030±0.007	0.027±0.006	> 0.05
K_{12} (min ⁻¹)	0.05±0.02	0.04±0.03	0.020±0.009	0.016±0.005	0.024
K_{21} (min ⁻¹)	0.016±0.007	0.014±0.006	0.009±0.003	0.010±0.003	> 0.05
$t_{1/2,\alpha}$ (min)	7.91±3.07	10.2±4.66	10.3±3.37	15.1±3.47	0.046
$t_{1/2,\beta}$ (min)	131±41	129±48	109±34	126±37	> 0.05
CL (l min ⁻¹ kg ⁻¹)	0.010±0.001	0.013±0.001	0.017±0.001	0.018±0.002	0.0001
AUC _{0→∞} (mg l ⁻¹ min)	2568±378	3998±148	5881±546	8597±1362	–
MRT (min)	128±37	113±31	91±23	98±24	> 0.05
Urinary recovery (%)	5.3±0.9	3.1±0.6	4.4±0.6	3.8±0.6	> 0.05
CL _r (l min ⁻¹ kg ⁻¹)×10 ⁻⁴	6.6±1.7	5.0±1.0	8.3±1.7	6.7±1.5	> 0.05
Faeces recovery (%)	1.02±0.16	0.69±0.07	0.58±0.23	0.65±0.13	< 0.05

Table 3. Pharmacokinetic parameter estimates of ECG following i.v. dosing ($n = 5$).

Parameter estimates	ECG in tea catechin-fraction				p
Dose (mg kg ⁻¹)	6.5	13	26	39	–
$C_{\max}$ (mg l ⁻¹)	20.2±5.4	40.5±24	41.7±11.9	64.5±21.9	–
V_e (l kg ⁻¹)	0.34±0.10	0.41±0.19	0.66±0.16	0.66±0.21	0.014
V_{ss} (l kg ⁻¹)	2.08±0.57	2.25±0.61	2.89±0.73	3.19±1.24	> 0.05
K_{10} (min ⁻¹)	0.020±0.006	0.034±0.020	0.028±0.007	0.031±0.011	> 0.05
K_{12} (min ⁻¹)	0.064±0.023	0.071±0.066	0.029±0.007	0.028±0.009	> 0.05
K_{21} (min ⁻¹)	0.012±0.003	0.012±0.007	0.008±0.002	0.008±0.003	> 0.05
$t_{1/2,\alpha}$ (min)	8.1±2.9	9.4±5.3	11.9±2.9	12.0±4.1	0.046
$t_{1/2,\beta}$ (min)	282±81	210±74	189±37	208±80	> 0.05
CL (l min ⁻¹ kg ⁻¹)	0.0063±0.0004	0.011±0.001	0.017±0.001	0.019±0.002	0.0001
AUC _{0→∞} (mg l ⁻¹ min)	1032±63	1212±102	1518±130	2082±170	–
MRT (min)	334±102	212±67	168±39	170±67	0.007
Urinary recovery (%)	4.3±3.4	4.6±1.5	2.3±0.4	3.5±1.8	> 0.05
CL _r (l min ⁻¹ kg ⁻¹)×10 ⁻⁴	3.3±1.6	5.0±1.4	5.0±1.0	6.6±3.3	> 0.05
Faeces recovery (%)	2.01±0.76	1.55±0.16	0.61±0.13	0.51±0.10	< 0.01

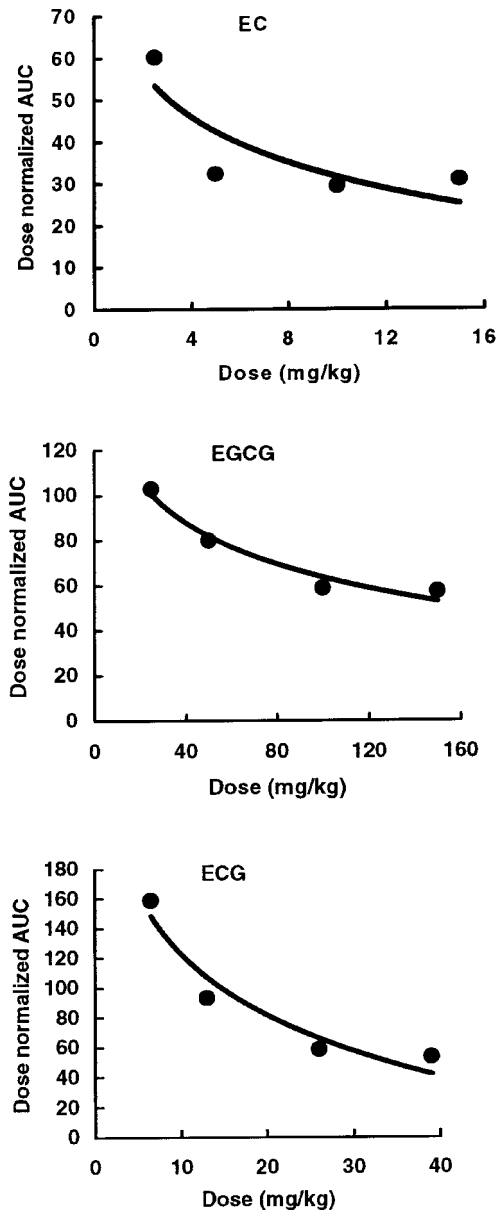


Figure 3. Dose-normalized mean $AUC_{0 \rightarrow \infty}$ versus dose for EC (top), EGCG (middle) and ECG (bottom). Note: trend lines were included for a better graphic depiction of the non-linear pharmacokinetic systems.

dose-dependent increase was observed (tables 1–3). Renal excretion of unchanged EC was 21–31% of dose, and those for EGCG and ECG were ~ 2 –5%. By comparing renal clearance (CL_r) to CL , the former for EGCG and ECG were 3.7–6.6% and 2.9–5.2%, respectively, of later, but that for EC was 21–49%. Therefore, the non-renal clearance for elimination of EGCG and ECG was more important than that of EC. The faecal recovery was 3–5% for EC which was slightly higher than that of EGCG (0.6–1%) and ECG (0.5–2%).

Table 4. Plasma protein binding and red blood cell partitioning of tea catechins ($n = 3$ for each study condition).

	Concentration tested ($\mu\text{g ml}^{-1}$)			Bound (%)		
	EC	EGCG	ECG	EC	EGCG	ECG
Catechin-Fraction	37	364	82	66 ± 7	94 ± 2	95 ± 1
	18	181	40	79 ± 2	97 ± 2	96 ± 1
	4	36	9	81 ± 2	97 ± 2	100 ± 0
p (ANOVA)				0.03	0.016	< 0.001
	Concentration tested ($\mu\text{g ml}^{-1}$)			Red blood cell partitioning (%)		
	EC	EGCG	ECG	EC	EGCG	ECG
Catechin-Fraction	11	105	19	43 ± 5	23 ± 3	11 ± 3
	2	21	4	29 ± 7	17 ± 3	9 ± 1
	1	10	2	39 ± 13	15 ± 8	11 ± 7
p (ANOVA)				> 0.05	> 0.05	> 0.05

As the dose range differed among EC, EGCG and ECG when they were administered in the fraction form, a nominal 10 mg kg^{-1} dose was employed to facilitate comparison. Pharmacokinetic parameter estimates obtained for the three catechins at doses closest to the nominal dose were normalized. At this nominal dose, maximum plasma concentrations (C_{max}) and areas under the plasma concentration curve ($\text{AUC}_{0 \rightarrow \infty}$) for EC, EGCG and ECG were $35, 38, 31 \text{ mg l}^{-1}$ and $294, 1027, 932 \text{ mg min l}^{-1}$, respectively. EGCG and ECG showed similar central distribution volume yet higher systemic exposure in comparison with EC at this nominal dose.

To evaluate further the linearity of the pharmacokinetic system exhibited by the catechins, $\text{AUC}_{0 \rightarrow \infty}$ and C_{max} estimates obtained at different doses were assessed for dose-dependency. Dose normalized $\text{AUC}_{0 \rightarrow \infty}$ were plotted against dose (using the dose range of EC as a reference) for the three catechins are shown in figure 3. A clear reduction in the dose-normalized systemic exposure was observed for all three catechins as the test dose was increased (figure 3). A similar observation was confirmed for dose normalized C_{max} . Further statistical tests showed significant increases ($p < 0.05$) in V_e and CL estimates when the dose was increased over the 6-fold range for the three compounds (tables 1–3). Although not reaching a degree of statistical significance ($p > 0.05$), there was a dose-related increase in the V_{ss} estimates for the three catechins.

Plasma protein binding of tea catechins

The degree of plasma protein binding was high for all three catechins (table 4). The percentage of catechins bound to plasma proteins ranged from 81 to 100% at the lowest concentration tested. A significant decrease in protein binding with increase of concentration was observed for the three compounds ($p < 0.05$), and free fractions of the catechins increased by two fold or higher at higher concentrations.

Partitioning of tea catechins into red blood cells

The three catechins displayed variable degrees of red blood cell partitioning ranging between 29–43%, 15–23% and 9–11% for EC, EGCG, and ECG, respectively (table 4). No statistically significant differences were detected among the three concentrations studied.

Discussion

Tea catechins are hydrophilic in nature and are readily soluble in water; the highest achievable concentration of the catechin fraction was 250 mg ml^{-1} in the present study. The high aqueous solubility of these compounds corresponds well with the V_c estimates that are in the order of magnitude similar to total body water. As a result of the good solubility, the maximum plasma concentration (normalized to the 10 mg kg^{-1} dose) for the catechins, under the current dosing conditions, was relatively high, i.e. $30\text{--}40 \text{ } \mu\text{g ml}^{-1}$.

A bi-exponential disposition profile was clearly demonstrated for the three tea catechins indicating a strong tendency for tissue distribution. Biodistribution data (Chen *et al.* 1997) revealed that uptake of catechins by the intestine, kidney, lung and liver is extensive following a single i.v. dosing of a catechin extract. The ability for these catechins to partition into tissues is in line with the current V_{ss} estimates ($1\text{--}3 \text{ l kg}^{-1}$). Moreover, these catechins appear readily to partition into red blood cells suggesting that the compounds can also partition into or through a lipophilic environment, i.e. lipid bilayers of a cell membrane. In fact, the 2–6-fold higher K_{12} estimates (rate constant from central- to tissue-compartment) relative to K_{21} (rate constant from tissue- to central-compartment) supports the above interpretation (tables 1–3). In general, except for ECGC, the two microrate constants, i.e. K_{12} and K_{21} , were not significantly altered ($p > 0.05$) with the increase of dose. The extensive tissue distribution also impacted on the elimination of these catechins; K_{21} estimates for the three compounds were in general 2–4-fold lower than the corresponding central elimination rate constants (K_{10}). Therefore, elimination of the catechins from the systemic circulation is likely to be limited by the slower rate of tissue redistribution. Thus, this phenomenon would offer some degree of shielding effect on the perturbations of metabolic and excretory processes once the catechins reside in the peripheral tissues.

From the perspective of drug elimination, the hepatic and renal routes are most important. The relatively low degree of urinary recovery observed for the three catechins suggests the domination of non-renal elimination. Estimated by subtracting the CL_r estimate from that of CL at the highest dose, the respective values of hepatic clearance (CL_H) for EC, EGCG, and ECG were 0.026 , 0.017 and $0.018 \text{ l min}^{-1} \text{ kg}^{-1}$. Therefore, CL_H represents 79–98% of total CL for the three catechins. Although the degree of biliary excretion of these catechins was not assessed, the low faecal recovery of unchanged catechins, i.e. 1–5% of dose, did not support significant involvement of this excretion route.

The decline in dose normalized $AUC_{0 \rightarrow \infty}$ and C_{max} as a function of dose suggests the existence of a non-linear kinetic system for the three catechins. The dose-dependent increase in V_c is in agreement with the non-linearity observed. Among all the factors affecting V_c , protein binding is a most prominent possibility. Owing to the fact that the phenolic catechins are slightly acidic at physiological pH, the compounds are likely to bind to plasma albumin (Haslam 1996, Ritschel and Kearns 1999). The increase in free fractions at higher doses would favour a more effective diversion of these catechins out of the central compartment, and thereby resulting in a reduction in the availability of catechins in plasma and thus an increase in V_c . In addition, similarity in their molecular structures (figure 1) increases the chance of mutual displacement from the competitive binding sites. Indeed, the magnitude of changes in free fractions was different for the three catechins at higher plasma concentrations (table 4).

Tea catechin fraction was used in the present study because this is the most common form available in practice. Among the three catechins studied (dose ratio; EC:ECG:ECGC = 1:2.6:10), EC was lowest in content and showed a short $t_{1/2\beta}$. On the contrary, ECGC was high in content and possessed relatively long $t_{1/2\beta}$ and high systemic exposure. Therefore, ECGC can potentially serve as a surrogate marker, if correlation between its presence and bioactivity can be made, and as a chemical marker for quality control of tea derived herbal products.

For all the research performed on tea catechins thus far, their pharmacokinetics and system characteristics have not been fully elucidated. However, information pertaining to all pharmacokinetic variables and their relations to the desired biological response is critical for estimating the needed level of tea consumption or dosage requirement. Data obtained in the present study show the complexity of the pharmacokinetics of these catechins and will be used as a basis for designing further studies in animals and humans.

References

- CHEN, L. S., LEE, M. J., LI, H. and YANG, C. S., 1997, Absorption, distribution and elimination of tea polyphenols in rats. *Drug Metabolism and Disposition*, **25**, 1045–1050.
- HASLAM, E., 1996, Natural polyphenols (vegetable tannins) as drugs: possible modes of action. *Journal of Natural Products*, **59**, 205–215.
- LEE, M. J., WANG, Z. Y., HE, L., CHEN, L., YANG, S., GOBBO, S., BALENTINE, D. A. and YANG, C. S., 1995, Analysis of plasma and urinary tea polyphenols in human subjects. *Cancer Epidemiology Biomarkers and Prevention*, **4**, 393–399.
- RITSCHEL, W. A. and KEARNS, G. L., 1999, *Handbook of Basic Pharmacokinetics: Including Clinical Application*, 5th edn (Washington, DC: American Pharmaceutical Association), pp. 119–121.
- SETO, R., NAKAMURA, H., NANJO, F. and HARA, Y., 1997, Preparation of epimers of tea catechins by heat treatment. *Bioscience, Biotechnology and Biochemistry*, **61**, 1434–1439.
- STEELE, V. E., BAGHERI, D. and BALENTINE, D. A., 1999, Preclinical efficacy studies of green and black tea extracts. *Proceeding of the Society for Experimental Biology and Medicine*, **220**, 210–212.
- VAN HET HOF, K. H., KIVITS, G. A. A., WESTSTRATE, J. A. and TIJBURG, L. B. M., 1998, Bioavailability of catechins from tea: the effect of milk. *European Journal of Clinical Nutrition*, **52**, 356–359.
- WEISBURGER, J. H., 1999, Tea and health: the underlying mechanisms. *Proceeding of the Society for Experimental Biology and Medicine*, **220**, 271–275.
- YAMAMOTO, T., 1997, *Chemistry and Applications of Green Tea* (Boca Raton: CRC Press).
- YANG, C. S., CHEN, L. S., LEE, M. J., BALENTINE, D., KUO, M. C. and SCHANTZ, S. P., 1998, Blood and urine levels of tea catechins after ingestion of different amounts of green tea by human volunteers. *Cancer Epidemiology Biomarkers and Prevention*, **7**, 351–354.
- YANG, C. S., LEE, M. J. and CHEN, L. S., 1999, Human salivary tea catechin levels and catechin esterase activities: implication in human cancer prevention studies. *Cancer Epidemiology Biomarkers and Prevention*, **8**, 83–89.
- ZHANG, A., CHAN, P. T., LUK, Y. S., HO, W. K. K. and CHEN, Z. Y., 1997, Inhibitory effect of jasmine green tea epicatechin isomers on LDL-oxidation. *Nutritional Biochemistry*, **8**, 334–340.
- ZHU, M. and XIAO, P. G., 1991, Quantitative analysis of active constituents in green tea. *Phytotherapy Research*, **5**, 239–240.

Metabolism of styrene in the human liver *in vitro*: interindividual variation and enantioselectivity

M. A. M. WENKER†*, S. KEŽIĆ†, A. C. MONSTER†
and F. A. DE WOLFF†§

† Coronel Institute, Academic Medical Center, PO Box 22700, 1100 DE Amsterdam, The Netherlands

‡ Toxicology Laboratory, Leiden University Medical Center, Leiden, The Netherlands

§ Research Institute Neurosciences Amsterdam, Chair of Human Toxicology, Academic Medical Center, University of Amsterdam, The Netherlands

Received 31 July 2000

1. The interindividual variation and enantioselectivity of the *in vitro* styrene oxidation by cytochrome P450 have been investigated in 20 human microsomal liver samples. Liver samples were genotyped for the *CYP2E1**6 and *CYP2E1**5B alleles.

2. Kinetic analysis indicated the presence of at least two forms of styrene-metabolizing cytochrome P450. The enzyme constants for the high-affinity component were subject to appreciable interindividual variation, i.e. $V_{\max 1}$ ranged from 0.39 to 3.20 nmol mg protein⁻¹ min⁻¹ (0.96 ± 0.63) and K_{m1} ranged from 0.005 to 0.03 mM (0.011 ± 0.006). Inhibition studies with chemical inhibitors of CYP2E1, CYP1A2, CYP2C8/9 and CYP3A4 demonstrated that CYP2E1 was the primary enzyme involved in the high-affinity component of styrene oxidation. No relationship between the interindividual variation in $V_{\max 1}$ and K_{m1} and the genetic polymorphisms of the *CYP2E1* gene was found.

3. Cytochrome P450-mediated oxidation of styrene demonstrated a moderate enantioselectivity, with an enantiomeric excess (ee) of (*S*)-styrene oxide of 15% (range 4–27%) at low styrene concentration and an ee of (*R*)-styrene oxide of 7% (range –11 to +22%) at high styrene concentration. This points towards the involvement of at least two cytochrome P450, with different enantioselectivities.

4. The data indicate that cytochrome P450-mediated styrene oxidation is subject to considerable interindividual variation, but only to a moderate product enantioselectivity.

Introduction

Styrene is extensively used in a wide range of industrial processes, particularly in the production of reinforced plastics, polyester resins and styrene butadiene rubber. Styrene exhibits low-to-moderate acute toxicity in both laboratory animals and humans, and the two major effects of acute exposure to styrene in humans and laboratory animals are irritation (both skin and respiratory tract) and central nervous system depression (Bond 1989).

Chronic exposure to styrene has been linked to cytotoxic, genotoxic and neurotoxic effects, in both humans and laboratory animals. Neurological symptoms in laboratory animals exposed to styrene include tremor, loss of equilibrium, hearing loss and slowing of motor conduction velocity (Arlie-Soborg 1992). In rats exposed to 320 ppm styrene for 3 months, increased glial fibrillary acidic protein concentrations have been found in the hippocampus and sensory motor cerebral cortex, which could indicate astrogliosis in these regions (Rosengren and Haglid 1989). In humans, occupational exposure to styrene has been associated with chronic toxic encephalopathy (Lilis *et al.* 1978, Mutti *et al.* 1984, Cherry and Gautrin 1990), although the evidence is not entirely consistent (Rebert and Hall 1994). In a cohort

* Author for correspondence; e-mail: M.Wenker@amc.uva.nl

study of 35 443 workers employed in the reinforced plastics industry, the mortality rate from diseases of the central nervous system increases with time since first exposure, duration of exposure, average level of exposure and cumulative exposure to styrene (Welp *et al.* 1996).

Styrene can induce chromosome aberrations and sister chromatid exchanges in several experimental assays and in peripheral human lymphocytes, but it requires metabolic activation to produce the observed genotoxic effects (Barale 1991). Based on available animal data the International Agency for Research on Cancer (IARC) has classified styrene as possible carcinogenic to humans (group 2B) (IARC 1994).

Styrene is oxidized by cytochrome P450 (CYP) to styrene-7,8-oxide (SO). This reactive intermediate is, in humans, mainly detoxified by microsomal epoxide hydrolase (mEH) to styrene glycol (SG) (Sumner and Fennell 1994). In rats, *in vivo* exposure to styrene results in high SO levels in liver, lung, kidney and brain; hence, most of the adverse effects of styrene have been attributed to accumulation of SO in tissues (Bond 1989).

Several cytochrome P450 isoforms may contribute to the oxidation of styrene in rodents. In man, CYP2E1 is the main isoform responsible for styrene metabolism (Guengerich *et al.* 1991, Nakajima *et al.* 1994, Kim *et al.* 1997); some studies have reported that at high substrate concentrations CYP2B6 is the most active isozyme (Kim *et al.* 1997). CYP2E1 is the key enzyme in metabolism of numerous organic solvents and it also plays a significant role in the metabolism of ethanol in chronic alcohol abusers (Wenker *et al.* 2000). Several studies have shown considerable, up to 50-fold, individual variability in the expression of CYP2E1 (Guengerich and Turvy 1991, Carriere *et al.* 1996, Powell *et al.* 1998) and a number of polymorphisms of the *CYP2E1* gene have been identified (Hayashi *et al.* 1991, Uematsu *et al.* 1991). *In vitro*, the *CYP2E1**5B allele has been shown to increase transcription by up to 10-fold (Watanabe *et al.* 1994). Thus, a substantial potential exists for interindividual variability in the metabolism of styrene in humans. Since SO is the metabolite responsible for the adverse effects of styrene, different rates of CYP2E1 activity between humans may correspond to variation in susceptibility to styrene toxicity.

Another important aspect of styrene oxide toxicity is chirality. Oxidation of styrene leads to chiral SO. The (*R*)-enantiomer of SO is more mutagenic to *Salmonella typhimurium* TA 100 in the Ames' test (Seiler 1990). Furthermore, in mice the (*R*)-enantiomer is more toxic to lung and liver than the (*S*)-enantiomer (Gadberry *et al.* 1996). The oxidation of styrene by cytochrome P450 represents a prochiral recognition process (product enantioselectivity) and the product enantioselectivity will be determined by the preferential orientation of the oxygen attack at the C = C double bond. Differences in product enantioselectivity of styrene oxidation between humans may also play an important role in the susceptibility to styrene toxicity.

Until now, little information is available in humans concerning variability and product enantioselectivity of styrene oxidation, which may be important parameters for the risk assessment of styrene. Therefore, the purpose of this study was to assess the interindividual variation in the cytochrome P450-mediated oxidation of styrene and enantioselectivity in 20 human liver samples. Furthermore, the contribution of hepatic CYP2E1 to styrene oxidation and the possible contribution of CYP2E1 polymorphism to the interindividual variability in styrene oxidation have been studied.

Materials and methods

Chemicals

Styrene, (R)-(-)-styrene-7,8-oxide (98%, 97% ee), (S)-(+)-styrene-7,8-oxide (98%, 97% ee), (R)-(-)-styrene glycol (98%, 97% ee), (S)-(+)-styrene glycol (98%, 97% ee) and pentafluoropropionic anhydride (PFPA, 98%) were from Fluka (Buchs, Switzerland). (1S, 2S)-(-)-1-phenylpropylene oxide (PPO) and sodium methoxide (0.5 M) were from Aldrich (WI, USA). DL-Dithiothreitol, phenylmethylsulphonyl fluoride (99%), glucose 6-phosphate, glucose 6-phosphate dehydrogenase (G-7877), β -NADP⁺, pentafluorobenzoyl chloride (PFBCl), 4-methylpyrazole, α -naphthoflavone, sulphaphenazole, *p*-nitrophenol and 4-nitrocatechol were from Sigma (St Louis, MO, USA). Pyridine (silylation grade) was from Pierce (Rockford, IL, USA), and methanol-dried from J. T. Baker (Deventer, The Netherlands). *Dra*I and *Rsa*I restriction enzymes, *Taq* DNA polymerase and dNTPs were from Boehringer-Mannheim (Mannheim, Germany). Trichloropropylene oxide (TCPO) was a generous gift from P. J. Boogaard (Shell, The Netherlands). Polypropylene glycol (PPG) was synthesized by acid-catalysed hydrolysis of PPO as follows: 100 μ l PPO was treated with 1 ml 0.5 M sulphuric acid in *n*-hexane. The reaction mixture was mixed for 60 min at room temperature and then neutralized with NaOH. The formed PPG was extracted with 2 $\times$ 5 ml ethyl acetate. The chemical purity was 97%. All other chemicals were at least reagent grade and were from Merck-Schuchardt (Darmstadt, Germany).

Microsomal preparations

Human liver samples were from the International Institute for the Advancement of Medicine (Scranton, USA). All liver samples were from healthy Caucasian males without any indication of liver disease, varying in age between 20 and 60 years ($n = 5$ per decade) and is considered to be a sample representative for the occupationally exposed population. Half of the samples were from smokers; six of 20 liver samples were from persons abstaining from alcohol; the other 14 were moderate alcohol consumers.

Microsomes were prepared by homogenizing ~ 10 g liver tissue in 100 mM Tris-HCl, pH 7.4, buffer containing 1.15% KCl, 1 mM dithiothreitol (DTT), 2.5 mM EDTA and 1 mM phenylmethylsulphonyl fluoride (PMSF) on ice, using a Potter-Elvehjem homogenizer. The homogenate was centrifuged for 10 min at 600g at 4 °C and the resulting pellet was homogenized and centrifuged again. Supernatants of both centrifugation steps were combined and centrifuged at 12000g for 20 min at 4 °C. The resulting supernatant was again centrifuged at 37 000g for 3 h at 4 °C. The pellet containing the microsomes was resuspended in 100 mM Tris-HCl, pH 7.4, storage buffer containing 20% glycerol, 1 mM DTT and 2.5 mM EDTA. Microsomes were stored at -80 °C until use. Protein content was measured according to the method of Pierce with BSA as standard (Pierce, Rockford, IL, USA).

Activity of cytochrome P450-mediated styrene oxidation

Since CYP2E1 as well as other CYP450 isoforms are inhibited by many organic solvents, the stock solutions of styrene were prepared in water at a concentration below the solubility limit. For each liver preparation a new stock solution was used and the exact styrene concentration of the used stock solution was determined by GC with flame ionization detection (FID). Styrene oxidation was assessed by incubating 0.5 mg microsomal protein with varying amounts of the styrene stock solution (4–665 μ l) and measuring styrene glycol formation. Incubations were carried out with a NADPH-generating system; the reaction mixture consisted of 50 mM potassium phosphate buffer (pH 7.4), 1 mM NADP⁺, 20 mM glucose 6-phosphate, 2 U glucose 6-phosphate dehydrogenase and 5 mM MgCl₂. The final volume of the reaction mixture was 1 ml. Incubations were carried out in a 37 °C shaking water bath for 10 min. Heat-denatured microsomal protein (10 min, 100 °C) was added to control tubes to determine the extent of non-enzymatic conversion. Reactions were stopped by adding 0.25 ml 0.6 N H₂SO₄. After addition of 25 μ l PPG (0.1 mg ml⁻¹) as internal standard, tubes were shaken for 1 min to convert the SO present to SG. To neutralize the acid 0.25 ml 0.6 N NaOH was added. The formed SG was extracted with 5 ml ethyl acetate and an aliquot of 1 ml was evaporated under nitrogen. To each sample, 0.5 ml 0.4% pyridine in toluene and 20 μ l 10% PFBCl in toluene was added. Derivatization was carried out at 70 °C for 30 min. After cooling to room temperature, samples were dried under nitrogen. The residues were dissolved in 0.5 ml 90% methanol and extracted with 1 ml hexane for analysis. The hexane extracts were kept at -20 °C till analysis.

Enantioselectivity of cytochrome P450-mediated styrene oxidation

The product enantioselectivity of cytochrome P450 was determined by incubations with styrene at two substrate concentrations: 16 and 1100 μ M. TCPO (50 μ M) was used to inhibit mEH, since hydrolysis of SO by mEH is an enantioselective process (Watabe *et al.* 1983). The percentage of mEH inhibition was determined by performing control experiments without inhibitor. After 10 min incubation, reactions were stopped by adding 2.5 ml hexane containing 25 μ l PPO (0.1 mg ml⁻¹) as internal standard. To

establish the efficiency of mEH inhibition, SG was determined in the remaining water phase. After removing the hexane extract containing SO and PPO, 5 ml ethyl acetate was added to the remaining water phase to extract the SG. Before extraction, 25 μ l PPG (0.1 mg ml⁻¹) was added as internal standard.

To analyse the SO enantiomers, an enantiospecific GC method was used based on base-catalysed methanolysis (Kezić *et al.* 2000). Briefly, the *n*-hexane extracts were reduced to ~ 100 μ l under a stream of nitrogen, 0.5 ml sodium methoxide was added and the samples were heated at 125 °C for 30 min. After cooling to room temperature, the samples were extracted with 2 $\times$ 3 ml *n*-pentane. Pentane was evaporated to dryness and to each sample 0.5 ml 0.5% pyridine in dichloromethane and 10 μ l PFPA were added. Derivatization was carried out at 60 °C for 60 min. After cooling to room temperature, samples were evaporated under a stream of nitrogen and the residues dissolved in 0.5 ml hexane.

To determine the amount of formed SG, 2.5 ml of the ethyl acetate fraction was evaporated under a stream of nitrogen; the residue was derivatized with PFPA as described above.

Inhibition of styrene oxidation

Studies with chemical inhibitors of several CYP450 isozymes were performed at 16 μ M styrene with microsomal samples from 10 different livers. Since the inhibitors are not soluble in water, they were dissolved in acetonitrile. The final concentration of acetonitrile in the incubation was $\leq 0.1\%$, a concentration which did not inhibit the oxidation of styrene under the conditions employed. The CYP2E1 inhibitor, 4-methylpyrazole, was used alone or in combination with three other inhibitors, sulphaphenazole (CYP2C8/9), α -naphthoflavone (CYP1A2) and troleandomycin (CYP3A4). The reason for combining these inhibitors with 4-methylpyrazole was the following: contribution of other cytochrome P450 isoforms beside CYP2E1 to styrene oxidation was expected to be low. Therefore, slight inhibition of the other isozymes would not result in a statistically significant inhibition of total styrene oxidation activity. A statistically significant effect of the other inhibitors on the residual activity (after inhibiting CYP2E1 with 4-methylpyrazole), however, is expected to be demonstrable if other isoforms of CYP450 are involved in styrene oxidation.

Sulphaphenazole (20 μ M), 4-methylpyrazole (50 μ M) and α -naphthoflavone (1 μ M) were added directly to the reaction mixture without pre-incubation. Incubations were carried out with the NADPH-generating system for 10 min at 37 °C. The mechanism-based inhibitor troleandomycin (100 μ M) was pre-incubated for 30 min with the microsomes and the NADPH-generating system. Then substrate and fresh NADPH-generating system were added and the reaction was carried out for 10 min at 37 °C.

Reactions were stopped by adding 0.6 N H₂SO₄, formed SG was extracted with ethyl acetate and samples derivatized with PFBCl, as described previously.

CYP2E1 activity

CYP2E1 activity in liver samples was measured according to Koop *et al.* (1986) with *p*-nitrophenol as substrate. Briefly, the reaction mixture consisted of microsomal protein (0.4 mg ml⁻¹), 0.1 M potassium phosphate, pH 6.8, 0.1 mM *p*-nitrophenol, 1 mM ascorbic acid, 1 mM NADP and 20 mM glucose 6-phosphate. Samples were incubated at 37 °C for 3 min before the addition of glucose 6-phosphate dehydrogenase to start the reaction. After 10 min, the reaction was stopped with 0.6 N perchloric acid. The 4-nitrocatechol formed was determined spectrally in 1.0 ml supernatant at 480 nm after the addition of 0.17 ml 10 N NaOH.

Polymorphisms of CYP2E1

DNA from 0.25 mg human liver was extracted with a DNA tissue extraction kit (Qiagen GmbH, Hilden, Germany). The CYP2E1*5B allele (c1/c2 genotype) and the CYP2E1*6 allele (C/D genotype) were analysed by PCR-RFLP, as described by Hildesheim *et al.* (1995) and Pirmohamed *et al.* (1995).

GC analysis

Unless otherwise indicated, analysis of the samples was performed on a Hewlett Packard 5890 GC (Hewlett Packard, USA) equipped with a ⁶³Ni electron capture detector (ECD), with injector temperature 250 °C and detector temperature 300 °C and a nitrogen gas flow rate of 30 ml min⁻¹.

The PFBCl esters (1 μ l) were analysed on an AT 35 column (10 m, 0.32 mm i.d., 0.5 μ m thickness; Alltech). Initial column temperature was 170 °C, and the column head pressure was 138 kPa.

The enantiomers of SO after PFPA derivatization (5 μ l) were chromatographed using two connected columns: CP Chirasil-Dex CB (25 m, 0.25 mm i.d., 0.25 μ m film thickness; Chrompack, The Netherlands) and CP Cyclodextrin B 236 M-19 (25 m, 0.25 mm i.d., 0.25 μ m film thickness; Chrompack). Column temperature was maintained isothermally at 105 °C; head pressure was 135 kPa.

The PFPA esters of SG (1 μ l) were analysed on a Carlo-Erba GC equipped with a ⁶³Ni ECD and a CP Chirasil-Dex-CB column (25 m, 0.25 mm i.d., 0.25 μ m film thickness; Chrompack). Column temperature was maintained isothermally at 110 °C; head pressure was 125 kPa.

The concentration of the styrene stocks were measured on a Hewlett Packard 5890 GC equipped with a FID and a DB 1701 column (30 m, 0.53 mm i.d., 1 μ m film thickness; J&W Scientific). Temperature

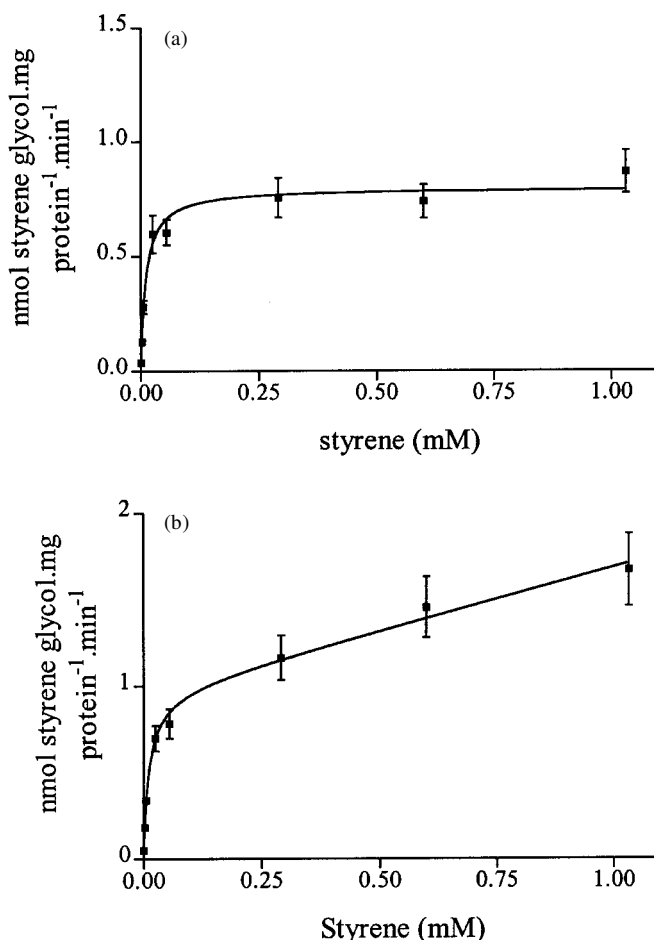


Figure 1. Michaelis-Menten plot of hepatic styrene oxidation. Each microsomal preparation was incubated with styrene (0.53–1030 μM) in duplicate, for 10 min at 37 °C. Errors bars represent SEM. (a) Plot of four livers, the curve was best described by a single Michaelis-Menten equation; (b) plot of 16 livers, the curve was best described by addition of two Michaelis-Menten equations.

was maintained isothermally at 90 °C. The column head pressure was 70 kPa. The flow of the nitrogen make-up gas was 30 ml min⁻¹, the FID hydrogen flow was 30 ml min⁻¹ and the air flow was 300 ml min⁻¹. The injector and detector temperature were 250 °C.

Enzyme kinetics and statistical analysis

Michaelis-Menten constants (V_{max} , K_m) were determined by analysing the data using non-linear regression. Enzyme constants were evaluated with respect to smoking, alcohol use and age using Student *t*-test and analysis of variance. Distributions of the Michaelis-Menten constants were assessed by histograms, normal plots, the Shapiro-Wilk test and the Kolmogorov-Smirnov test. If data were not normally or log-normally distributed, non-parametric tests were used. Correlation was assessed by using Spearman's rank correlation coefficient (r_s). All statistical tests were performed in SPSS (SPSS, Inc., Chicago, IL, USA). Differences were considered statistically significant when $p \leq 0.05$.

Results

Styrene concentrations in the incubations ranged from 0.53 to 1030 μM . As shown in figure 1a, the activity of the cytochrome P450-mediated styrene oxidation in four of 20 livers plateaued at a concentration of $\sim 50 \mu\text{M}$. However, the activity of

Table 1. Enzyme constants for the high- and low-affinity component of styrene oxidation determined in 20 human liver microsomes *in vitro*.

	Styrene metabolism	
	Mean±SD	Range
$V_{\max 1}^a$	0.962±0.628	0.392–3.204
K_{m1}^b	0.011±0.006	0.005–0.031
CLi_1^c	0.093±0.047	0.028–0.164
CLi_2^d	0.673±0.629	0.174–2.748

Styrene (0.53–1030 μM) was incubated with 0.5 mg microsomal protein for 10 min at 37 °C.
^a $V_{\max}$ for the high-affinity component, expressed as nmol.mg protein^{−1}.min^{−1}.
^b K_m for the high-affinity component, expressed in mM.
^c CLi for the high-affinity component, $V_{\max 1}/K_{m1}$, expressed as ml.min^{−1}.mg^{−1}.
^d CLi for the low-affinity component, in $\mu\text{l.min}^{-1}.\text{mg}^{-1}$.

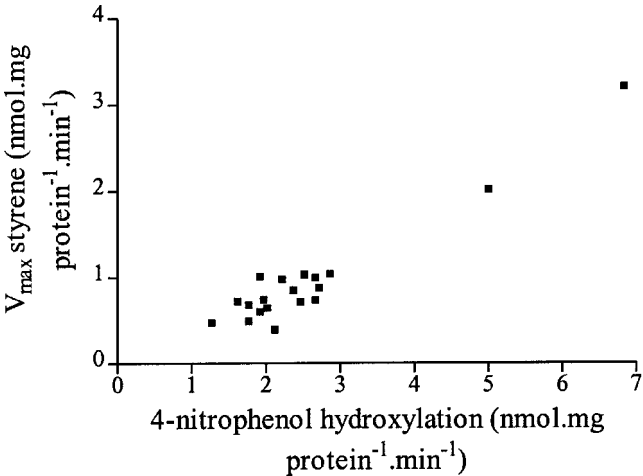


Figure 2. Relationship between the $V_{\max}$ of the high-affinity component of styrene oxidation and the 4-nitrophenol hydroxylation activity, in 20 human liver samples. Spearman's rank correlation (r_s) was 0.75, $p < 0.001$. Individual data were derived from duplicate determinations.

the cytochrome P450-mediated styrene oxidation in the other 16 livers increased again at higher concentrations (figure 1b). Eadie-Hofstee plots of these 16 livers showed clear curvature. In this case the obtained hyperbolical Michaelis-Menten plots are best described by addition of two Michaelis-Menten equations. Since the low-affinity component was not saturated in the present study, the equation was simplified to:

$$v = \frac{V_{\max 1} \cdot [S]}{K_{m1} + [S]} + CLi_2 \cdot [S],$$

where CLi_2 represents the intrinsic clearance ($V_{\max 2}/K_{m2}$) for the low-affinity component (Clarke 1998). This formula was used to derive the enzyme constants for the high-affinity component ($V_{\max 1}$, K_{m1}) and the intrinsic clearance (CLi_2) for the

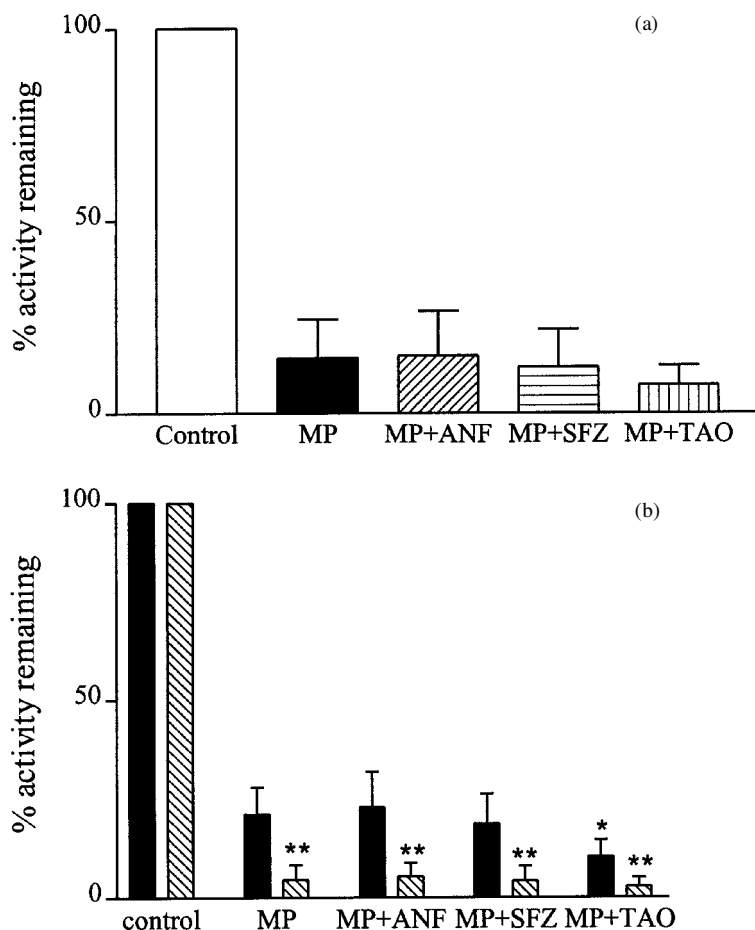


Figure 3. Inhibition of styrene oxidation activity; MP 4-methylpyrazole ($50 \mu\text{M}$), ANF α -naphthoflavone ($1 \mu\text{M}$), SFZ sulphaphenazole ($20 \mu\text{M}$) and TAO troleandomycin ($100 \mu\text{M}$). Error bars represent SD. Styrene ($16 \mu\text{M}$) was incubated with 0.5 mg microsomal protein for 10 min at 37°C with the inhibitors. MP, ANF and SFZ were added together with styrene; TAO was pre-incubated with 0.5 mg microsomal protein for 30 min at 37°C , after which styrene was added. (a) Overall inhibition in 10 human liver samples; (b) inhibition compared between two groups: 1, low enantiomeric excess ($n = 5$, black bars); 2, moderate enantiomeric excess ($n = 5$, hatched bars). *Significantly different from MP alone, by Mann-Whitney U-test, $p < 0.05$; **significantly different from group 1, by Mann-Whitney U-test, $p < 0.05$.

low-affinity component (table 1). Enzyme constants (V_{max1} , K_{m1}) for the other four livers were derived from a single Michaelis-Menten equation. V_{max1} was not normally distributed; K_{m1} , CLi_1 and CLi_2 were normally distributed, as judged from normal plots and statistical tests.

To assess the involvement of CYP2E1 in styrene metabolism, the correlation coefficient of V_{max1} against 4-nitrophenol hydroxylation activity, a model substrate for CYP2E1, was calculated (figure 2). A strong significant correlation ($r_s = 0.75$, $p < 0.001$) was obtained between 4-nitrophenol hydroxylation activity and the V_{max} of the high-affinity component. The involvement of CYP2E1 in the high-affinity component of styrene metabolism was supported by using specific inhibitors of cytochrome P450 isoforms (figure 3a). Extensive inhibition ($85 \pm 10\%$) was observed with 4-methylpyrazole, a specific inhibitor of CYP2E1 (Newton *et al.* 1995, Clarke 1998). No additional inhibition was observed with α -naphthoflavone,

Table 2. Styrene oxidation stratified by smoking, alcohol status and age.

Group	$V_{\max 1}^a$	K_{m1}^b
	Mean±SD	Mean±SD
Smoking (<i>n</i> = 10)	0.93±0.410	0.010±0.0036
Non-smoking (<i>n</i> = 10)	0.99±0.813	0.013±0.0071
Alcohol (<i>n</i> = 14)	0.88±0.377	0.011±0.0067
No alcohol (<i>n</i> = 6)	1.16±1.031	0.012±0.0031
Age:		
20–30 (<i>n</i> = 5)	1.07±0.557	0.010±0.0033
31–40 (<i>n</i> = 5)	1.25±1.102	0.008±0.0035
41–50 (<i>n</i> = 5)	0.81±0.268	0.015±0.0097
51–60 (<i>n</i> = 5)	0.72±0.237	0.012±0.0026

Styrene (0.53–1030 μM) was incubated with 0.5 mg microsomal protein for 10 min at 37 °C.

^a $V_{\max}$ for the high-affinity component of styrene oxidation, expressed as nmol.mg protein⁻¹.min⁻¹.

^b K_m for the high-affinity component of styrene oxidation, expressed in mM.

Table 3. Product enantioselectivity of cytochrome P450-mediated styrene oxidation with low (16 μM) and high styrene (1100 μM) concentration (*n* = 20).

Concentration	Enantiomeric excess (ee) ^a	
	Mean±SD	Range
Low (16 μM):		
All (<i>n</i> = 20)	14.8 (S)±6.9	4–27
Low ee (<i>n</i> = 12) ^b	9.8 (S)±3.1	4–12
Moderate ee (<i>n</i> = 8)	22.3 (S)±2.8	20–27
High (1100 μM):		
All (<i>n</i> = 20)	7.0 (R)±8.9	–11–22
Low ee (<i>n</i> = 8) ^b	2.3 (S)±5.4	–11–4
Moderate ee (<i>n</i> = 12)	13.1 (R)±4.0	8–22

Styrene (16 and 1100 μM) was incubated together with TCPO (50 μM , mEH inhibitor) for 10 min at 37 °C with 0.5 mg microsomal protein.

^a %. Absolute configuration of formed excess SO enantiomer in parentheses.

^b Significantly different from moderate ee by Mann–Whitney U-test.

an inhibitor of CYP1A2 (Newton *et al.* 1995, Ono *et al.* 1996), sulphaphenazole, an inhibitor of CYP2C8/9 (Newton *et al.* 1995, Ono *et al.* 1996, Hickman *et al.* 1998) or troleandomycin, an inhibitor of CYP3A4 (Chang *et al.* 1994, Newton *et al.* 1995).

Large interindividual differences were found in $V_{\max 1}$ and K_{m1} (table 1). The $V_{\max 1}$ differed eight-fold between the livers and the K_{m1} six-fold. No statistically significant differences were found in $V_{\max 1}$ and K_{m1} between smokers and non-smokers and alcohol users and abstainers or between the age groups (table 2). All livers were homozygote wild-type (c1/c1) for the *CYP2E1**5B allele and two livers were genotyped as heterozygotes (C/D) for the *CYP2E1**6 allele. No differences in enzyme constants were found between these two heterozygotes and the wild-type (data not shown).

Product enantioselectivity of cytochrome P450 was measured at two substrate concentrations: 16 and 1100 μM . The results are summarized in table 3. A moderate

enantioselectivity was found, with an enantiomeric excess (ee) of (*S*) of 15% at 16 μM . At 1100 μM , ee = 7%, with (*R*) being the preferred product. Interindividual differences in the enantioselective oxidation were considerable, with the *R/S* ratio ranging from an ee = 4 to 27% at the low styrene concentration. Statistical analysis (Kolmogorov-Smirnov test, $p < 0.05$) indicated that these data were not normally distributed. Based on the frequency distribution, the data were tentatively divided in two groups (4–12, 20–27%), which differed significantly using the Mann-Whitney U-test (table 3), and analysed the data obtained from the inhibition experiments separately for each group. The results indicated that 4-methylpyrazole inhibited styrene oxidation activity in the group with the higher ee by 95%, but that in the group with the low ee this inhibition was reduced to 80%. In the latter group, addition of troleandomycin inhibited the styrene oxidation activity further, to 90% (figure 3b), indicating the involvement of CYP3A4 in the styrene oxidation in this group.

Discussion and conclusions

In light of the neurotoxic and genotoxic potential of styrene and the different mutagenic potentials of the two enantiomeric oxiranes, the variability in clearance and enantioselectivity of styrene oxidation may play an important role in metabolic activation and, consequently, may be related to individual susceptibility to styrene toxicity.

The Michaelis-Menten plot indicated the involvement of at least two CYP450 isoforms with different K_m and $V_{\max}$. This is in agreement with Nakajima *et al.* (Nakajima *et al.* 1994). The high-affinity component had at least a 100-fold higher clearance of styrene than the low-affinity component, indicating that styrene is a much better substrate for the high-affinity component.

Enzyme constants for styrene metabolism in humans have been reported in two other studies. The $V_{\max 1}$ found in the present study (i.e. 0.96 nmol mg protein⁻¹ min⁻¹) was similar to the 1.05 and 1.39 nmol mg protein⁻¹ min⁻¹ in Nakajima *et al.* (1994) and the 2.1 nmol mg protein⁻¹ min⁻¹ in Mendrala *et al.* (1993). However, there is a considerable difference between the K_m found in the present study (0.011 mM) and those of Nakajima *et al.* (0.04 and 0.10 mM) and Mendrala *et al.* (0.09 mM). One reason for this discrepancy may be that in both of the latter studies styrene was added as a solution in acetone or methanol, solvents known to be substrates themselves for CYP450. In Mendrala *et al.* (1993), the final concentration of acetone in the incubation was 1.8%, while Nakajima *et al.* (1994) probably used < 1% methanol although the exact amount could not be tracked. A recent study showed that acetone and methanol (1% v/v) inhibit the activity of a number of CYP450, in particular CYP2E1, up to 70% (Hickman *et al.* 1998). If acetone and methanol act as a competitive inhibitor of styrene metabolism, then the apparent K_m for styrene could increase, however without affecting $V_{\max}$.

The interindividual variability observed in $V_{\max 1}$ and K_{m1} could not be explained by alcohol or smoking and was not age-related, since no significant changes in $V_{\max}$ or K_m were found with smoking or alcohol status or between age groups. To explain the interindividual variability, knowledge about which cytochrome P450 isoform(s) is responsible for styrene oxidation is relevant. Most studies indicate CYP2E1 as the responsible enzyme for styrene oxidation (Guengerich *et al.* 1991, Kim *et al.* 1997) but as shown in a study with individual cDNA-expressed cytochrome P450 isoforms

in cultured cells, CYP2B6, CYP1A2, CYP2C8 and, to a lesser extent, CYP3A4 can also contribute to styrene oxidation (Nakajima *et al.* 1993). The results of the *p*-nitrophenol hydroxylation and the inhibition experiments in the present study strongly suggests that CYP2E1 is the main enzyme responsible for the high-affinity component of styrene metabolism. Therefore, environmental or genetic factors that influence CYP2E1 activity could contribute to the interindividual variation found in styrene oxidation. Genetic polymorphism of the *CYP2E1* gene did not explain the observed variation; however, only two heterozygotes for the *CYP2E1**6 mutated allele and no *CYP2E1**5B mutated alleles were found. The small number of livers used and the rare occurrence of the variant *CYP2E1* alleles, therefore, does not allow a definite conclusion about the influence of the polymorphism on styrene oxidation. Other explanations for the observed interindividual variability in styrene metabolism may be: (1) polymorphisms in the coding region of the *CYP2E1* gene, which change the amino acid sequence of the protein, although a recent study showed that the coding region of the gene is highly conserved (Hu *et al.* 1997); (2) different degree of induction of CYP2E1 per liver by environmental factors like drugs, food, environmental pollutants, etc.; (3) variable contributions per liver of other CYP450 isoforms, like CYP3A4 or CYP2B6; or (4) availability of co-factors like NADPH-cytochrome P450 reductase.

The styrene-metabolizing enzyme(s) showed a moderate enantioselectivity. An *ee* = 15% (*S*) SO found at the low concentration of styrene is in agreement with studies with rat and rabbit liver microsomes, in which a 15–20% product enantioselectivity in favour of the (*S*)-enantiomer of SO has been reported (Watabe *et al.* 1983, Foureman *et al.* 1989, Bellucci *et al.* 1994). The dependence of *ee* on substrate concentration suggests, just like the present kinetic data (figure 1a), that at high styrene concentrations one or more other CYP450 beside CYP2E1 are involved in the styrene oxidation. If only one isoform with a defined enantioselectivity catalyses styrene oxidation, then the enantioselectivity of the oxidation would not vary with the substrate concentration (Wistuba *et al.* 1989, Chiappe *et al.* 1998).

The high interindividual variability in product enantioselectivity of the styrene oxidation at 16 μ M seems to suggest that, although CYP2E1 is the main enzyme, it might not be the only enzyme that oxidizes styrene at low concentrations. The present inhibition experiments implied that CYP2E1 is the primary enzyme in the moderate excess group (*ee* = *S* 22%), while in the low excess group (*ee* = *S* 9%) CYP3A4 also seemed to play a limited additional role. In terms of product enantioselectivity this would mean that CYP2E1 has a preference for the formation of the (*S*)-enantiomer of SO, whereas CYP3A4 has a preference for the (*R*)-enantiomer of SO (thereby decreasing the overall excess found). However, purified enzymes would be the best option to determine the steric course of an individual cytochrome P450 enzyme. Factors that enhance or decrease the individual CYP450 content/activity could alter the ratio of SO formed, thereby modulating styrene toxicity.

Both enantioselective formation and enantioselective hydrolysis of SO are important for the risk assessment of styrene. Wenker *et al.* (2000) have shown that mEH is highly enantioselective, favouring hydrolysis of (*R*)- over (*S*)-SO in a racemic mixture. In the present study, only a moderate product enantioselectivity of cytochrome P450-mediated styrene oxidation has been shown. Hence, it seems that the mEH-mediated hydrolysis is the determining factor in assessing the risks associated with enantioselective metabolism of styrene.

In conclusion, the present study indicates that cytochrome P450-mediated styrene oxidation is subject to appreciable interindividual variation and that at least two cytochrome P450 isoforms with different enzyme constants and enantioselectivity contribute to the oxidation. It is very likely that the enzyme responsible for the high-affinity component of styrene oxidation is CYP2E1. Therefore, factors that modulate CYP2E1 activity should be considered as modifying factors in styrene toxicity. The oxidation of styrene proceeded with a moderate product enantioselectivity, with a preference for the less toxic enantiomer.

References

- ARLIEN-SOBORG, P., 1992, Styrene. In P. Arlien-Soborg, *Solvent Neurotoxicity* (Boca Raton: CRC Press), pp. 129–153.
- BARALE, R., 1991, The genetic toxicology of styrene and styrene oxide. *Mutation Research*, **257**, 107–126.
- BELLUCCI, G., CHIAPPE, C., CORDONI, A. and MARIONI, F., 1994, Enantioselectivity in the rabbit liver microsomal biotransformation of styrene and phenylpropenes. *Biocatalysis*, **10**, 149–157.
- BOND, J. A., 1989, Review of the toxicology of styrene. *CRC Critical Reviews in Toxicology*, **19**, 227–249.
- CARRIERE, V., BERTHOUD, F., BAIRD, S., BELLOC, C., BEAUNE, P. and WAZIERS DE, I., 1996, Human cytochrome P450 2E1 (CYP2E1): from genotype to phenotype. *Pharmacogenetics*, **6**, 203–211.
- CHANG, T. K. H., GONZALEZ, F. J. and WAXMAN, D. J., 1994, Evaluation of triacetyloleandomycin, a-naphthoflavone and diethyldithiocarbamate as selective chemical probes for inhibition of human cytochromes P450. *Archives of Biochemistry and Biophysics*, **311**, 437–442.
- CHERRY, N. and GAUTRIN, D., 1990, Neurotoxic effects of styrene: further evidence. *British Journal of Industrial Medicine*, **47**, 29–37.
- CHIAPPE, C., DE RUBERTIS, A., AMATO, G. and GERVASI, P. G., 1998, Stereochemistry of the biotransformation of 1-hexene and 2-methyl-1-hexene with rat liver microsomes and purified P450 of rats and humans. *Chemical Research in Toxicology*, **11**, 1487–1493.
- CLARKE, S. E., 1998, *In vitro* assessment of human cytochrome P450. *Xenobiotica*, **28**, 1167–1202.
- FOUREMAN, G. L., HARRIS, C., GUENGERICH, F. P. and BEND, J. R., 1989, Stereoselectivity of styrene oxidation in microsomes and in purified cytochrome P-450 enzymes from rat liver. *Journal of Pharmacology and Experimental Therapeutics*, **248**, 492–497.
- GADBERRY, M. G., DENICOLA, D. B. and CARLSON, G. P., 1996, Pneumotoxicity and hepatotoxicity of styrene and styrene oxide. *Journal of Toxicology and Environmental Health*, **48**, 273–294.
- GUENGERICH, F. P., KIM, D. H. and IWASAKI, M., 1991, Role of human cytochrome P-450 IIE1 in the oxidation of many low molecular weight cancer suspects. *Chemical Research in Toxicology*, **4**, 168–179.
- GUENGERICH, F. P. and TURVY, C. G., 1991, Comparison of levels of several human microsomal cytochrome P-450 enzymes and epoxide hydrolase in normal and disease states using immunochemical analysis of surgical liver samples. *Journal of Pharmacology and Experimental Therapeutics*, **256**, 1189–1194.
- HAYASHI, S., WATANABE, J. and KAWAJIRI, K., 1991, Genetic polymorphisms in the 5'-flanking region change transcriptional regulation of the human cytochrome P450IIE1 gene. *Journal of Biochemistry*, **110**, 559–565.
- HICKMAN, D., WANG, J.-P., WANG, Y. and UNADKAT, J. D., 1998, Evaluation of the selectivity of *in vitro* probes and suitability of organic solvents for the measurement of human cytochrome P450 monooxygenase activities. *Drug Metabolism and Disposition*, **26**, 207–215.
- HILDESHEIM, A., CHEN, C.-J., CAPORASO, N. E., CHENG, Y.-J., HOOVER, R. N., HSU, M.-M., LEVINE, P. H., CHEN, I.-H., CHEN, J.-Y., YANG, C. S., DALY, A. K. and IDLE, J. R., 1995, Cytochrome P4502E1 genetic polymorphisms and risk of nasopharyngeal carcinoma: Results from a case-control study conducted in Taiwan. *Cancer Epidemiology, Biomarkers and Prevention*, **4**, 607–610.
- HU, Y., OSCARSON, M., JOHANSSON, I., YUE, Q.-Y., DAHL, M.-L., TABONE, M., ARINCO, S., ALBANO, E. and INGELMAN-SUNDBERG, M., 1997, Genetic polymorphism of human CYP2E1: characterization of two variant alleles. *Molecular Pharmacology*, **51**, 370–376.
- IARC, 1994, Styrene-7,8-oxide. *IARC Monographs on the Evaluation of Carcinogenic Risk to Humans*, **60**, 321–346.
- KEŽIĆ, S., JAKASA, I., WENKER, M. A. M., BOOGAARD, P. J., MONSTER, A. C. and WOLFF DE, F. A., 2000, *Journal of Chromatography B*, **749**, 265–274.
- KIM, H., WANG, R. S., ELOVAARA, E., RAUNIO, H., PELKONEN, O., AOYAMA, T., VAINIO, H. and NAKAJIMA, T., 1997, Cytochrome P450 isoenzymes responsible for the metabolism of toluene and styrene in human liver microsomes. *Xenobiotica*, **27**, 657–665.
- KOOP, D. R., 1986, Hydroxylation of *p*-nitrophenol by rabbit ethanol-inducible cytochrome P-450 isoenzyme 3a. *Molecular Pharmacology*, **29**, 399–404.

- LILIS, R., LORIMER, W. V., DIAMOND, S. and SELIKOFF, I. J., 1978, Neurotoxicity of styrene in production and polymerization workers. *Environmental Research*, **15**, 133–138.
- LUCAS, D., MENEZ, C., GIRRE, C., BERTHOU, F., BODENEZ, P., JOANNET, I., HISPARD, E., BARDOU, L. and MENEZ, J., 1995, Cytochrome P450 2E1 genotype and chlorzoxazone metabolism in healthy and alcoholic Caucasian subjects. *Pharmacogenetics*, **5**, 298–304.
- MENDRALA, A. L., LANGVARDT, P. W., NITSCHKE, K. D., QUAST, J. F. and NOLAN, R. J., 1993, In vitro kinetics of styrene and styrene oxide metabolism in rat, mouse, and human. *Archives of Toxicology*, **67**, 18–27.
- MUTTI, A., MAZZUCCHI, A., RUSTICHELLI, P., GRAZIANO, F., ARFINI, G. and FRANCHINI, I., 1984, Exposure-effect and exposure-response relationships between occupational exposure to styrene and neuropsychological functions. *American Journal of Industrial Medicine*, **5**, 275–286.
- NAKAJIMA, T., ELOVAARA, E., GONZALEZ, F. J., GELBOIN, H. V., RAUNIO, H., PELKONEN, O., VAINIO, H. and AOYAMA, T., 1994, Styrene metabolism by cDNA-expressed human hepatic and pulmonary cytochromes P450. *Chemical Research in Toxicology*, **7**, 891–896.
- NAKAJIMA, T., ELOVAARA, E., GONZALEZ, F. J., GELBOIN, H. V., VAINIO, H. and AOYAMA, T., 1993, Characterization of the human cytochrome P450 isoenzymes responsible for styrene metabolism. *IARC Scientific Publications*, **127**, 101–108.
- NEWTON, D. J., WANG, R. W. and LU, A. Y. H., 1995, Cytochrome P450 inhibitors. Evaluation of specificities in the *in vitro* metabolism of therapeutic agents by human liver microsomes. *Drug Metabolism and Disposition*, **23**, 154–158.
- ONO, S., HATANAKA, T., HOTTA, H., SATOH, T., GONZALEZ, F. J. and TSUTSUMI, M., 1996, Specificity of substrate and inhibitor probes for cytochrome P450: evaluation of *in vitro* metabolism using cDNA-expressed human P450 and human liver microsomes. *Xenobiotica*, **26**, 681–693.
- PIRMOHAMED, M., KITTINGHAM, N. R., QUEST, L. J., ALLOTT, R. L., GREEN, V. J., GILMORE, I. T. and PARK, B. K., 1995, Genetic polymorphism of cytochrome P4502E1 and risk of alcoholic liver disease in Caucasians. *Pharmacogenetics*, **5**, 351–357.
- POWELL, H., KITTINGHAM, N. R., PIRMOHAMED, M., SMITH, D. A. and PARK, B. K., 1998, Expression of cytochrome P4502E1 in human liver: assessment by mRNA, genotype and phenotype. *Pharmacogenetics*, **8**, 411–421.
- REBERT, C. S. and HALL, T. A., 1994, The neuroepidemiology of styrene: a critical review of representative literature. *CRC Critical Reviews in Toxicology*, **24**, S57–S106.
- ROSENGREN, L. E. and HAGLID, K. G., 1989, Long term neurotoxicity of styrene. A quantitative study of glial acidic protein (GFA) and S-100. *British Journal of Industrial Medicine*, **46**, 316–320.
- SEILER, J. P., 1990, Chirality-dependent DNA reactivity as the possible cause of the differential mutagenicity of the two components in an enantiomeric pair of epoxides. *Mutation Research*, **245**, 165–169.
- SUMNER, S. J. and FENNEL, T. R., 1994, Review of the metabolic fate of styrene. *Critical Reviews in Toxicology*, **24**, S11–S33.
- UEMATSU, F., IKAWA, S., KIKUCHI, H., SAGAMI, I., KANAMARU, R., ABE, T., SATOH, K., MOTOMIYA, M. and WATANABE, M., 1994, Restriction fragment length polymorphism of the human CYP2E1 (cytochrome-P450IIE1) gene and susceptibility to lung cancer – possible relevance to low smoking exposure. *Pharmacogenetics*, **4**, 58–63.
- UEMATSU, F., KIKUCHI, H., MOTOMIYA, M., ABE, T., SAGAMI, I., OHMACHI, T., WAKUI, A., KANAMARU, R. and WATANABE, M., 1991, Association between restriction fragment polymorphism of the human P450IIE1 gene and susceptibility to lung cancer. *Japanese Journal of Cancer Research*, **82**, 254–266.
- WATABE, T., OZAWA, N. and HIRATSUKA, A., 1983, Studies on metabolism and toxicity of styrene – VI. regioselectivity in glutathione S-conjugation and hydrolysis of racemic, R- and S-phenyloxiranes in rat liver. *Biochemical Pharmacology*, **32**, 777–785.
- WATANABE, J., HAYASHI, S. and KAWAJIRI, K., 1994, Different regulation and expression of the human CYP2E1 gene due to the RsaI polymorphism in the 5'-flanking region. *Journal of Biochemistry*, **116**, 321–326.
- WELP, E., KOGEVINAS, M., ANDERSEN, A., BELLANDER, T., BIOCCA, M., COGGON, D., ESTEVE, J., GENARRO, V., KOLSTAD, H., LUNDBERG, I., LYNGE, E., PARTANEN, T., SPENCE, A., BOFFETTA, P., FERRO, G. and SARACCI, R., 1996, Exposure to styrene and mortality from nervous system diseases and mental disorders. *American Journal of Epidemiology*, **144**, 623–633.
- WENKER, M. A. M., KEŽIĆ, S., MONSTER, A. C. and WOLFF DE, F. A., 2000, Metabolism of styrene-7,8-oxide in human liver *in vitro*: interindividual variation and stereochemistry. *Toxicology and Applied Pharmacology*, **169**, 52–58.
- WISTUBA, D., NOWOTNY, H. P., TRÄGER, O. and SCHURIG, V., 1989, Cytochrome P-450-catalyzed asymmetric epoxidation of simple prochiral and chiral aliphatic alkenes: Species dependence and effect of enzyme induction on enantioselective oxirane formation. *Chirality*, **1**, 127–136.

Species difference in stereoselective involvement of CYP3A in the mono-*N*-dealkylation of disopyramide

L. ZHANG^{††}, J. F. FITZLOFF[†], L. C. ENGEL[†]
and C. S. COOK^{†*}

[†] Metabolism and Safety Evaluation, Pharmacia, Skokie, IL 60077, USA

^{††} Department of Medicinal Chemistry and Pharmacognosy, College of Pharmacy,
University of Illinois at Chicago, Chicago, IL 60612-7231, USA

Received 4 September 2000

1. To determine which CYP isoenzyme is involved in the *N*-dealkylation of disopyramide (DP) metabolism in human and dog, and to determine the stereoselectivity of DP metabolism with human CYP and dog CYP isoenzymes, the following *in vitro* metabolism studies of DP were conducted: correlation between human CYP isoenzyme activities and DP metabolism with human liver microsomes; inhibition of DP metabolism in human and dog liver microsomes with chemical inhibitors of CYP isoenzymes; inhibition of DP metabolism in human microsomes with human CYP antibodies; inhibition of DP metabolism in dog liver microsomes with human and dog CYP antibodies; metabolism of DP with human (CYP3A4) and dog (CYP3A12) cDNA-expressed isoenzymes; determination of K_m and V_{max} of DP enantiomers by using cDNA-expressed CYP3A4 and CYP3A12.

2. In human liver microsomes, the formation of the mono-*N*-dealkylated disopyramide (MNDP) metabolite was best correlated with CYP3A4 activities. DP metabolism was substantially inhibited by ketoconazole, troleandomycin (TA) and human CYP3A4 antibody. DP was metabolized by cDNA-expressed CYP3A isoenzymes. In dog liver microsomes, DP metabolism was inhibited by ketoconazole, TA and dog anti-CYP3A12. DP was also metabolized by cDNA-expressed CYP3A12.

3. CYP3A4 and CYP3A12 are the principal isoenzymes involved in DP metabolism in human and dog respectively. There was no stereoselectivity in *N*-dealkylation of DP by human CYP3A4. However, there was notable stereoselectivity in the *N*-dealkylation by dog CYP3A12.

Introduction

Disopyramide, a chiral compound having a single centre of optical activity, is a class IA anti-arrhythmic agent widely used for treatment of various types of arrhythmias (Broghden *et al.* 1987, Heel *et al.* 1978). In the human, disopyramide (DP) is only converted by *N*-dealkylation to MNDP, whereas in dog the MNDP is further metabolized to di-*N*-dealkylated disopyramide (Karim *et al.* 1972). Species differences in the stereoselective pharmacokinetics of disopyramide have been reported (Cook *et al.* 1982, Le Corre *et al.* 1992). The stereoselectivity of DP was primarily due to stereospecific aryl hydroxylation in rat, stereoselective *N*-dealkylation in dog, and stereoselective plasma protein binding and biliary excretion in human. More recently, it has been reported that metabolism of DP in human liver microsomes was inhibited by erythromycin (Echizen *et al.* 1994), suggesting that CYP3A may be involved in the metabolism of DP in human.

The purpose of the current study was to investigate which CYP isoenzyme is responsible for the mono-*N*-dealkylation of DP in human and dog, whether there is

* Author for correspondence; e-mail: chyung.s.cook@monsanto.com

a species difference in the stereoselectivity of *N*-dealkylation in human and dog, and if this is due to different subfamilies or within the same subfamily of CYP isoenzymes.

Materials and methods

Materials

[¹⁴C]disopyramide phosphate was prepared from phenyl(2-pyridyl)acetonitrile-1-¹⁴C (lot no. GDS4361-19, 98.5% pure, specific activity of 41.1 $\mu\text{Ci mg}^{-1}$) by the method of Cusic and Sause (1965) and unlabeled disopyramide (98.2% pure) was from G. D. Searle & Co. (Skokie, IL, USA). Pooled human liver microsomes and baculovirus-insect cell-expressed human CYP isoenzymes (CYP1A2, CYP2A6, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4) and human CYP antibodies (anti-CYP1A, anti-CYP2A6, anti-CYP2C, anti-CYP2D6, anti-CYP2E1, anti-CYP3A4) were from Gentest Corp. (Woburn, MA, USA) and stored at -80°C until use. The supplier provided protein concentrations and P450 contents. Human liver microsomes, which had been characterized for CYP isoenzyme activities with selective markers, were from XenoTech LLC (Kansas City, KS, USA). Dog liver microsomes (batch: 1003) were from In Vitro Technology, Inc. (Baltimore, MD, USA). Microsomes containing recombinant dog CYP isoenzymes (CYP3A12, CYP2C21, CYP2D15) were generated as described in Paulson *et al.* (1999). All other chemicals were commercially available unless otherwise stated.

Preparation of *R*-($-$)- and *S*-($+$)-[¹⁴C]DP and unlabeled DP

R-($-$)- and *S*-($+$)-[¹⁴C]DP and DP were prepared as follows: the optical resolution of *R,S*-($\pm$)-[¹⁴C]DP was achieved by using HPLC with a Chiral AGP column (100 $\times$ 4.0 mm, 5 μm , Chrom Tech, Sweden) with a radiochemical detector. A mobile phase containing 2-propanol:sodium phosphate buffer (0.02 M, pH 6.9, 11:89 v/v) under isocratic conditions was employed over a 16-min run at a flow rate of 0.9 ml min⁻¹ for each injection.

The chiral separation of unlabeled *R,S*-($\pm$)-DP was achieved by using a preparative HPLC method with a UV detector. The preparative liquid chromatography system consisted of a pump (P73, Rainin Instrument Co., Inc., Woburn, MA, USA), a UV detector (D-56, Rainin Instrument Co., Inc., USA), a Chiralpak AD column (250 $\times$ 20 mm, 10 μm , Regis Technologies, Inc., Morton Grove, IL, USA). The isocratic chromatography was carried out at a flow rate of 20 ml min⁻¹ using a mobile phase of ethanol:heptane:triethylamine (10:90:0.1 by vol.), with UV detection at 254 nm.

Preparation of polyclonal antibodies recognizing canine CYP3A12, CYP2C21 and CYP2D15

Polyclonal antibodies against dog CYP3A12, CYP2C21 and CYP2D15 were raised in rabbit against synthetic peptides (prepared by Sigma/Genysis, The Woodlands, TX, USA) corresponding to several regions of the following predicted protein sequences: DFFRKSVERMKESRLKDKQKHRVDFL-QLMIN for anti-CYP3A12, FKFISEKIKEHEESLNFSNPRDFIDY for anti-CYP2C21 and MIQ-EHRKTREDPTQPPR for anti-CYP2D15. The peptides correspond to the G/H loop region (haem-binding domain). Immunological grade peptides were monitored for purity using reverse-phase HPLC that included a VYDAC RP C-8 column (VYDAC, Hesperia, CA, USA) and a linear gradient system from 10 to 90% acetonitrile in water with a flow rate of 1.5 ml min⁻¹. Each peptide was conjugated to keyhole limpet haemocyanin (KLH) and suspended in complete Freund's adjuvant. The conjugated peptide was then injected subcutaneously at multiple sites into rabbits. Each conjugated peptide was injected into two rabbits. All subsequent immunizations were given in incomplete Freund's adjuvant. In general, five subsequent injections were given at 2-week intervals following the initial immunization. IgG fractions were affinity-purified using a Sepharose-protein A column. The IgG fractions from the two rabbits were pooled. The combined IgGs were each diluted 1:10 in an immunoblot analysis of canine microsomes. The IgG fraction of the anti-CYP3A12, anti-CYP2C21 or anti-CYP2D15 recognized a single band that migrated with an apparent molecular weight of 50 kD in microsomes that contained respective recombinant CYP3A12, CYP2C21 or CYP2D15. The anti-CYP3A12 antibodies did not recognize any proteins other than recombinant canine CYP3A12 in immunoblots of microsomes that contained recombinant canine CYP2D15, CYP2C21 or CYP2B11. Similarly, the anti-CYP2D15 antibodies recognized recombinant canine CYP2D15 but not CYP3A12, CYP2C21 or CYP2B11. The anti-CYP2C21 antibodies did not recognize recombinant canine CYP2D15, CYP3A12 or CYP2B11, except for CYP2C21.

Enzyme assays

DP was incubated in a sodium phosphate buffer media (Echizen *et al.* 1994). The basic incubation medium contained 2 mg human liver microsomes ml⁻¹, 5 mM NADP, 20 mM glucose 6-phosphate, 20 mM MgCl₂, 0.1 mM EDTA, 4 IU ml⁻¹ glucose 6-phosphate dehydrogenase, 0.1 M sodium

phosphate buffer (pH 7.4) and [^{14}C]DP or [^{14}C]DP enantiomers at various concentrations in a final volume of 200 μl . The mixture was prewarmed with the NADPH-generating system for 2 min at 37 °C. Reactions were initiated by adding each of the DP enantiomers, which were incubated for 45 min. Reactions were terminated by placing the incubation tubes on ice, immediately adding 200 μl ice-cold methanol and vortex-mixing. The samples were then centrifuged and the supernatant was injected into an HPLC as described below.

Incubation conditions used for the eight different recombinant human isoenzymes (CYP1A2, CYP2A6, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4) and three recombinant dog CYP isoenzymes (CYP2D15, CYP2C21, CYP3A12) were the same as those used for human liver microsomes, except for the incubation times (50 min for the human CYP isoenzymes; 60 min for the dog CYP isoenzymes). For all incubations using these recombinant isoenzymes, microsomal concentrations equivalent to 2 mg protein ml^{-1} were used.

A correlation study of the metabolic rates of DP and CYP isoenzyme activities with a respective marker substrate was conducted as follows: [^{14}C]DP phosphate salt (2.5 μM) was incubated with 15 individual human liver microsomes, which had been characterized for CYP isoenzyme activities with selective markers by XenoTech LLC. The metabolic activity of CYP1A2 was measured by the *O*-deethylation of 7-ethoxyresorufin, CYP2A6 by the 7-hydroxylation of coumarin, CYP2B6 by the deethylation of 7-ethoxy-4-trifluoro-methylcoumarin, CYP2C9 by the methylhydroxylation of tolbutamide, CYP2C19 by the 4'-hydroxylation of *S*-mephenytoin, CYP2D6 by the *O*-demethylation of dextromethorphan, CYP2E1 by the 6-hydroxylation of chlorzoxazone, and CYP3A4 by the *N*-demethylation of dextromethorphan.

Sample analyses

Concentrations of DP and the MNDP metabolite were determined by an HPLC method using radiochemical detection. The HPLC system consisted of a model 1050HP pump and 1050HP autosampler (Hewlett Packard, Wilmington, DE, USA) with a SupelcosilTM LC-18DB (3 μm , 33 $\times$ 4.6 mm) and a SupelcosilTM LC-18DB HPLC column (5 μm , 250 $\times$ 4.6 mm) (Supelco, Inc., Bellefonte, PA, USA). The radioactivity was detected by a Flo-One detector (Flo-One/Beta Model A-500, Packard, Meriden, CT, USA) programmed to monitor for ^{14}C . A mobile phase consisting of Milli-Q water/acetonitrile/methanol/dibutylamine phosphate buffer, pH 2.5 (70:19:10:1 v/v) was delivered at a flow rate of 1.0 ml min^{-1} .

Kinetics of DP enantiomers

The formation rate of MNDP was linear at 37 °C for incubation times of up to 45 min at concentrations of racemic DP or DP enantiomers of 5 or 250 μM and at a human microsomal concentration of 2 mg ml^{-1} . Since a linear relationship was observed between the formation rates of MNDP over 45 min and protein concentration of up to 2 mg ml^{-1} , the kinetic studies were performed at 37 °C with a 30-min incubation and at a protein concentration of 2 $\text{mg human liver microsomes ml}^{-1}$.

The kinetic parameters were estimated initially by the graphic analysis of Lineweaver-Burk plots, and the values obtained were used as the first estimate for non-linear regression analysis using WinNonlin (v.1.5; Scientific Consulting, Inc., USA), according to the following Michaelis-Menten equations for one (equation 1) and two (equation 2) binding sites:

$$V = V_{\max}[S]/([S] + [K_m]) \quad (1)$$

$$V = V_{\max 1}[S]/([S] + [K_{m1}]) + V_{\max 2}[S]/([S] + [K_{m2}]), \quad (2)$$

where V is the rate of formation of MNDP, $[S]$ is the concentration of *R*-(−)- or *S*-(+)-DP in the incubation mixture, K_m is the concentration of substrate at which the rate of metabolite formation is equal to one-half of $V_{\max}$, and $V_{\max}$ is the maximum enzyme rates. K_{m1} , $V_{\max 1}$ and K_{m2} , $V_{\max 2}$ are K_m and $V_{\max}$ for high- and low-affinity sites respectively.

Inhibition study

The effects of co-incubation of specific inhibitors for different human CYP isoenzymes on the microsomal metabolism of DP were studied separately. The formation rate of the MNDP metabolite from [^{14}C]DP (2.5 μM) was evaluated in the absence (control) and presence of the following known isoform-specific inhibitors (2.5 and 50 μM): α -naphthoflavone for CYP1A2, tranlylcypromine for CYP2A6, sulphaphenazole for CYP2C9, tranlylcypromine for CYP2C19, quinidine for CYP2D6, dihydrocapsaicin for CYP2E1 and ketoconazole and troleandomycin (TA) for CYP3A4. [^{14}C]DP was pre-incubated for 2 min with or without CYP isoform-specific inhibitor and with the NADPH-generating system. Human or dog liver microsomes were added to initiate the reaction and incubated for 50 and 60 min respectively, at 37 °C in a final incubation volume of 200 μl . All of isoform-specific inhibitors were studied at two different concentrations selected for the respective CYP isoforms on the

basis of a preliminary study. Inhibitors were dissolved in water where appropriate or in suitable organic solvents (ethanol, acetone or dimethyl sulphoxide) and then serially diluted with water to contain < 0.5% solvent in the final incubation mixture.

For the immuno-inhibition study, four monoclonal (anti-CYP2A6, anti-CYP2E1, anti-CYP2D6, anti-CYP3A4) and two polyclonal antibodies (anti-CYP1A, anti-CYP2C) were used with the human liver microsomes. Each anti-CYP IgG (5–20 μ l) was mixed with 20 μ l pooled human liver microsomes (400 μ g protein) and placed in an ice bath for 30 min. The final volume of the incubation mixture was brought to 200 μ l using sodium phosphate buffer. For the dog microsomal inhibition study, pooled dog liver microsomes were incubated with and without human anti-CYP IgG or dog anti-CYP IgG (anti-CYP2C21, anti-CYP2D15 and anti-CYP3A12). The concentration of racemic DP was 2.5 μ M. All other incubation conditions were the same as described above.

Results

Correlation with CYP marker activities

Rates of MNDP formation were correlated with specific CYP isoenzyme activities in human liver microsomes (figure 1). The rates of [^{14} C]DP *N*-dealkylation were best correlated with the dextromethorphan *N*-demethylation activities (CYP3A4) when compared with *O*-deethylation of 7-ethoxyresorufin (CYP1A2), 7-hydroxylation of coumarin (CYP2A6), deethylation of 7-ethoxy-4-trifluoromethylcoumarin (CYP2B6), methylhydroxylation of tolbutamide (CYP2C9), 4'-hydroxylation of *S*-mephenytoin (CYP2C19), *O*-demethylation of dextromethorphan (CYP2D6) and 6-hydroxylation of chlorzoxazone (CYP2E1).

Metabolism by microsomes from CYP cDNA-expressed cells

When racemic [^{14} C]DP was incubated with microsomes from cDNA-expressed human CYP isoenzymes, [^{14} C]DP was metabolized only by the CYP3A4 isoenzyme and $22.3 \pm 1.2\%$ of the initial [^{14} C]DP concentration (2.5 μ M) was MNDP. There was no notable metabolism with CYP1A2, CYP2A6, CYP2B6, CYP2C9, CYP2C19 and CYP2E1 isoenzymes. On incubation of [^{14} C]DP with microsomes prepared from cDNA-expressed dog CYP isoenzymes, the formation of MNDP was confined to CYP3A12 with $11.6 \pm 0.8\%$ of the initial [^{14} C]DP concentration observed as MNDP. There was no metabolism observed with CYP2C21 and CYP2D15.

Inhibition of MNDP formation

The inhibition of [^{14} C]DP metabolism in human liver microsomes was investigated at a [^{14} C]DP concentration of 2.5 μ M and inhibitor concentrations of 2.5 and 50 μ M (figure 2). In the presence of ketoconazole and TA, inhibition of [^{14} C]DP metabolism was > 60% regardless of inhibitor concentrations. With other inhibitors, DP metabolism was only marginally reduced (< 20%). Inhibition of [^{14} C]DP metabolism was also examined with human CYP antibodies (figure 3). The formation of the MNDP metabolite with human liver microsomes was extensively inhibited by monoclonal CYP3A4 antibody. The inhibition activity of monoclonal anti-CYP3A4 was $\sim 83\%$ compared with the control group. There were little or no inhibition activities of MNDP metabolite formation when human liver microsomes were incubated with other monoclonal antibodies (anti-CYP2A6, anti-CYP2D6 and anti-CYP2E1). The same results were observed when microsomes were incubated with polyclonal antibodies to CYP1A and CYP2C.

With dog liver microsomes, MNDP was the only metabolite in contrast to the *in vivo* metabolism. In dog (*in vivo*), MNDP is further metabolized to di-*N*-dealkylated metabolite (Karim *et al.* 1972). The inhibition of DP metabolism by ketoconazole

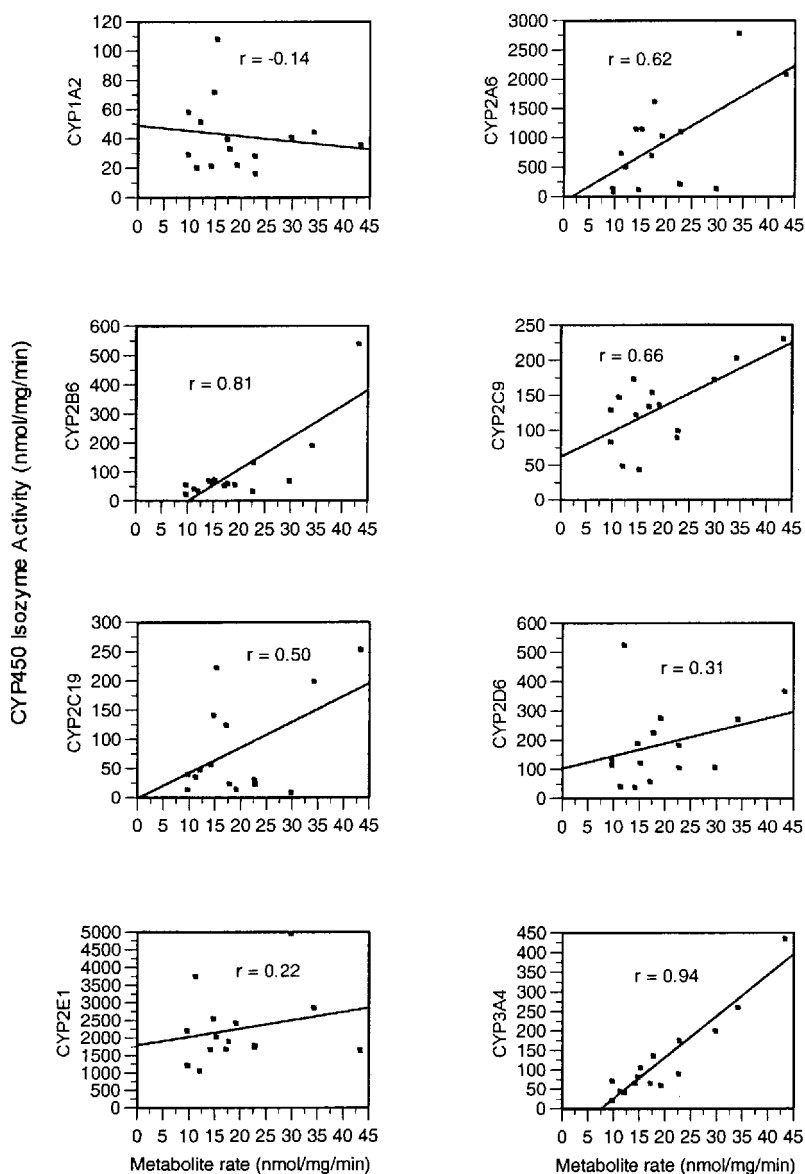


Figure 1. Relationship between CYP isoenzyme activity and metabolic rate of DP.

and TA was up to 55 and 36% respectively, in a concentration-dependent manner (figure 2). With other inhibitors, reduction of DP metabolism was minimal ($< 15\%$).

When DP metabolism in dog liver microsomes was examined with and without human polyclonal anti-CYP1A and anti-CYP2C, and monoclonal anti-CYP2A6, anti-CYP2D6, anti-CYP2E1 and anti-CYP3A4, none of human CYP antibodies exhibited inhibition activity for the mono-*N*-dealkylation of [^{14}C]DP when compared with the control group (figure 3). In the immuno-inhibition study with dog anti-CYP2C21, anti-CYP2D15 and anti-CYP3A12 (figure 3), the anti-

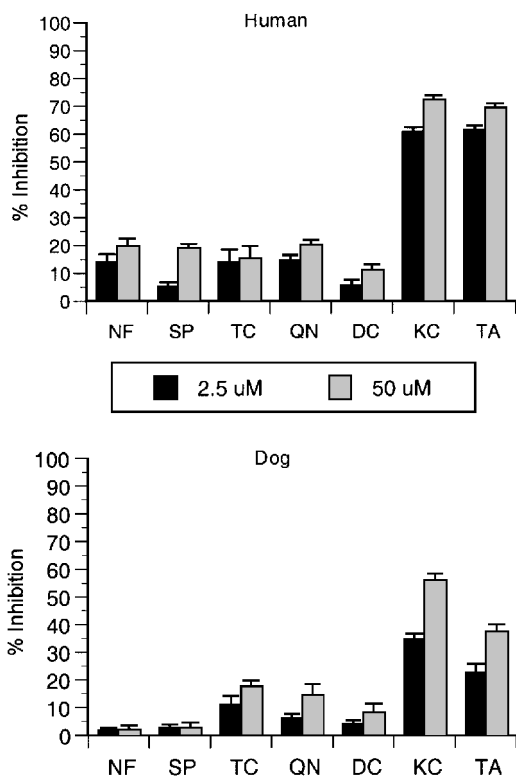


Figure 2. Metabolic activity of [^{14} C]DP in the presence of various CYP inhibitors in human (upper) and dog (lower) liver microsomes. Data are the mean ($\pm$ SEM) of percentage inhibition determinations. NF, naphthoflavone; SP, sulphaphenazole; TC, tranlycypromine; QN, quinidine; DC, dihydrocapsaicin; KC, ketoconazole; TA, troleandomycin.

CYP3A12 showed the inhibition activity (53%) for the formation of MNDP compared with the control group. However, the anti-CYP2C21 and anti-CYP2D15 exhibited no notable inhibition activity.

Kinetics of DP metabolism

Racemic [^{14} C]DP was metabolized to [^{14} C]MNDP in an NADPH-dependent manner upon incubation with human and dog liver microsomes, and cDNA-expressed CYP3A4 and CYP3A12. Figure 4 shows plots of the MNDP formation rate versus [^{14} C]DP concentrations in human liver microsomes and cDNA-expressed CYP3A4 microsomes, with Lineweaver-Burk transformations of the same data (Figure 4, inserts). The Lineweaver-Burk plot demonstrates that the formation of MNDP with human liver microsomes exhibited biphasic enzyme kinetics. In the concentration range examined, Michaelis-Menten parameters $V_{\max 1}$ and $V_{\max 2}$ for racemic MNDP were 30.3 ± 12.2 and 699 ± 22 nmol min $^{-1}$ mg protein $^{-1}$ respectively, and K_{m1} and K_{m2} were 12.2 ± 1.7 and 2504 ± 321 μ M respectively. The hepatic intrinsic clearances of racemic DP for high- and low-binding components of human liver microsomes calculated from $V_{\max}/K_m$ were 0.15 and 0.02 l h $^{-1}$ mg $^{-1}$ protein respectively.

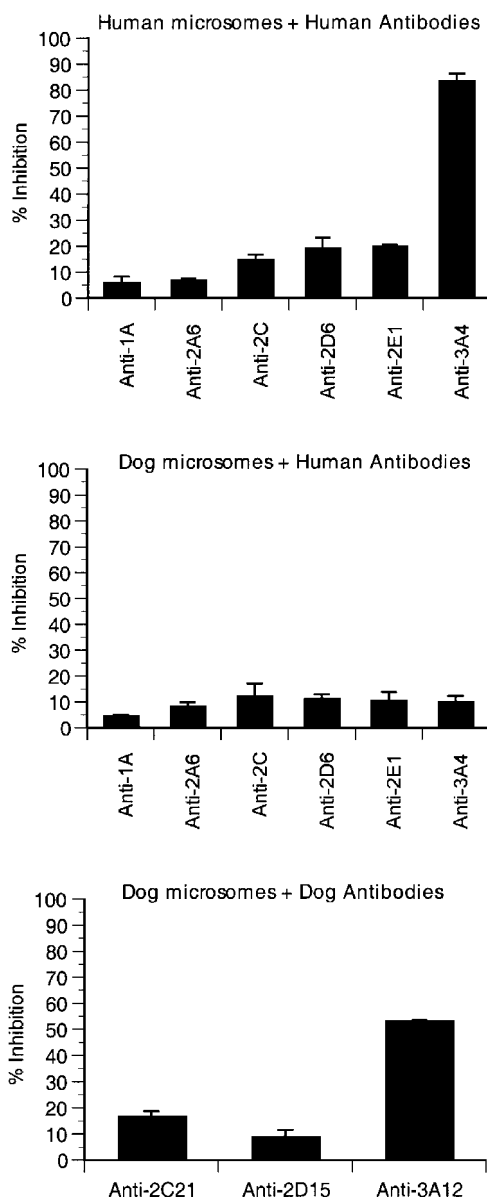


Figure 3. Metabolic activities of DP in human liver microsomes in the presence of various human CYP antibodies (upper), dog liver microsomes in the presence of various human CYP antibodies (middle) and dog CYP antibodies (bottom). Data are the mean ($\pm$ SEM) of metabolic activities of DP determinations.

With cDNA-expressed CYP3A4, enzyme kinetics were monophasic, hence $V_{\max}$ for racemic MNDP was $637 \pm 22 \text{ nmol min}^{-1} \text{ mg}^{-1} \text{ protein}$, and K_m was $1637 \pm 22 \mu\text{M}$. The hepatic intrinsic clearance of racemic DP by CYP3A4 calculated from $V_{\max}/K_m$ was $0.02 \text{ l h}^{-1} \text{ mg}^{-1} \text{ protein}$, which was similar to the clearance of the low-affinity component of human liver microsomes.

Stereoselectivity of MNDP formation from *R*-(-)- and *S*-(+)-DP was examined with cDNA-expressed CYP3A4 (figure 5). The formation rates of

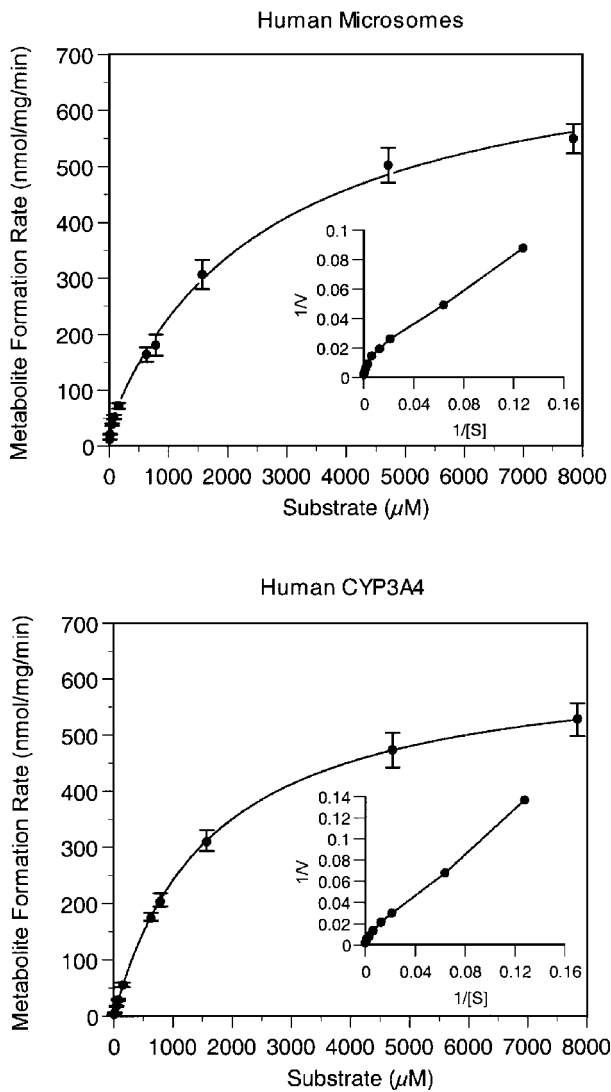


Figure 4. Formation rate of MNDP from DP in human liver microsomes (upper) and cDNA-expressed CYP3A (lower). The inset shows the Lineweaver-Burk plot. Data are the mean ($\pm$ SEM) of formation rate of MNDP determinations.

MNDP from *R*-($-$)- and *S*-($+$)-[^{14}C]DP were almost identical, indicating no stereoselectivity in *N*-dealkylation of [^{14}C]DP by human CYP3A4. V_{max} and K_{m} for the *S*-($+$)-enantiomer were $286 \pm 9 \text{ nmol min}^{-1} \text{ mg}^{-1} \text{ protein}$ and $330 \pm 32 \mu\text{M}$ respectively. V_{max} and K_{m} for the *R*-($-$)-enantiomer were $288 \pm 8 \text{ nmol min}^{-1} \text{ mg}^{-1} \text{ protein}$ and $355 \pm 32 \mu\text{M}$, which is similar to the respective values for the *S*-($+$)-enantiomer.

In contrast to human CYP3A4, the formation rate of MNDP was stereoselective with dog cDNA-expressed CYP3A12 (figure 5). V_{max} and K_{m} for the *R*-($-$)-enantiomer were $316 \pm 11 \text{ nmol min}^{-1} \text{ mg}^{-1} \text{ protein}$ and $574 \pm 55 \mu\text{M}$ respectively. V_{max} and K_{m} for the *S*-($+$)-enantiomer were $398 \pm 8 \text{ nmol min}^{-1} \text{ mg}^{-1} \text{ protein}$ and $450 \pm 16 \mu\text{M}$ respectively. The hepatic intrinsic clearances for the *S*-($+$)- and

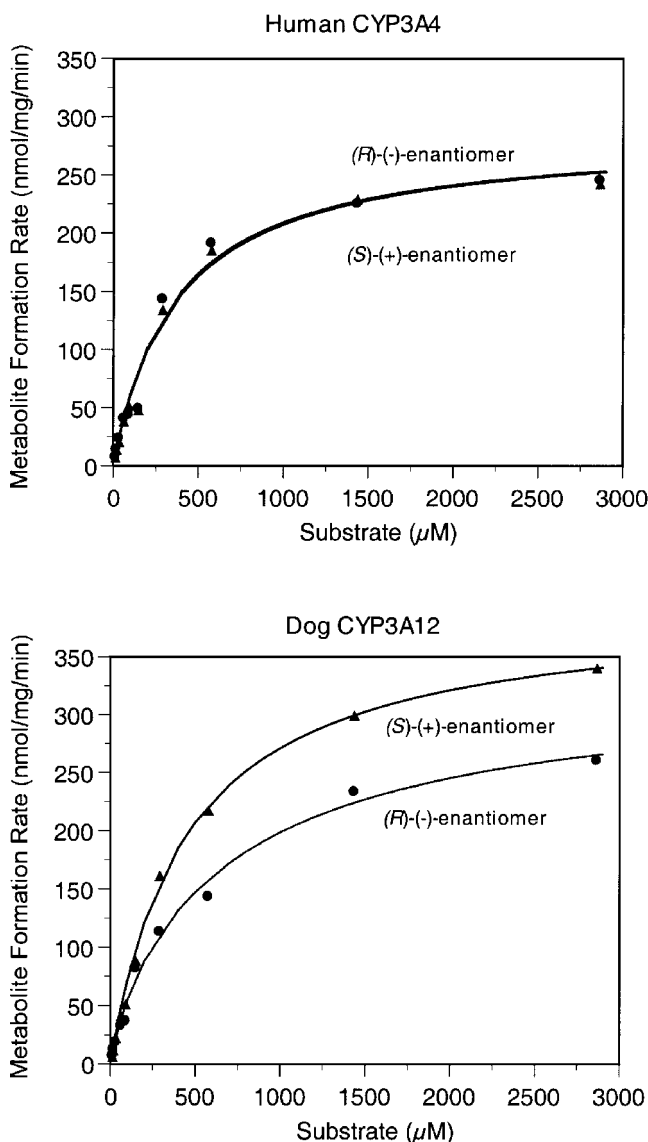


Figure 5. Comparison of metabolic rate of *R*-(-)- and *S*-(+)-DP by human CYP3A4 (upper) and dog CYP3A12 (lower). Data are the mean of metabolic rate of *R*-(-)- and *S*-(+)-DP determinations.

R-(-)-enantiomers calculated from $V_{\max}/K_m$ for CYP3A12 were 0.05 and 0.03 l h⁻¹ mg⁻¹ protein respectively.

Discussion

The present study has demonstrated that mono-*N*-dealkylation of [¹⁴C]DP in both human and dog was primarily mediated by CYP3A isoenzymes. In human, the metabolism of [¹⁴C]DP was best correlated with dextromethorphan *N*-dealkylation activity, which is a typical marker for CYP3A4 activity and was extensively inhibited by ketoconazole, TA and human CYP3A4 antibody. In addition, DP was metabolized by human cDNA-expressed CYP3A4, which is consistent with these

findings. In dog, the metabolism of [^{14}C]DP was also inhibited by ketoconazole, TA and dog CYP3A12 antibody. Metabolism of [^{14}C]DP was observed with dog cDNA-expressed CYP3A12 whereas there was no notable metabolism with dog CYP2C21 and CYP2D15.

Metabolism of [^{14}C]DP by human CYP3A4 was not stereoselective whereas [^{14}C]DP metabolism by dog CYP3A12 was stereoselective. These results are consistent with *in vivo* previous findings (Cook *et al.* 1982, Lima *et al.* 1985, Giacomini *et al.* 1986, Le Corre *et al.* 1991). The pharmacokinetics of total DP (plasma protein unbound plus bound) was reported as non-stereoselective in human. However, plasma protein binding was stereoselective and, consequently, renal clearance of unbound DP was stereoselective. Metabolic clearances (total plasma clearance minus renal clearance) of the *S*-(+)- and *R*-(-)-DP enantiomers were about the same at 226 and 219 ml min⁻¹ respectively. Thus, there were little differences in the *in vivo* metabolism of DP enantiomers in the human. However, in dog, metabolism of the *S*-(+)-enantiomer was more extensive than in the *R*-(-)-enantiomer. Total plasma clearance of *S*-(+)-enantiomer was 1.22 l h⁻¹ kg⁻¹ and 1.6 times higher than that of *R*-(-)-enantiomer (0.76 l h⁻¹ kg⁻¹) in the dog. The hepatic intrinsic clearance of the *S*-(+)-enantiomer, calculated from V_{max}/K_m for dog CYP3A12, was also 1.6 times greater than that of the *R*-(-)-enantiomer. These results indicate that the stereoselective pharmacokinetics of DP observed in the dog is primarily due to stereoselective *N*-dealkylation of DP by CYP3A12. Furthermore, it can be concluded that species differences in stereoselective pharmacokinetics (metabolic clearance) of DP observed in the *in vivo* studies were due to species differences in stereoselective metabolism within the same subfamily of the CYP isoenzymes between human (CYP3A4) and dog (CYP3A12) rather than due to involvement of different subfamilies of CYP isoenzymes in the *N*-dealkylation pathway.

CYP3A4 and CYP3A12 share 89% amino acid sequence similarity when calculated using The Wisconsin Package (v.7, Genetic Computer Group, Madison, WI, USA). However, the active sites of these two isoenzymes appeared to be substantially different. With dog CYP3A12, the extent of inhibition by ketoconazole and TA was not as high as with human CYP3A4. In addition, [^{14}C]DP metabolism by the CYP3A12 isoenzyme was not inhibited by the human CYP3A4 antibody showing that despite the sequence similarity there was a significant species difference between CYP3A4 and CYP3A12. This was further supported by the species difference in stereoselectivity observed in the *N*-dealkylation of DP by these isoenzymes, as mentioned above. According to a three-dimensional model of CYP3A4 proposed by Szklarz and Halpert (1998), the active site of CYP3A4 is surrounded by two helical domains, namely amino acid residues 211–214 and 301–309. In CYP3A12, Lys replaces the residue 212 Arg of CYP3A4, and Thr and Ser replace residues 403 (Ser) and 404 (Val) respectively. Both of these two helical portions of CYP3A4 are in the middle of highly conserved sequences, as is the adjacent β -strand in their proposed model (residues 369–373). In dog, residues 367 (Tyr) and 371 (Gly) were substituted for Phe and Met residues in CYP3A4 respectively, in the β -strand area. In addition, in the top loop around Ile 119, there were two residue changes (Ile $\rightarrow$ Leu and Ala $\rightarrow$ Ser) at the N+2 and N+3 positions respectively. These changes around the active site may account for the species difference in the stereoselectivity of CYP3A as well as in observed inhibition of [^{14}C]DP metabolism by ketoconazole, TA and CYP antibodies.

References

- BROGDEN, R. N. and TODD, P. A., 1987, Disopyramide. A reappraisal of its pharmacodynamic and pharmacokinetic properties, and therapeutic use in cardiac arrhythmias. *Drugs*, **34**, 151–187.
- COOK, C. S., KARIM, A. and SOLLMAN, P., 1982, Stereoselectivity in the metabolism of disopyramide enantiomers in rat and dog. *Drug Metabolism and Disposition*, **10**, 116–121.
- CUSIC, J. W. and SAUSE, H. W., 1965, 4-Dipropylamino-2-aryl-2-pyridyl-alkanamides. *United States Patent*, 3, 225,054.
- ECHIZEN, H., MOCHIZUKI, K., TANI, M. and ISHIZAKI, T., 1994, Interspecies differences in enantioselective mono-*N*-dealkylation of disopyramide by human and mouse liver microsomes. *Journal of Pharmacology and Experimental Therapeutics*, **268**, 1518–1525.
- GIACOMINI, K. M., NELSON, W. L., PERSHE, R. A., VALDIVIESO, L., TURNER-TAMIYASU, K. and BLASCHKE, T. F., 1986, *In vivo* interaction of the enantiomers of disopyramide in human subjects. *Journal of Pharmacokinetics and Biopharmaceutics*, **14**, 335–356.
- HEEL, R. C., BROGDEN, R. N., SPEIGHT, T. M. and AVERY, G. S., 1978, Disopyramide: A review of its pharmacological properties and therapeutic use in treating cardiac arrhythmias. *Drugs*, **15**, 331–368.
- KARIM, A., RANNEY, R. E. and KRAYCHY, S., 1972, Species differences in the biotransformation of a new antiarrhythmic agent. *Journal of Pharmaceutical Sciences*, **61**, 888–893.
- LE CORRE, P., GIBASSIER, D., SADO, P. and LE VERGE, R., 1992, Human pharmacokinetics and metabolism of disopyramide enantiomers. *Laboratoire de Pharmacia Galenique et Biopharmacie, Université de Rennes I, France*, **4**, 80–83.
- LE CORRE, P., MALLEDANT, Y., CHEVANNE, F., SADO, P. and LE VERGE, R., 1991, Stereoselective biliary elimination of disopyramide and mono-*N*-desisopropylidisopyramide in humans. *Laboratoire de Pharmacia Galenique et Biopharmacie, Université de Rennes I, France*, **3**, 233–237.
- LIMA, J. J., BOUDOULAS, H. and SHIELDS, B. J., 1985, Stereoselective pharmacokinetics of disopyramide enantiomers in man. *Drug Metabolism and Disposition*, **13**, 572–577.
- PAULSON, S. K., ENGEL, L. C., REITZ, B., BOLTEN, S., BURTON, E. G., MAZIASZ, T. J., YAN, B. and SCHOENHARD, G. L., 1999, Evidence for polymorphism in the canine metabolism of the cyclooxygenase 2 inhibitor, celecoxib. *Drug Metabolism and Disposition*, **27**, 1133–1142.
- SZKLARZ, G. D. and HALPERT, J. R., 1998, Molecular basis of P450 inhibition and activation, implications for drug development and drug therapy. *Drug Metabolism and Disposition*, **26**, 1179–1184.

PCB methyl sulphones in rat liver after exposure to PCB (Clophen A50): analysis and radiosynthesis of selected methylsulphonyl-PCBs

C. LARSSON and Å. BERGMAN*

Department of Environmental Chemistry, Stockholm University, S-106 91 Stockholm, Sweden

Received 4 May 2000

1. The hepatic metabolism of polychlorinated biphenyls (PCBs) and formation of PCB methyl sulphone metabolites (MeSO_2 -PCBs) was determined in the male Sprague–Dawley rat 1, 2, 4 or 8 weeks after dosage with Clophen A50 (a commercial PCB mixture).

2. The total concentration of the PCB congeners examined (ΣPCB) decreased during the experimental period, from $40 \mu\text{g g}^{-1}$ lipid (l.w.) after 1 week to $4 \mu\text{g g}^{-1}$ l.w. after 8 weeks. A 50% decrease of PCB in the liver were estimated to be 28, 13 and 11 days for 2,2',4,4',5,5'-hexachlorobiphenyl (CB153), 2,2',3,3',4,4',5-heptaCB (CB170) and 2,2',3,4',5,5',6-heptaCB (CB187), respectively.

3. The total MeSO_2 -PCB (ΣMeSO_2 -PCB) concentration increased from 800 to 1020 ng g^{-1} l.w. during the first 2 weeks of treatment and thereafter a decrease to 120 ng g^{-1} l.w. after 8 weeks. The relative concentration of both 3'- MeSO_2 -2,2',4,4',5-pentaCB (3'- MeSO_2 -CB101) and 3'- MeSO_2 -2,2',3,4,5'-pentaCB (3'- MeSO_2 -CB87) in rat liver showed significant increases during the 8 weeks. In contrast, the relative concentrations of 4'- MeSO_2 -2,4',5,5'-tetraCB (4'- MeSO_2 -CB64), 3'- MeSO_2 -2,3',4',5'-tetraCB (3'- MeSO_2 -CB70) and 4'- MeSO_2 -2,2',3,4,5',6'-hexaCB (4'- MeSO_2 -CB132) decreased significantly.

4. A route for the synthesis of radiolabelled MeSO_2 -PCBs was developed employing the 'Pummerer reaction' to convert methylthio-PCBs (MeS -PCBs) to the corresponding mercapto-PCBs (SH -PCB). The SH -PCBs were methylated with radiolabelled methyl iodide and the resulting sulphides oxidized to yield the corresponding MeSO_2 -PCBs. Using this approach, 3'- ^{14}C - MeSO_2 -2,2',4',5,5',6'-hexaCB (3'- ^{14}C - MeSO_2 -CB149), 4'- ^{14}C - MeSO_2 -2,2',4',5,5',6'-hexaCB (4'- ^{14}C - MeSO_2 -CB149), 3'- ^{14}C - MeSO_2 -CB101, 4'- ^{14}C - MeSO_2 -2,2',4,5,5'-pentaCB (4'- ^{14}C - MeSO_2 -CB101) and 4'- ^3H - MeSO_2 -CB101 were synthesized in quantitative radiochemical yields.

Introduction

Polychlorinated biphenyls (PCBs) are ubiquitous environmental pollutants first detected in 1966 (Jensen 1972). Shortly thereafter, hydroxylated metabolites (OH -PCBs) were reported from wildlife (Jansson *et al.* 1975) and experimental studies (for a review, see Sundström *et al.* 1976, Letcher *et al.* 2000). In 1976, the occurrence of methylsulphonyl-PCBs (MeSO_2 -PCBs) and 2-(4-chloro-3-methylsulphonylphenyl)-2-(4-chlorophenyl)-1,1-dichloroethene (3'- MeSO_2 -DDE) in blubber from the Baltic grey seal were determined (Jensen and Jansson 1976). At this time MeSO_2 -PCB metabolites were also detected in the faeces from mouse dosed with 2,2',5,5'-tetrachlorobiphenyl (Mio *et al.* 1976) and in the lung tissue of mouse administered individual radiolabelled PCB congeners (Brandt *et al.* 1976).

MeSO_2 -PCBs are lipophilic PCB metabolites with high chemical stability. The MeSO_2 -PCBs are at least as persistent as their parent compounds (Letcher *et al.* 2000) and in most cases even more persistent since the insertion of the sulphone

* Author for correspondence; e-mail: ake.bergman@mk.su.se

group reduces the rate of further metabolism. To date, as many as 30 different MeSO₂-PCB congeners have been detected in wildlife and the majority of these have been structurally identified. All these MeSO₂-PCBs originate from PCB congeners containing at least one phenyl ring substituted with chlorine atoms at the 2,5- or 2,3,6-positions and having the second phenyl ring substituted with a chlorine atom in the 4-position. For an extensive review on MeSO₂-PCBs, see Letcher *et al.* (2000).

PCBs containing vicinal hydrogen atoms are oxidized to arene oxides by the cytochrome P450 (CYP) system (Preston and Allen 1980). The arene oxide formed may subsequently react with glutathione (GSH) at either the 3- or 4-position in the 2,5-dichloro- or 2,3,6-trichloro-substituted phenyl ring (Bakke 1989). After dehydration, glutathione conjugates are transformed via the mercapturic acid pathway (MAP) to cysteine conjugates (Bakke and Gustafsson 1986). PCB-cysteine conjugates are excreted with the bile into the gastrointestinal tract and cleaved there by C-S lyase to produce the corresponding thiol-substituted PCB (Bakke *et al.* 1982, Bakke and Gustafsson 1986). A MeSO₂-PCB is formed when an aryl thiol is methylated in a reaction involving *S*-adenosylmethionine as a methyl group donor to give the corresponding methyl sulphide, which is in turn oxidized to an aryl methyl sulphone in a two-step CYP-catalysed reaction (Bakke 1989).

MeSO₂-PCBs accumulate primarily in adipose tissue because of their high degree of lipophilicity, but these compounds are also known to bind non-covalently to proteins in the lung and liver of mammals (Lund *et al.* 1985, Larsen *et al.* 1992). High-affinity binding of MeSO₂-PCBs to certain proteins results in highly selective tissue retention. Depending on the species and structure of the metabolite, MeSO₂-PCBs may be retained in lung bronchial mucosa (Bergman *et al.* 1979, Lund *et al.* 1985), kidney cortex (Bergman *et al.* 1979), intraluminal uterine fluid (Brandt *et al.* 1982) and liver (Bergman *et al.* 1994). *Para*-substituted MeSO₂-PCBs (4-MeSO₂-PCBs) bind with a high affinity and selectivity to an uteroglobin-like protein, known as 'the PCB-binding protein', in Clara cells (Brandt *et al.* 1985, Lund *et al.* 1985). *Meta*-substituted MeSO₂-PCBs (3-MeSO₂-PCBs) are retained to a great extent in the liver, although the mechanism underlying this retention is still unclear (Larsen and Bergman 1994, Lund *et al.* 1999). Knowledge concerning the possible toxicity of MeSO₂-PCB is limited, but these metabolites have been linked to respiratory insufficiency observed in the Yusho victims (Nakanishi *et al.* 1985). More recently, certain 3-MeSO₂-PCBs have been reported to induce CYP 450 isozymes strongly (Kato *et al.* 1997, Lund *et al.* 1999). In fact, some of these sulphones are as much as three orders of magnitude more potent as inducers than are their parent PCB congeners.

The aim of the present study was to synthesize radiolabelled MeSO₂-PCBs to promote mechanistic studies on the retention of these metabolites in rat hepatic tissue. To ensure that relevant MeSO₂-PCBs were selected for synthesis, rats were administered a commercial mixture of PCBs (Clophen A50). The rat livers were analysed for the presence of PCBs and MeSO₂-PCBs at different times after treatment. A pathway for synthesis, omitting the use of radiolabelled chloanilines or chlorobenzenes but inserting the label at a late stage in the process, was selected and worked out.

More than 100 different PCB methyl sulphones have hitherto been synthesized (Letcher *et al.* 2000), primarily employing three different methods: (1) nucleophilic aromatic substitution of a chlorine atom in the *para*-position of a PCB containing at

least one 2,4,5-trichloro-substituted phenyl ring with a methane thiolate group, using methanol as solvent (Bergman and Wachtmeister 1978); (2) a Cadogan diaryl coupling reaction between a methylsulphonyl-substituted chlorinated aniline and a chlorinated benzene (Haraguchi *et al.* 1987a); or (3) a Cadogan diaryl coupling reaction between a chlorinated aniline and a chlorothioanisole (Haraguchi *et al.* 1987b). A procedure involving palladium catalysis of the coupling between an aryl iodide and an aryl trimethylstannane was more recently successfully employed by Mortimer and Newsome (1996) for the synthesis of MeSO₂-PCBs. Only a small number of radiolabelled 4-MeSO₂-PCBs has so far been prepared (Bergman and Wachtmeister 1978, Haraguchi and Bergman 1991). None of the methods used previously was suitable in the present study due to the low specific activity of the commercially available chlorobenzenes and chloroanilines to be potentially used as starting materials.

Materials and methods

Chemicals

Clophen A50 (Bayer, Leverkusen, Germany) was administered to the rats. All individual MeSO₂-PCBs used as analytical standards were synthesized according to Haraguchi *et al.* (1987a, b). 2,3,3',4,4',5,5'-Heptachlorobiphenyl (CB189) and 3-methylsulphonyl-4-methyl-2',3',4',5,5'-pentachlorobiphenyl were used as internal standards (IS) in the analysis of PCBs and MeSO₂-PCBs, respectively. [³H]-methyl iodide and [¹⁴C]-methyl iodide were from Amersham Life Science (Solna, Sweden). 2,4,5-Trichloroaniline (Fluka, Buchs, Switzerland), 2,5-dichlorophenyl methyl sulphide (a kind gift from Dr Koichi Haraguchi, Daiichi College of Pharmaceutical Sciences, Fukuoka, Japan), 3-methylbutyl nitrite (Sigma-Aldrich, Steinheim, Germany) and tetrabutylammonium hydrogen sulphate (Labkemi AB, Stockholm, Sweden) were employed in the syntheses. Silica gel (Kieselgel 60, 40–63 µm, Merck, Darmstadt, Germany) was activated at 180 °C overnight prior to use. Thin-layer chromatography was performed on silica gel plates (Kieselgel 60 F₂₅₄; Merck), on RP-18 plates (RP-18 F₂₅₄S; Merck) or on silica gel plates impregnated with tetrabutylammonium hydrogen phosphate (0.8 M) (Bergman *et al.* 1976). Gel permeation chromatography (GPC) was carried out using BioBeads SX-3 gel (200–400 Mesh, 50 g; Bio-Rad Laboratories, Richmond, CA, USA). All solvents used were of analytical grade.

The abbreviated names of the PCB methyl sulphones used here are based on the structure of the PCB congener structure using the PCB numbers suggested by Ballschmitter *et al.* (1993) and adding the actual position (3-, 3'-, 4- or 4'-) of the sulphone substituent in front of the prefix 'MeSO₂-' as in, for example, 3-methylsulphonyl-2,2',4',5,5'-pentachlorobiphenyl, which will then be abbreviated 3'-MeSO₂-CB101 (Letcher *et al.* 2000).

Instruments

Liver samples were homogenized using an Ultra-Turrax IKAT25 homogenizer (Labassco AB, Partille, Sweden). GPC was performed as described by Klasson Wehler *et al.* (1987). The GPC column was connected on-line to a Gilson 112 UV/VIS detector (254 nm).

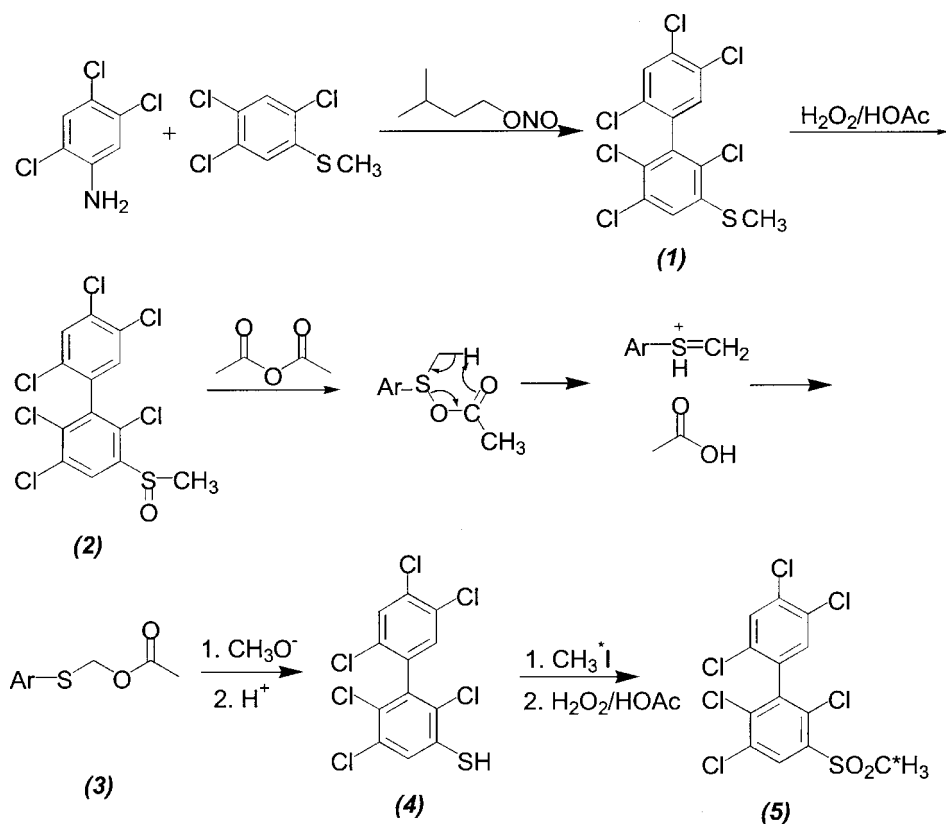
Gas chromatographic (GC) analyses were performed on a Varian GC 3400 (Varian Associates, Palo Alto, CA, USA) equipped with an electron-capture detector (ECD) maintained at 360 °C. The gas chromatograph was fitted with a fused silica capillary column (DB-5, 30 m × 0.25 mm i.d.; 0.25-µm film thickness; J&W Scientific, Folsom, CA, USA). Injections were made with a Varian 8200 autosampler in the splitless mode at 280 °C. Hydrogen was used as the carrier gas and nitrogen as make-up gas. The oven temperature was programmed as follows: 80 °C (2 min); 20 °C min⁻¹ to 230 °C; 3 °C min⁻¹ to 300 °C (10 min). Data collection and processing were achieved utilizing the ELDS-Pro lab data system (Chromatography Data System AB, Svartsjö, Sweden).

Gas chromatography-mass spectrometry (GC-MS) was performed employing a Finnigan ITS40 mass spectrometer (Finnigan MAT, San Jose, CA, USA) connected to a Varian GC 3400 equipped with a split-splitless injector, maintained at 270 °C and used in the splitless mode. This GC was equipped with a fused silica capillary DB-5 column (30 m × 0.25 mm i.d.; 0.25-µm film thickness; J&W Scientific) and helium was the carrier gas. The ITS40 was operated with an EI energy of 70 eV and an ion source temperature of 220 °C.

Radioactivity was measured utilizing a Wallac 1409 Emulsifier Scintillator 299, with OptiScint 'HiSafe' (all Wallac Oy, Turku, Finland) as the scintillation cocktail. The radiochemical purity of labelled compounds was analysed with a radioactivity scanner and the computer software RITA (all Raytek Scientific Ltd, Sheffield, UK).

Table 1. Starting materials and references for the syntheses of some PCB methyl sulphides (MeS-PCBs).

Starting material	Product (step 1 in figure 1)	Reference
2,2',4,4',5,5'-Hexachloro-biphenyl (CB153)	4'-MeS-2,2',4',5,5'-pentaCB	Bergman <i>et al.</i> (1978)
2,5-Dichloromethylthiobenzene and 2,4,5-trichloroaniline	3'-MeS-2,2',4,4',5-pentaCB and 4'-MeS-2,2',4',5,5'-pentaCB	Haraguchi <i>et al.</i> (1987)
2,4,5-Trichloromethylthiobenzene and 2,4,5-trichloroaniline	3-MeS-2,2',4',5',6-hexaCB	Haraguchi <i>et al.</i> (1987)
2,2',3,4,4',5,6'-Hepta-chlorobiphenyl (CB 183)	4-MeS-2,2',3,4',5',6-hexaCB	Bergman <i>et al.</i> (1978)

Figure 1. Route for the synthesis of [^{14}C]-3-MeSO $_2$ -CB149.

Treatment of the animals

Sixteen 180 g male Sprague-Dawley rats were administered orally a technical PCB mixture, Clophen A50, dissolved in corn oil (0.2 ml/rat = 25 mg kg $^{-1}$ b.w.). Two control rats received corn oil only. The rats had free access to a pellet diet (Ewos AB, Södertälje, Sweden) and water. Four rats were sacrificed at each time point, 1, 2, 4 and 8 weeks after treatment; while the two control rats were killed after 4 weeks. Livers were removed and maintained frozen at -20°C until analysis.

Analyses

The wet weight of the liver was determined prior to homogenization and extraction as described by Jensen *et al.* (1983). The surrogate standard (IS) was added to the organic phase after extraction and, after evaporation of the organic solvent, the extracted lipid was weighed.

Table 2. Starting materials for the synthesis of ³H- and ¹⁴C-labelled methylsulphonyl-PCBs prepared. The product yield is shown.

Starting materials (step 1 in figure 1)	Methyl iodide	Final product	Abbreviation	Yield
4'-MeS-2,2',4',5,5'-pentaCB	[³ H]-MeI (12 mCi, 85 Ci mmol ⁻¹)	4'-[³ H]-MeSO ₂ -2,2',4',5,5'- pentaCB	4'-MeSO ₂ -CB 101	12 mCi
3'-MeS-2,2',4,4',5-pentaCB and 4'-MeS-2,2',4',5,5'- pentaCB	[¹⁴ C]-MeI (1.0 mCi, 53.0 mCi mmol ⁻¹)	3'-[¹⁴ C]-MeSO ₂ -2,2',4,4',5- pentaCB and 4'-[¹⁴ C]-MeSO ₂ - 2,2',4',5,5'-pentaCB	3'-MeSO ₂ -CB 101 and 4'-MeSO ₂ -CB 101	740 µCi of a mixture of 3'-101 and 4'-101
3-MeS-2,2',4',5,5',6-hexaCB	[¹⁴ C]-MeI (1.0 mCi, 55.0 mCi mmol ⁻¹)	3-[¹⁴ C]-MeSO ₂ -2,2',4',5,5',6- hexaCB	3-MeSO ₂ -CB 149	490 µCi
4-MeS-2,2',3,4',5',6-hexaCB	[¹⁴ C]-MeI (1.0 mCi, 55.0 mCi mmol ⁻¹)	4-[¹⁴ C]-MeSO ₂ -2,2',3,4',5',6- hexaCB	4-MeSO ₂ -CB 149	480 µCi

Lipids were subjected to GPC as described by Klasson Wehler *et al.* (1987) using a 1:1 mixture of hexane (Hx) and dichloromethane (DCM) as the mobile phase, and a flow-rate of 4 ml min⁻¹. Three fractions were collected, i.e. a first fraction containing the bulk of the lipids (at an elution volume of 40–90 ml), an intermediate fraction (90–104 ml) and a third fraction (104–200 ml) containing the xenobiotics of interest (Olsson and Bergman 1995). The xenobiotics in this third fraction were further separated from small amounts of co-eluting, matrix-related material using a silica gel column (1 g), from which PCBs were eluted with Hx (7 ml), followed by the elution of MeSO₂-PCBs with DCM (10 ml). The DCM was carefully evaporated and fractions that still contained > 1 mg endogenous material were further purified utilizing sulphuric acid (Klasson Wehler *et al.* 1998). Identification and quantitation of PCBs and MeSO₂-PCBs was based on comparison with authentic reference standards (Klasson Wehler *et al.* 1998).

Syntheses

The first step in the synthesis of [¹⁴C]-3-MeSO₂-2,2',3,4',5',6-hexachlorobiphenyl was a Cadogan coupling reaction between 2,4,5-trichloromethylthiobenzene and 2,4,5-trichloroaniline (table 1; Haraguchi *et al.* 1987a, b). After purification, the 3-methylthio-2,2',3,4',5',6-hexaCB obtained (**1**) (figure 1) was selectively oxidized employing 30% hydrogen peroxide (0.5 ml) in acetic acid (1 ml) at room temperature for 30 min, to yield the corresponding 3-methylsulphonyl-2,2',3,4',5',6-hexaCB (**2**).

The 3-methylsulphonyl-2,2',3,4',5',6-hexaCB (20 mg) was transferred to an argon-filled ampoule, to which acetic acid anhydride (100 µl) was added prior to sealing (Pummerer 1910, Parham *et al.* 1968). This ampoule was subsequently maintained at 100 °C for 11 days after which the reaction was terminated by addition of water and the product extracted with DCM and identified by GC/MS. MS (EI) % relative abundance: 420 [16%, M⁺], 422 [34%, M+2]⁺, 406 [100%, M⁺-CH₃]⁺, 385 [11%, M⁺-(Cl)]⁺, 373 [13%, M⁺-SCH₃]⁺.

The isolated product (**3**) was treated with sodium methoxide in methanol (1 ml, 2 M) at 65 °C for 4 h under an argon atmosphere in a new ampoule. Thereafter, the 3-mercapto-2,2',3,4',5',6-hexaCB (**4**) produced was extracted and identified by GC/MS. MS (EI) relative abundance: 462 [23%, M⁺], 464 [43%, M + 2]⁺, 432 [6%, M⁺-CH₂O]⁺, 390 [100%, M⁺-(CHOCOCH₃)]⁺, 354 [27%, M⁺-(CHOCOCH₃+HCl)]⁺.

The 3-mercapto-2,2',3,4',5',6-hexaCB (**4**) was methylated with [¹⁴C]-methyl iodide (1.0 mCi; 55.0 mCi mmol⁻¹; table 2) in a phase-transfer catalysis (PTC) reaction as described by Bergman *et al.* (1987), yielding the [¹⁴C]-labelled methyl sulphide. The product 3-[¹⁴C]-methylthio-2,2',3,4',5',6-hexaCB was purified on silica gel plates and identified by GC/ECD by comparison with an authentic unlabelled external standard. Finally the [¹⁴C]-labelled methylthio-PCB was oxidized to the corresponding 3-[¹⁴C]-methylsulphonyl-2,2',3,4',5',6-hexaCB (**5**) using 30% hydrogen peroxide in acetic acid (1.5 ml; 1:2) as described by Bergman and Wachtmeister (1978). The product was identified by comparison with an unlabelled authentic reference standard. The specific radioactivity was determined utilizing GC/ECD quantification of the compound, relative an authentic unlabelled standard, and measurements of the radioactivity of the compound. The radiopurity was confirmed to be 100% using a TLC-scanner after separation on both silica gel and RP-18 plates.

The 3'-MeS-2,2',4',5',5'-pentaCB (3'-MeS-CB101) was synthesized similarly using the starting materials presented in table 1. The *para*-substituted methylthio-PCBs, e.g. 4'-MeS-2,2',4',5',5'-pentaCB (4'-MeS-CB101) and 4-MeS-2,2',3,4',5',6-hexaCB (4-MeS-CB149), were synthesized via nucleophilic aromatic substitution of a chlorine atom in the *para*-position of the corresponding PCB congeners (table 1) with a methane thiolate group (2 M in methanol; Bergman and Wachtmeister 1978). The methylthio-PCBs formed were reacted as described above and as shown in figure 1 to produce the corresponding polychlorobiphenylthiols (SH-PCB); 3'-SH-CB101, 4'-SH-CB101 and 4-SH-CB149, respectively.

Finally, radiolabelled MeS-PCBs were prepared, as described above, via PTC reactions of each SH-PCB with methyl iodide, as characterized in table 2. The MeSO₂-PCBs formed from 2,5-dichloroanisole and 2,4,5-trichlorobenzene, i.e. 3'-[¹⁴C]-MeSO₂-CB101 and 4'-[¹⁴C]-MeSO₂-CB101, were separated on silica gel plates impregnated with tetrabutylammonium hydroxide (Bergman *et al.* 1976).

Results

The PCB and MeSO₂-PCB congener profiles in rat liver were determined at 1, 2, 4 and 8 weeks after administration of the PCB product, Clophen A50 (data not shown). The total amount of PCB congeners quantified (ΣPCB) decreased significantly and continuously from the first week to the end of the experimental period (figure 2). Three weeks after administration the ΣPCB was half that observed at week 1, but this pattern differed for the individual PCB congeners. In detail, the concentrations of CB153, CB170 and CB187 decreased by 50% within 28, 13 and 11

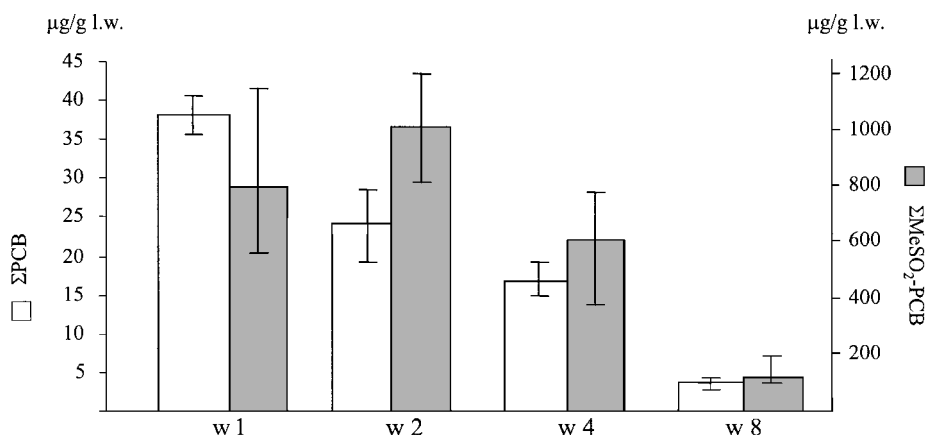


Figure 2. Σ PCB and Σ MeSO₂-PCB concentrations in rat liver 1, 2, 4 and 8 weeks after treatment with a mixture of a commercial PCB product, Clophen A50. Bars represent SD. $n = 4$ in all cases.

Table 3. Estimated 50% decrease (days) for the retention of certain PCB congeners in rat liver after administration of the technical PCB product, Clophen A50.

PCB congener	Abbreviation	50% decrease of retention (days)
2,2',3,4',5,5',6-HeptaCB	CB187	11
2,3,3',4,4'-PentaCB/ 2,2',3,3',4',6-HexaCB	CB105/132	33
2,2',4,4',5,5'-HexaCB	CB153	28
2,2',3,3',4,4',5-HeptaCB	CB170	13
2,3',4,4',5-PentaCB/ 2,2',3,4',5',6-HexaCB/ 2',3,4,4',5-PentaCB	CB118/149/123	13
2,2',4,5,5'-PentaCB	CB101	10

days, respectively (table 3). CB105/132 and CB118/149/123 were found to demonstrate a 50% decrease in the rat liver in 33 and 13 days, respectively, whereas a corresponding decrease of CB101 was only 10 days (table 3).

The total amount of all the MeSO₂-PCB congeners analysed (Σ MeSO₂-PCB) in rat liver is also documented in figure 2. The Σ MeSO₂-PCB increased at the first two sampling times, i.e. at 1 and 2 weeks, and thereafter the Σ MeSO₂-PCB levels decreased (figure 2). The relative concentrations for most of the MeSO₂-PCB congeners (i.e. concentration of the individual MeSO₂-PCB congener/ Σ MeSO₂-PCBs) remained constant throughout the experimental period (figure 3). However, the relative concentrations of 3'-MeSO₂-CB101 and 3'-MeSO₂-CB87 in rat liver increased from 0.10 to 0.21 and from 0.071 to 0.13, respectively, during the 8 weeks. In contrast, 4-MeSO₂-CB64, 3-MeSO₂-CB70 and 4'-MeSO₂-CB132 all demonstrated a decrease in relative concentration, from 0.11 to 0.057, 0.15 to 0.066 and 0.079 to 0.033, respectively (figure 3).

Applying the synthetic strategy depicted in figure 1, 3-[¹⁴C]-MeSO₂-CB149, 4-[¹⁴C]-MeSO₂-CB149, 3'-[¹⁴C]-MeSO₂-CB101, 4'-[¹⁴C]-MeSO₂-CB101 and 4'-[³H]-MeSO₂-CB101 (table 1 and 2) were synthesized as described in the Materials

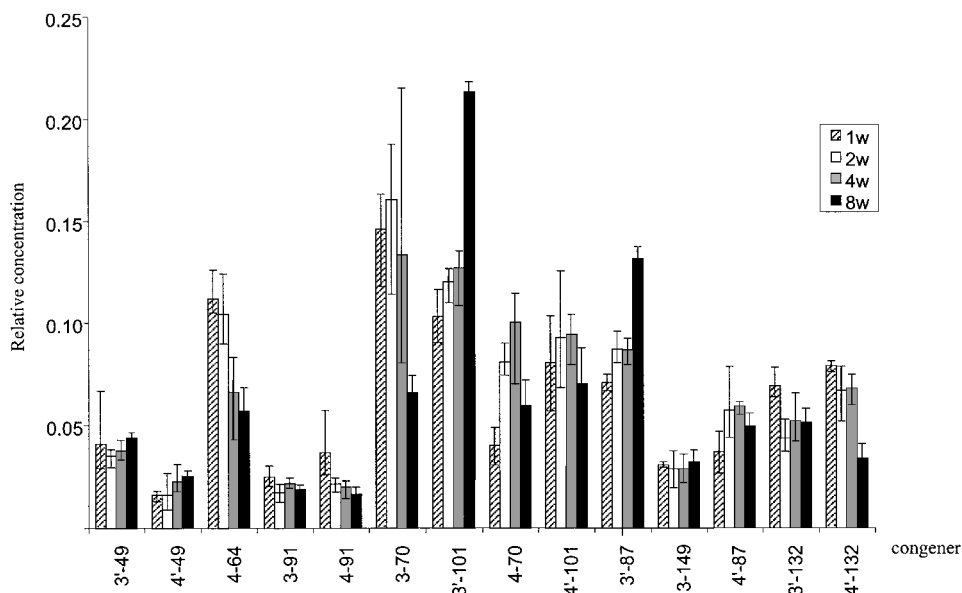


Figure 3. Relative concentrations of individual MeSO₂-PCB congeners (concentration of each congener/ Σ MeSO₂-PCB) 1, 2, 4 and 8 weeks after treatment with the commercial PCB product, Clophen A50. Bars represent SD. $n = 4$ in all cases.

and methods. The products were shown to give similar mass spectra as described (Bergman *et al.* 1980) and to have relative retention times corresponding to the authentic standards (Haraguchi *et al.* 1987a).

Discussion

The total amount of PCB congeners analysed (Σ PCB) in the liver of rat administered the technical PCB mixture, Clophen A50, decreased from $40 \mu\text{g g}^{-1}$ l.w at week 1, after treatment, to $4 \mu\text{g g}^{-1}$ l.w 7 weeks later (figure 2). The mean half-life of Σ PCB in rat liver was calculated as 21 days. Tanabe *et al.* (1981) treated male Wistar rats with a technical PCB product and found the PCB half-life in the whole body to be >90 days. However, the hepatic level of PCB has been reported to decrease more rapidly than in adipose tissue (Hori *et al.* 1971). Biological half-lives of PCBs vary widely, both between individuals and between species, ranging, for example, from 3.3 days in the guppy (Gooch and Hamdy 1982) to 7.1 years in the blood of children whose mothers were occupationally exposed to PCB (Yakushiji *et al.* 1984) and as long as 14 years in mothers' milk (Norén and Meironyté 2000). This wide range of biological half-lives reflects metabolic differences between species, interindividual differences and also differences in the metabolic capacities of different tissues.

Commercial PCB products are complex mixtures of many PCB congeners, each one with their own half-life. The persistency, or rate of metabolism, between different PCB congeners is highly different (Letcher *et al.* 2000). For example, in the blood of the female Rhesus monkey, the half-lives of CB105, CB118 and CB153 were determined to be 146, 474 and 256 days, respectively (Mes *et al.* 1995). In another study, a man voluntarily ingested a mixture of [¹⁴C]-labelled PCBs similar to Arochlor 1254 and the half-lives of CB138, CB153 and CB180 in his blood were calculated to be 321, 338 and 124 days, respectively (Bühler *et al.* 1988). In the study

of mothers' milk, half-lives for CB153, CB138 and CB118 were calculated to be 17, 14 and 11 years, respectively (Norén and Meironyté 2000). The present study reports that a 50% decrease for 101, CB105/132, CB118/149/123 and CB153 in the rat liver are estimated to be 10, 33, 14 and 28 days, respectively. In Haraguchi *et al.* (1999) rats were administrated with either CB101 or CB149 and the formation of metabolites and rates of excretion were monitored. CB101 was shown to have half-lives of 24 days in liver and 26 days in fat; while CB149 had half-lives of 20 and 35 days in liver and adipose tissue, respectively. The minor differences in the half-lives found in these two studies may be due to an increase in the metabolism rate depending on enzyme induction of the complex PCB mixture that is not obtained by single PCB congener administration and/or metabolic differences between the rat strains employed.

The continuous decreases in the levels of PCBs reflect metabolism and excretion. It has been demonstrated that PCB congeners with chlorine atoms at positions 2 and 5 or 2, 3 and 6 in at least one of the phenyl rings are easily metabolized (Bergman *et al.* 1992, Letcher *et al.* 2000). Thus, as expected, PCB concentrations were highest at the beginning of our experimental period, soon after the rats were administered the PCB mixture.

As illustrated in figure 2, the hepatic $\Sigma\text{MeSO}_2\text{-PCB}$ concentration is at a maximum of ~ 14 days after treatment. Determination of the exact time of this maximum would require sampling at additional time-points. From 2 to 8 weeks after treatment, the hepatic $\Sigma\text{MeSO}_2\text{-PCB}$ decreased from $1000 \mu\text{g g}^{-1}$ l.w. to $< 200 \mu\text{g g}^{-1}$ l.w., with the half-maximal being observed at 5 weeks.

With respect to individual $\text{MeSO}_2\text{-PCB}$ congeners, 3- $\text{MeSO}_2\text{-CB101}$ appears to be most avidly retained in rat liver, its relative concentration ratio increasing more than twofold (i.e. from 0.1 to 0.22) during the experimental period. The actual concentrations of methyl sulphone metabolites did, however, decrease during the experimental period from $127 \mu\text{g g}^{-1}$ l.w. at 2 weeks after treatment to $31 \mu\text{g g}^{-1}$ l.w. at 8 weeks. Analysis of $\text{MeSO}_2\text{-PCB}$ in free-living organisms has revealed that 3'-/4'- $\text{MeSO}_2\text{-CB101}$, 3'-/4'- $\text{MeSO}_2\text{-CB87}$ and 3- $\text{MeSO}_2\text{-CB149}$ are present at the highest levels (Bergman *et al.* 1993, Letcher *et al.* 2000). However, in the present study we could not see any predominance of 3- $\text{MeSO}_2\text{-CB149}$. This is probably due to species differences in formation, again emphasizing the importance of performing investigations on various species.

Specific hepatic retention of 3- $\text{MeSO}_2\text{-PCBs}$ has been observed in the grey seal (*Halichoerus grypus*) and otter (*Lutra lutra*) (Bergman *et al.* 1994), polar bear (*Ursus maritimus*) (Letcher 1996) and human (Weistrand and Norén 1997). In contrast, no specific retention of 3- $\text{MeSO}_2\text{-PCBs}$ in rat liver has yet been reported (Haraguchi *et al.* 1999, present study). In the present investigation both 3'- $\text{MeSO}_2\text{-CB87}$ and 3'- $\text{MeSO}_2\text{-CB101}$ demonstrates large increases during the 4–8 weeks after treatment; whereas after 8 weeks the relative concentration of 3- $\text{MeSO}_2\text{-CB70}$, 4- $\text{MeSO}_2\text{-CB64}$, 4'- $\text{MeSO}_2\text{-CB132}$ had all decreased to approximately half of the corresponding value 1 week after treatment. There are no obvious similarities in the structures between these latter three $\text{MeSO}_2\text{-PCB}$ congeners. Nor is there any similarity between the $\text{MeSO}_2\text{-PCB}$ profile observed in the present study and that observed in mink treated with Clophen A50 (Bergman *et al.* 1992). In the mink the 3- $\text{MeSO}_2\text{-PCBs}$ appear to be retained in the liver, with 3'- $\text{MeSO}_2\text{-CB87}$ and 3'- $\text{MeSO}_2\text{-CB132}$ being most abundant. Such differences may again be explained by species differences.

The changes in Σ PCB and Σ MeSO₂-PCB demonstrate similar time-courses, although Σ MeSO₂-PCB is at a maximum 2 weeks after treatment, while Σ PCB decreases continuously throughout the experimental period. The first two sampling times in figure 2 reflect the rates of PCB metabolism and excretion and of MeSO₂-PCB formation; while later values mainly reflect the relative rates of MeSO₂-PCB excretion. Haraguchi *et al.* (1999) reported that maximal levels of both MeSO₂-CB101 and MeSO₂-CB149 occur 4 days after administration. The fact that the present authors obtained maximal levels of MeSO₂-PCBs 14 days after treatment may reflect their use of oral administration, whereas Haraguchi *et al.* treated their animals intraperitoneally.

On the basis of the results described above, 3'- and 4'-MeSO₂-CB101 were selected for radiosynthesis and further studies. In addition, 3- and 4-MeSO₂-CB149 were synthesized. These radiolabelled compounds will be used to examine the mechanism underlying selective retention of MeSO₂-PCBs in the liver.

When performing radiosynthesis, it is desirable to introduce the radioisotope as late as possible in the synthetic sequence in order to obtain as high isotopic yield as possible, as well as for safety reasons. In previously described syntheses of radiolabelled MeSO₂-PCBs, the radioisotope has been introduced in the very first step, since uniformly labelled chloroaniline was used (Haraguchi and Bergman 1991). This first synthetic step, a Cadogan-coupling reaction between the [¹⁴C]-chloroaniline and the chlorobenzene, provides poor yields; a large amount of the radioactivity is lost in by-products, only 3.5–40% of the initial ¹⁴C being recovered in the products desired. The high cost of the starting material, the tediousness of the work and the limited commercial availability of labelled chloroanilines made the synthesis of certain MeSO₂-PCB congeners virtually impossible.

This situation motivated us to develop another route for the synthesis of PCB methyl sulphones. This new approach is based on the 'Pummerer reaction', i.e. reaction of sulphoxides with acetic anhydrides (DeLucci *et al.* 1991), followed by hydrolysis to form SH-PCBs. This procedure for the conversion of thioanisoles to the corresponding aryl thiols is particularly simple, mild and selective and results in high yields (Young *et al.* 1984). It has been employed for the synthesis of a variety of compounds (for details, see DeLucchi *et al.* 1991) but not previously for the synthesis of PCB methyl sulphones.

Since the present authors wished to work on a smaller scale, i.e. only 1/500 as much starting material as used by Parham and Edwards (1968), it was necessary to modify the reaction conditions. The large excess of acetic acid anhydride utilized by Parham and Edwards diluted the present reactants far too extensively and actually gave no products. By decreasing the amount of acetic acid anhydride to one-tenth and using a small (1 ml) sealed ampoule with an argon atmosphere as the reaction vessel, a small amount of the desired product could be obtained after 20 h, i.e. the reaction time employed in the original procedure (Parham and Edwards 1968). Upon extending the reaction time to 11 days, the desired product was formed in quantitative yield, thus enabling the preparation of MeSO₂-PCB congeners not previously synthesized.

It was chosen to introduce the radioisotope as part of the methyl group after carefully considering the risk for methyl and/or H exchange. The methylsulphone group has been shown to be chemically stable and to the present authors' knowledge it is not participating in any methyl exchange.

In conclusion, MeSO₂-PCB congeners have been identified that are selectively

retained in rat liver. One of these congeners, 3'-MeSO₂-CB101, the isomeric 4'-MeSO₂-CB101 and two other MeSO₂-PCBs were synthesized in ¹⁴C-labelled form. The estimated hepatic half-lives for the different PCB congeners varied widely, ranging from 10 to 33 days. There is no obvious structural basis for this large differences in their half-life.

Acknowledgement

Ioannis Athanasiadis, Anders Olsson and Lotta Hovander are gratefully acknowledged for their technical assistance. The authors also thank Anna Mörck and Eva Klasson Wehler for comments on the manuscript.

References

- BAKKE, J. E., 1989, Metabolites derived from glutathione conjugation. In D. H. Hutson *et al.* (eds), *Intermediary Xenobiotic Metabolism in Animals* (London: Taylor & Francis), pp. 205–224.
- BAKKE, J. E., BERGMAN, Å. L. and LARSEN, G. L., 1982, Metabolism of 2,4',5-trichlorobiphenyl in the mercapturic acid pathway. *Science*, **217**, 645–647.
- BAKKE, J. E. and GUSTAFSSON, J. Å., 1986, Role of intestinal microflora in metabolism of polychlorinated biphenyls. In B. Hallgren *et al.* (ed.), *Diet and Prevention of Coronary Heart Disease and Cancer* (New York: Raven), pp. 47–54.
- BALLSCHMITTER, K., MENNEL, A. and BUYTEN, J., 1993, Long chain alkyl-polysiloxanes as non-polar stationary phases in capillary gas chromatography. *Fresenius Journal of Analytical Chemistry*, **346**, 396–402.
- BERGMAN, Å., ATHANASIADOU, M., BERGEG, S., HARAGUCHI, K., JENSEN, S. and KLASSON WEHLER, E., 1992, PCB and PCB methyl sulfones in mink treated with PCB and various PCB fractions. *Ambio*, **21**, 570–576.
- BERGMAN, Å., BRANDT, I. and JANSSON, B., 1979, Accumulation of methylsulfonyl derivatives of some bronchial-seeking polychlorinated biphenyls in the respiratory tract of mice. *Toxicology and Applied Pharmacology*, **48**, 213–220.
- BERGMAN, Å., GÖTHE, R. and WACHTMEISTER, C. A., 1976, Impregnation of silica gel with tetraalkylammonium salts in adsorption chromatography of neutral aromatic compounds. *Journal of Chromatography*, **123**, 231–236.
- BERGMAN, Å., HARAGUCHI, K., ATHANASIADOU, M. and LARSSON, C., 1993, Selective retention of PCB methyl sulfones in liver of mammals. *Organohalogen Compounds*, **14**, 199–201.
- BERGMAN, Å., JANSSON, B. and BAMFORD, I., 1980, Methylthio and methylsulfonylpolychlorobiphenyls: synthesis and studies of correlation between structure and fragmentation pattern on electron impact. *Biomedical Mass Spectrometry*, **7**, 20–27.
- BERGMAN, Å., NORSTROM, R. J., HARAGUCHI, K., KUROKI, H. and BÉLAND, P., 1994, PCB and DDE methyl sulfones in mammals from Canada and Sweden. *Environmental Toxicology and Chemistry*, **13**, 121–128.
- BERGMAN, Å. and WACHTMEISTER, C. A., 1978, Synthesis of methylthio- and methylsulfonyl polychlorobiphenyls via nucleophilic aromatic substitution of certain types of polychlorobiphenyls. *Chemosphere*, **12**, 949–956.
- BERGMAN, Å. and WACHTMEISTER, C. A., 1987, Phase transfer mediated synthesis of radiolabelled alkyl aryl ethers and sulphides. *Journal of Labelled Compounds and Radiopharmaceuticals*, **8**, 925–930.
- BRANDT, I., BERGMAN, Å. and WACHTMEISTER, C. A., 1976, Distribution of polychlorinated biphenyls. Structural requirements for accumulation in the mouse bronchial mucosa. *Experimenta*, **32**, 497–498.
- BRANDT, I., DARNERUD, P. O., BERGMAN, Å. and LARSSON, Y., 1982, Metabolism of 2,4',5-trichlorobiphenyl: enrichment of hydroxylated and methyl sulfone metabolites in the uterine luminal fluid of pregnant mice. *Chemico-Biological Interactions*, **40**, 45–56.
- BRANDT, I., LUND, J., BERGMAN, Å., KLASSON WEHLER, E., POELLINGER, L. and GUSTAFSSON, J. Å., 1985, Target cells for the polychlorinated biphenyl metabolite 4,4'-bis(methylsulfonyl)-2,2',5,5'-tetrachlorobiphenyl in lung and kidney. *Drug Metabolism and Disposition*, **13**, 490–496.
- BÜHLER, F., SCHMID, P. and SCHLATTER, C., 1988, Kinetics of PCB elimination in man. *Chemosphere*, **17**, 1717–1726.
- DELUCCI, O., MIOTTI, U. and MODENA, G., 1991, The Pummerer reaction of sulfinyl compounds. In L. A. Paquette *et al.* (eds), *Organic Reactions* (New York: Wiley), pp. 157–405.
- GOOCH, J. A. and HAMDY, M. K., 1982, Depuration and biological half-life ¹⁴C-PCB in aquatic organisms. *Bulletin of Environmental Contamination and Toxicology*, **28**, 305–312.

- HARAGUCHI, K. and BERGMAN, Å., 1991, Synthesis of ^{14}C -labelled PCB methyl sulfones. *Chemosphere*, **23**, 1837–1843.
- HARAGUCHI, K., KATO, Y., KIMURA, R. and MASUDA, Y., 1999, Tissue distribution of methylsulfonyl metabolites derived from 2,2',4,5,5'-penta- and 2,2',3,4',5',6-hexachlorobiphenyls in rats. *Archives of Environmental Contamination and Toxicology*, **37**, 135–142.
- HARAGUCHI, K., KUROKI, H. and MASUDA, Y., 1987a, Synthesis and characterisation of tissue-retainable methylsulfonyl polychlorinated biphenyls isomers. *Journal of Agricultural and Food Chemistry*, **35**, 178–182.
- HARAGUCHI, K., KUROKI, H. and MASUDA, Y., 1987b, Synthesis of PCB methylsulfone: Some differences in mass and proton magnetic resonance spectroscopy. *Chemosphere*, **16**, 2299–2313.
- HORI, S., MIYATA, H., MAEDA, K., MURAKAMI, Y. and KASHIMOTO, T., 1971, *Proceedings of Osaka Prefectural Institute of Public Health*, ed. of Food Sanitation, 101.
- JANSSON, B., JENSEN, S., OLSSON, M., REHNBERG, L., SUNDBSTRÖM, G. and VAZ, R., 1975, Identification by GC-MS of phenolic metabolites of PCB and *p,p'*-DDE isolated from Baltic guillemot and seal. *Ambio*, **4**, 93–97.
- JENSEN, S., 1972, The PCB story. *Ambio*, **1**, 45–53.
- JENSEN, S. and JANSSON, B., 1976, Anthropogenic substances in seal from the Baltic: methyl sulfone metabolites of PCB and DDE. *Ambio*, **5**, 257–260.
- JENSEN, S., REUTEGÅRD, L. and JANSSON, B., 1983, *Analytical Methods for Measuring Organochlorines and Methyl Mercury by Gas Chromatography*. FAO Fisheries Technical Paper no. 212, pp. 21–33.
- KATO, Y., HARAGUCHI, K., TOMIYASU, K., SAITO, H., ISOGAI, M., MASUDA, Y. and KIMURA, R., 1997, Structure-dependent induction of CYP2B1/2 by 3-methylsulfonyl metabolites of polychlorinated biphenyl congeners in rats. *Environmental Toxicology and Pharmacology*, **3**, 137–144.
- KLASSON WEHLER, E., BERGMAN, Å., ATHANASIADOU, M., LUDWIG, J. P., AUMAN, H. J., KANNAN, K., VAN DEN BERG, M., MURK, A. J., FEYK, L. A. and GIESY, J. P., 1998, Hydroxylated and methylsulfonyl polychlorinated biphenyl metabolites in albatrosses from Midway atoll, North Pacific ocean. *Environmental Toxicology and Chemistry*, **17**, 1620–1625.
- KLASSON WEHLER, E., BERGMAN, Å., KOWALSKI, B. and BRANDT, I., 1987, Metabolism of 2,3,4',6-tetrachlorobiphenyl: formation and tissue localization of mercapturic acid pathway metabolites in mice. *Xenobiotica*, **17**, 477–486.
- LARSEN, G. L. and BERGMAN, Å., 1994, Interaction of methylsulfonyl-containing PCB with mammalian carrier proteins. *Organohalogen Compounds*, **25**, 451–454.
- LARSEN, G. L., HUWE, J. K., BERGMAN, Å., KLASSON WEHLER, E. and HARGIS, P., 1992, Methylsulfonyl metabolites of xenobiotics can serve as ligands for fatty acid binding proteins in chicken liver and intestinal mucosa. *Chemosphere*, **25**, 1189–1194.
- LETCHER, R. J., 1996, The ecological and analytical chemistry of chlorinated hydrocarbon contaminants and methyl sulfonyl-containing metabolites in the polar bear (*Ursus maritimus*) food chain. PhD thesis, Carleton University, Ottawa.
- LETCHER, R. J., KLASSON WEHLER, E. and BERGMAN, Å., 2000, Methyl sulfone and hydroxylated metabolites of polychlorinated biphenyls. In J. Paasivirta (ed.), *The Handbook of Environmental Chemistry* (Berlin: Springer-Verlag), pp. 315–359.
- LUND, B. O., ÖRBERG, J., BERGMAN, Å., LARSSON, C., BERGMAN, A., BÄCKLIN, B. M., HÅKANSSON, H., MADEJ, A., BROUWER, A. and BRUNSTRÖM, B., 1999, Chronic and reproductive toxicity of a mixture of 15 methylsulfonyl-polychlorinated biphenyls and 3-methylsulfonyl-2,2-bis-(4-chlorophenyl)-1,1-dichloroethane in mink (*Mustela vison*). *Environmental Toxicology and Chemistry*, **18**, 292–298.
- LUND, J., BRANDT, I., POELLINGER, L., BERGMAN, Å., KLASSON WEHLER, E. and GUSTAFSSON, J. Å., 1985, Target cells for the polychlorinated biphenyl metabolite 4,4'-bis(methylsulfonyl)-2,2',5,5'-tetrachlorobiphenyl. *Molecular Pharmacology*, **27**, 314–323.
- MES, J., ARNOLD, D. L. and BRYCE, F., 1995, The elimination and estimated half-lives of specific polychlorinated biphenyl congeners from the blood of female monkeys after discontinuation of daily dosing with Aroclor 1254. *Chemosphere*, **30**, 789–800.
- MIO, T., SUMINO, K. and MIZUTANI, T., 1976, Sulfur-containing metabolites of 2,2',5,5'-tetrachlorobiphenyl, a major component of commercial PCBs. *Chemical and Pharmaceutical Bulletin*, **24**, 1958–1960.
- MORTIMER, R. D. and NEWSOME, W. H., 1996, An alternative synthesis of chlorinated biphenyl methylsulphonyl metabolites. *Chemosphere*, **32**, 935–946.
- NAKANISHI, Y., SHIGEMATSU, N., KURITA, Y., MATSUBA, K., KANEGAE, H., ISHIMURA, S. and KAWAZOE, Y., 1985, Respiratory involvement and immune status in Yusho patients. *Environmental Health Perspectives*, **154**, 217–227.
- NORÉN, K. and MEIRONYTYÉ, D., 2000, Certain organochlorine and organobromine contaminants in Swedish human milk in perspective of past 20–30 years. *Chemosphere*, **40**, 1111–1123.
- OLSSON, A. and BERGMAN, Å., 1995, A new persistent contaminant detected in Baltic wildlife: Bis-(4-chlorophenyl)sulfone. *Ambio*, **24**, 119–123.

- PARHAM, W. E. and EDWARDS, L. D., 1968, The scope of the Pummerer reaction. *Journal of Organic Chemistry*, **33**, 4150–4154.
- PRESTON, B. D. and ALLEN, J. R., 1980, 2,2',5,5'-Tetrachlorobiphenyl: isolation and identification of metabolites generated by rat liver microsomes. *Drug Metabolism and Disposition*, **8**, 197–204.
- PUMMERER, R., 1910, Über Phenylsulfoxy-essigsäure (II), 1401–1412.
- SUNDSTRÖM, G., HUTZINGER, O. and SAFE, S., 1976, The metabolism of chlorobiphenyls – a review. *Chemosphere*, **5**, 267–298.
- TANABE, S., NAKAGAWA, Y. and TATSUKAWA, R., 1981, Absorption efficiency and biological half-life of individual chlorobiphenyls in rats treated with Kanechlor products. *Agricultural and Biological Chemistry*, **45**, 717–726.
- WEISTRAND, C. and NORÉN, K., 1997, Methylsulfonyl metabolites of PCBs and DDE in human tissues. *Environmental Health Perspectives*, **105**, 644–649.
- YAKUSHIJU, T., WATANABE, I., KUWABARA, K., TANAKA, R., KASHIMOTO, T., KUNITA, N. and HARA, I., 1984, Rate of decrease and half-life of polychlorinated biphenyls (PCBs) in the blood of mothers and their children occupationally exposed to PCBs. *Archives of Environmental Contamination and Toxicology*, **13**, 341–345.
- YOUNG, R. N., GAUTHIER, J. Y. and COOMBS, W., 1984, The methyl group as a protecting group for arylthiols: a mild and efficient method for conversion of methyl aryl sulfides to arylthiols. *Tetrahedron Letters*, **25**, 1753–1756.

Bioactivation to free radicals and cytotoxicity of 1,1-dichloro-1-fluoroethane (HCFC-141b)

A. ZANOVELLO, R. TOLANDO, R. FERRARA,
S. BORTOLATO and M. MANNO*

Department of Environmental Medicine & Public Health, University of Padua Medical School, Padua, Italy

Received 15 July 2000

1. The *in vitro* bioactivation by rat liver microsomes and the cytotoxicity in rat hepatocytes of 1,1-dichloro-1-fluoroethane (HCFC-141b), a replacement for some ozone depleting chlorofluorocarbons (CFC), have been investigated.

2. Anaerobic incubations of liver microsomes from pyridine-induced rats with HCFC-141b in the presence of the spin-trapping agent *N*-*t*-butyl- α -phenylnitrone (PBN) resulted in the formation of a typical ESR radical signal.

3. In the presence of HCFC-141b, a dose-dependent formation of conjugated dienes was observed that was partially inhibited by PBN, glutathione (GSH) and vitamin C. Moreover, HCFC-141b increased the release of lactate dehydrogenase (LDH) and the depletion of cellular glutathione in isolated rat hepatocytes under both normoxic and hypoxic conditions.

4. HCFC-141b-dependent cytotoxicity was completely prevented by PBN under both conditions and it was partially prevented under normoxic conditions by the broad-spectrum P450 inhibitor metyrapone, the P4502E1 specific inhibitor 4-methylpyrazole and the P4503A-specific inhibitor troleandomycin. Interestingly, HCFC-141b-dependent glutathione depletion was not prevented by PBN, metyrapone, 4-methylpyrazole or troleandomycin, whereas two glutathione depletors, 2,6-dimethyl-2,5-heptadien-4-one (phorone) and diethylmaleate, partially prevented LDH release.

5. The present results indicate that HCFC-141b is reductively metabolized *in vitro* to free radical intermediates by P450, in particular by the CYP2E1 and, to a lower extent, CYP3A isoforms, leading to peroxidative membrane damage and glutathione-independent cytotoxicity.

Introduction

Hydrochlorofluorocarbons (HCFC) are being developed and used as replacements for the ozone-depleting chlorofluorocarbons (CFC) for both industrial and domestic applications (solvents, cleaning fluids, refrigerants, propellants, blowing agents in the production of foam plastics) resulting in occupational and environmental human exposure. CFC do not decompose in the troposphere and thus rise to the stratosphere where ultraviolet radiation catalyses the release of their chlorine atoms, which in turn destroy stratospheric ozone. The depletion of stratospheric ozone by CFC is thought to lead to increased amounts of UV radiation that reach the earth's surface (Molina and Rowland 1974) resulting in adverse effects on human health, including an increase in cataract formation (Taylor *et al.* 1988) and skin cancer (Longstreth *et al.* 1988). The Montreal Protocol, signed by 24 nations and the European Economic Community, agreed a schedule to control the production and consumption of CFC and halons. Because of the ubiquitous use and benefits of CFC and halons, a search for safe replacements of these compounds was undertaken (Jarabek *et al.* 1994).

* Author for correspondence; e-mail: manno@ux1.unipd.it

HCFC possess many of the beneficial properties of CFC but with a lower ozone-depleting potential due to the presence of the C-H bond(s), which increase(s) not only HCFC tropospheric lability and, therefore, reduce their migration to the stratosphere (Jarabek *et al.* 1994) but also their biotransformation. HCFC, unlike CFC, have been demonstrated to undergo metabolic bioactivation by various enzyme systems both in animals and humans. They may undergo P450-catalyzed oxidation of the C-H σ bond or, in the absence of oxygen, P450-catalyzed reductive dehalogenation reactions resulting, in some cases, in the formation of reactive metabolites, as demonstrated for 2,2-dichloro-1,1,1-trifluoroethane (HCFC-123) (Harris *et al.* 1991, Huwyler *et al.* 1992, Ferrara *et al.* 1997), 2-chloro-1,1,1,2-tetrafluoroethane (HCFC-124) and 1,1,1,2-tetrafluoroethane (HCFC-134a) (Dekant 1996), as well as for other polyhalogenated alkanes such as halothane (Manno *et al.* 1991, Lind and Gandolfi 1993).

1,1-Dichloro-1-fluoroethane (HCFC-141b) is a potential substitute for CFC-11 in foam-blowing operations, and the mixture of HCFC-141b and HCFC-123, an effective substitute for CFC-113, is used as a cleaning agent in the computer industry. HCFC-141b has a low ozone-depleting potential of 0.10–0.12 and a low global warming potential of 0.12 relative to the corresponding CFC-11 references of 1.0 (ECETOC 1994). HCFC-141b is oxidatively metabolized by P450 both *in vitro* (Loizou and Anders 1993) and *in vivo* (Loizou and Anders 1993, Tong *et al.* 1998) to 2,2-dichloro-2-fluoroethanol and it was reductively activated to reactive metabolites by rat liver microsomes resulting in P450 loss (Tolando *et al.* 1996).

The aims of the present study were (1) to characterize the reactive metabolites produced by rat liver microsomes, (2) to investigate the P450 isoforms involved and (3) to clarify further the mechanism of toxicity of HCFC-141b *in vitro*. The formation of free radical species was studied by the ESR technique. The toxic effects of the compound were studied by measuring (1) the levels of conjugated dienes, in anaerobic liver microsomal incubations from pyridine-treated rats and (2) the release of cellular LDH or the depletion of total cellular glutathione, in isolated rat hepatocytes in suspension. Preliminary summary reports of parts of the present study have already been published (Manno *et al.* 1998, Ferrara *et al.* 1999, Zanollo *et al.* 1999).

Materials and methods

Chemicals and biochemicals

HCFC-141b (>99.9% purity) was from PCR, Inc. (Gainesville, Florida, USA). Glucose 6-phosphate, glucose 6-phosphate dehydrogenase, glucose oxidase, catalase, reduced glutathione (GSH), glutathione reductase, NADP⁺, NADPH, *N*-*t*-butyl- α -phenylnitron (PBN) and vitamin C were from Sigma Chemical Co. Ltd (Poole, UK). Collagenase H was from Boehringer-Mannheim (Mannheim, Germany). Other reagents were of cellular culture or analytical grade.

Treatment of animals

Since HCFC-141b is metabolized by CYP2E1, male Wistar albino rats (290–390 g) received an i.p. injection of the CYP2E1 inducer pyridine (PYR, 200 mg kg⁻¹ b.w., 100 mg ml⁻¹ aqueous solution, adjusted to pH 7.4 with 1 M HCl) once daily for 4 consecutive days and were sacrificed 24 h after the last injection.

Preparation of microsomes

Rats were starved for 18 h before being killed by cervical dislocation. Livers were perfused *in situ* through the inferior vena cava with ~50 ml ice-cold 0.9% (w/v) NaCl, weighed and homogenized in 3 vols 0.25 M sucrose by a Polytron homogenizer (1 min at speed 5). From these homogenates the microsomal fraction was prepared by a modification of the method of Kamath *et al.* (1971) as described by Manno *et al.* (1991).

Anaerobic microsome incubations

Incubations were performed in glass vials sealed with a Teflon-lined rubber stopper. To ensure anaerobic conditions throughout the incubation, care was taken to remove oxygen by bubbling O₂-free N₂ through the buffer for 1 h, as described by Cooper *et al.* (1982), and flushing the incubation vials with the same gas through the Teflon-lined rubber septum before injection of the microsomal suspension. Residual O₂ was removed by including in the incubation mixture the following oxygen-scavenging system: catalase (600 U ml⁻¹), glucose oxidase (12.5 units ml⁻¹) and D-glucose (60 mM). Under these conditions the O₂ concentration in the incubation mixture was kept below the lower limit of detection of the O₂ electrode (~0.2 µM). A typical incubation contained 1.5–3 mg microsomal protein ml⁻¹ and a NADPH-regenerating system consisting of glucose 6-phosphate (4 mM), glucose 6-phosphate dehydrogenase (0.2 U ml⁻¹), NADP⁺ (0.2 mM) and MgCl₂ (6 mM), in 3 ml 0.1 M Na₂HPO₄ buffer, pH 7.4. The reaction was started by the addition of HCFC-141b dissolved in methanol. Values given indicate the final calculated concentrations of substrate in the incubation mixture. Where relevant, the spin-trapping agent PBN or GSH or vitamin C were added to the incubation mixture at the indicated concentrations, before injecting the substrate in methanol and/or the NADPH-regenerating system.

Hepatocyte isolation

Hepatocytes were prepared from untreated male Wistar rats, 150–350 g body weight, anaesthetized with sodium pentobarbital i.p. (150 mg kg⁻¹ b.w.) by the perfusion method described by Williams *et al.* (1977), using EGTA-supplemented Hanks' balanced salt solution (HBSS) as the perfusing medium, and collagenase as the dissociating agent. After isolation, hepatocytes were washed and resuspended in ice-cold Williams' medium E or L-15 medium Leibovitz (hypoxic condition) containing 10% foetal calf serum, 2 mM L-glutamine and penicillin/streptomycin (24 units ml⁻¹ and 24 µg ml⁻¹, respectively). The final cell suspension contained 3–6 × 10⁶ cells ml⁻¹, and viability was between 70 and 88%.

Incubation of isolated hepatocytes

Incubations (2.5 ml cell suspension with 10⁶ viable cells ml⁻¹) were usually carried out at 37 °C in 5 ml glass tubes sealed with rubber/Teflon septa and aluminium caps, in the medium supplemented as described above. Where relevant, hypoxic conditions (~4% O₂ by the oxygen electrode) were obtained by bubbling O₂-free N₂ through the medium for 1 h before adding the cells, and by flushing N₂ into the sealed vials before addition of the final cell suspension. The incubations contained the indicated final concentrations of HCFC-141b added in dimethylsulphoxide (DMSO) (5 µl total volume) and control incubations were performed adding DMSO alone (5 µl). At the end of the incubation, 0.5 ml cell suspension was removed from the incubation and immediately centrifuged at 50g for 5 min to sediment the cells. The supernatant was transferred to a clean plastic tube and stored at -20 °C until the LDH release assay was performed. For the glutathione assay, the cell pellet was washed with Dulbecco's phosphate-buffered saline (PBS) and centrifuged again at 50g. The supernatant was removed and the cells were lysated in 10% trichloroacetic acid (TCA), centrifuged at 5800g for 2.5 min to sediment the precipitate and, finally, the cell lysate was stored at -80 °C until the glutathione assay was performed.

Assays

To detect the formation of free radical intermediates in the microsomal incubation, samples were removed with an equal volume of a 2:1 (v/v) chloroform/methanol mixture, shaken twice and then the chloroform layer was removed for electron spin resonance (ESR) analysis. ESR spectra were obtained at room temperature on a Bruker ESP 300E spectrometer, the instrument settings being the following: modulation frequency = 100 kHz, microwave power = 20 mW, modulation amplitude = 1 G, receiver gain = 10⁵, sweep width = 100 G, sweep time = 168 s and time constant = 41 ms. For the measurement of conjugated dienes, at the end of the incubation, 1 ml samples were mixed, centrifuged twice at 260g for 10 min with 10 ml of a 2:1 (v/v) chloroform/methanol mixture, and the chloroform layer was transferred into a flask. Water (2 ml) was added to this organic phase and mixed gently to remove the residual methanol. The chloroform was then removed from this organic phase with a rotary evaporator. Finally, chloroform-free lipids were dissolved in 1 ml pure cyclohexane (Wills 1987). Conjugated dienes were measured spectrophotometrically by the peak formed at λ = 233 nm in the second derivative spectrum according to the method of Corongiu *et al.* (1986) as described by Halliwell and Gutteridge (1989). Cell viability was determined by the trypan blue exclusion test. Lactate dehydrogenase (LDH) activity released in the medium from hepatocytes was assayed spectrophotometrically by a reagent kit (LDH Optimized, Sigma Diagnostics). Total cellular glutathione was determined in the cell lysate in the presence of 10% TCA by the method of Griffith (1985). Statistical analysis of the results was carried out by the one-way analysis of variance and the Student's *t*-test.

Results

After incubation of pyridine-induced rat liver microsomes with 10 mM HCFC-141b in the presence of the spin-trapping agent PBN, an ESR signal was observed

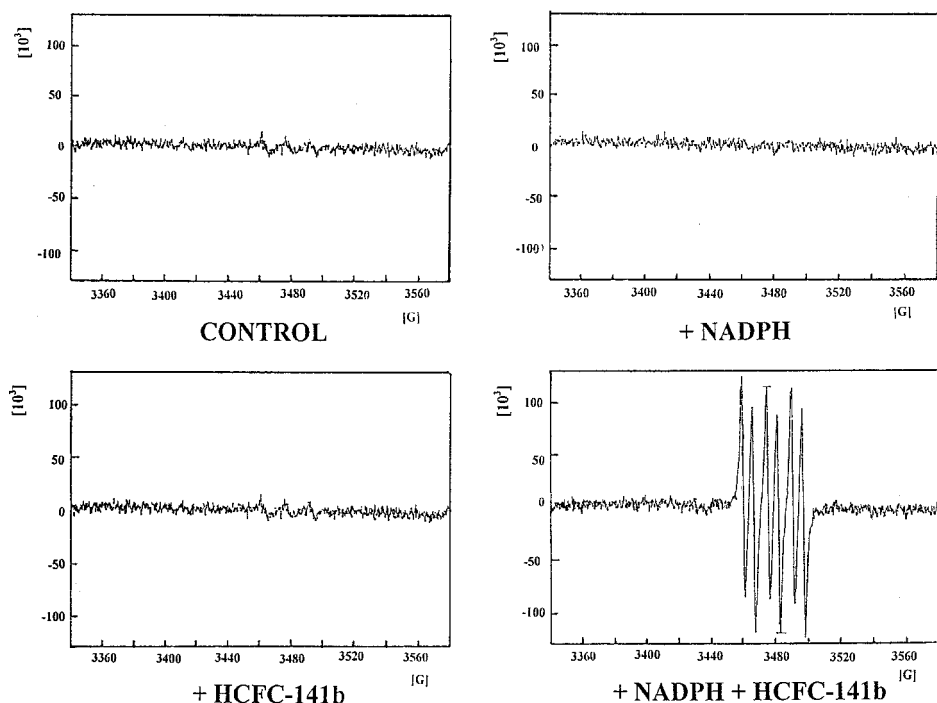


Figure 1. Electron spin resonance (ESR) of HCFC-141b free radicals produced during anaerobic incubation with NADPH-reduced liver microsomes from pyridine-induced rats. Anaerobic incubations were at 37 °C and contained 3 mg microsomal protein ml⁻¹ in phosphate buffer, pH 7.4, in the presence of 20 mM PBN and the O₂-scavenging system described in the Materials and methods. After pre-incubation for 10 min, 10 μ l methanol alone (control) or the NADPH-regenerating system (+NADPH) or 10 mM HCFC-141b in methanol (+HCFC-141b) or the NADPH-regenerating system+10 mM HCFC-141b in methanol (+NADPH+HCFC-141b) were injected. After a 60-min incubation, the ESR assay was carried out as described in the Materials and methods.

that showed a characteristic six-line spectrum consisting of a doublet of triplets, suggesting the formation of a carbon-centred free radical intermediate. No signal appeared in any of the control incubations (microsomes alone, microsomes and NADPH-regenerating system, or microsomes and HCFC-141b) (figure 1).

The ability of HCFC-141b to stimulate lipid peroxidation was investigated in anaerobic incubations of pyridine-induced rat liver microsomes by measuring the formation of conjugated dienes by the UV absorption spectra of phospholipids extracted from microsomes. Incubation of microsomes with increasing HCFC-141b concentrations (0, 0.1, 0.5, 1.0, 5.0 mM) in the presence of an NADPH-regenerating system resulted in a statistically significant concentration-dependent formation of conjugated dienes (figure 2). To obtain information on the role played in this process by the reactive species formed, the effects of the spin-trapping agent PBN and that of the antioxidants glutathione (GSH) and vitamin C were investigated. HCFC-141b-dependent conjugated dienes formation was partially prevented by PBN, GSH or vitamin C (figure 3).

The cytotoxic effect of HCFC-141b on isolated rat hepatocytes was evaluated by monitoring LDH release in the cell suspension medium. Isolated rat hepatocytes incubated for 60 min with increasing concentrations of HCFC-141b (0, 1, 5, 10 mM) under normoxic or hypoxic conditions showed a concentration-dependent LDH

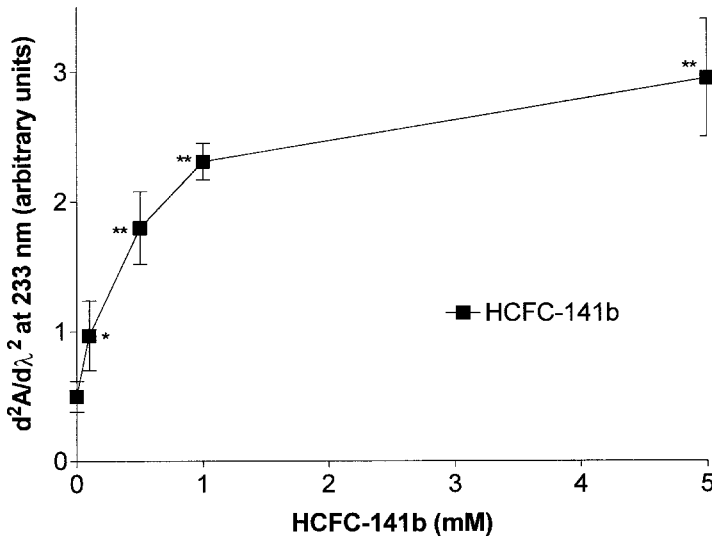


Figure 2. Formation of conjugated dienes during the reductive anaerobic incubation of pyridine-induced rat liver microsomes with HCFC-141b. Anaerobic incubations were at 37 °C and contained 1.5 mg protein ml⁻¹ in 1.0 ml phosphate buffer, pH 7.4, in the presence of the O₂-scavenging system. After pre-incubation for 10 min, the reaction was started by the addition of the NADPH-regenerating system and the indicated concentrations of HCFC-141b in methanol. After 2 h of incubation the reaction mixture was extracted by a chloroform/methanol mixture (see the Materials and methods for details). Conjugated dienes were measured by recording the second derivative spectral signal and are expressed as $d^2A/d\lambda^2$ at $\lambda = 233$ nm, as described in the Materials and methods. Values are mean $\pm$ SD from four determinations. * $p < 0.05$, ** $p < 0.001$ versus control (Student's *t*-test).

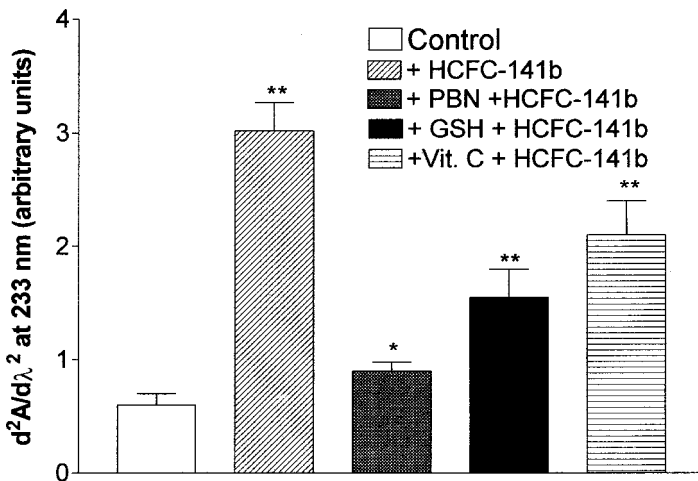


Figure 3. Protection by PBN, GSH and vitamin C against the formation of conjugated dienes during the reductive anaerobic incubation of pyridine-induced rat liver microsomes with HCFC-141b. Incubations were as described in figure 2. Where indicated, PBN (1 mM), GSH (10 mM) or vitamin C (10 mM) were injected into the incubation mixture. After pre-incubation, the NADPH-regenerating system (control) or the NADPH-regenerating system and 5 mM HCFC-141b in methanol were added to start the reaction. After 2 h, determinations were as described for figure 2. Values are mean $\pm$ SD from four determinations. * $p < 0.05$, ** $p < 0.001$ versus control (Student's *t*-test).

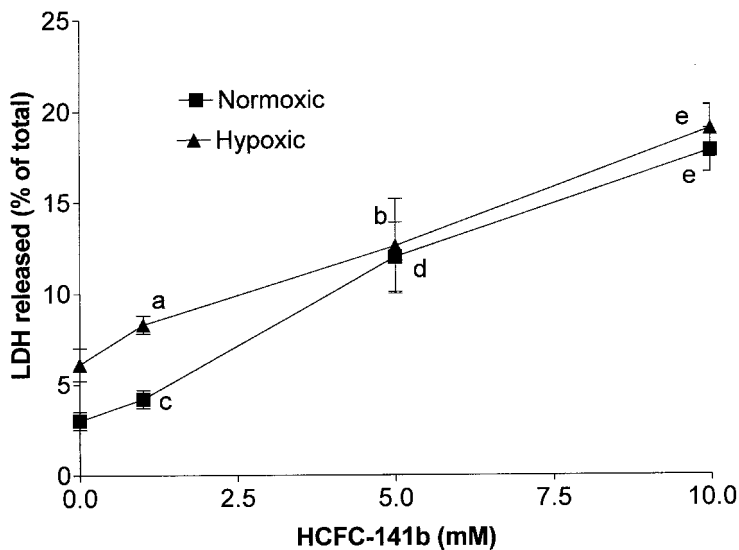


Figure 4. Effect of increasing concentrations of HCFC-141b on LDH release from hepatocytes under normoxic or hypoxic conditions. The hepatocyte incubations contained 10^6 cells ml^{-1} and were performed at 37°C as described in the Materials and methods. After pre-incubation for 10 min, HCFC-141b was added in DMSO, in a total volume of $5\text{ }\mu\text{l}$, to achieve the indicated final concentrations. DMSO alone ($5\text{ }\mu\text{l}$) was added to control incubations. After 60 min, 0.5 ml cell suspensions were transferred to plastic tubes and centrifuged at $50g$ to sediment cells. The supernatant was then separated and stored for LDH determination. Values are mean $\pm$ SD from three incubations. (a) Not significant; (b) $p < 0.05$; (c) $p < 0.02$; (d) $p < 0.01$ and (e) $p < 0.001$ versus the respective control in the absence of HCFC-141b (Student's t -test).

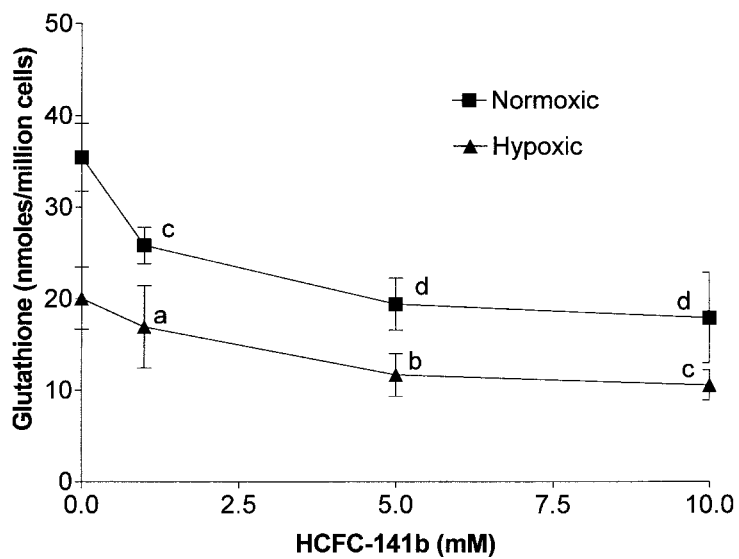


Figure 5. Glutathione depletion in hepatocytes incubated with various concentrations of HCFC-141b under normoxic or hypoxic conditions. Determinations were carried out on the same incubations used for the LDH assay (figure 4) and were as described in the Materials and methods. Values are mean $\pm$ SD from three incubations. (a) Not significant; (b) $p < 0.05$; (c) $p < 0.02$ and (d) $p < 0.01$ versus the respective control in the absence of HCFC-141b (Student's t -test).

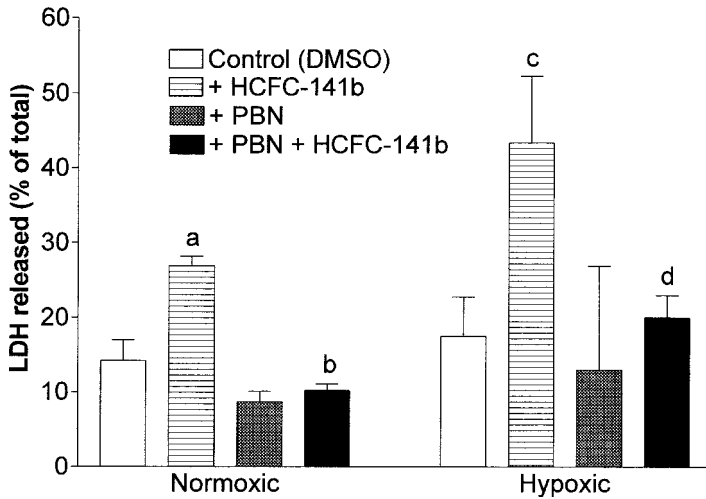


Figure 6. Protective effect of PBN on LDH release from hepatocytes incubated with HCFC-141b under normoxic or hypoxic conditions. Incubations and determination were as for figure 4. Before pre-incubation, PBN was added in methanol at 30 mM final concentration. After pre-incubation, HCFC-141b was added in DMSO, for a total volume of 5 μ l, to a final concentration of 10 mM; 5 μ l DMSO were added to control incubations. (a) $p < 0.01$ versus control; (b) not significant versus '+PBN' and $p < 0.001$ versus '+HCFC-141b'; (c) $p < 0.02$ versus control; (d) not significant versus '+PBN' and $p < 0.02$ versus '+HCFC-141b' (Student's *t*-test).

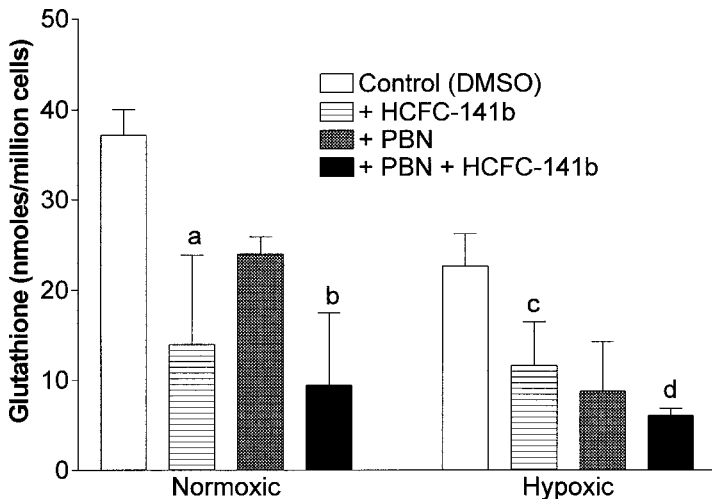


Figure 7. Lack of effect of PBN on glutathione depletion in hepatocytes incubated with HCFC-141b under normoxic or hypoxic conditions. Determinations were carried out on the same incubations used for the LDH assay (figure 6) and were as described in the Materials and methods. Values are mean $\pm$ SD from three incubations. (a) $p < 0.02$ versus control; (b) $p < 0.05$ versus '+PBN' and not significant versus '+HCFC-141b'; (c) $p < 0.05$ versus control; (d) not significant versus '+PBN' or '+HCFC-141b' (Student's *t*-test).

release (figure 4). A concentration-dependent depletion of total intracellular glutathione was also observed in the same incubations (figure 5). Interestingly, no effect on HCFC-141b-dependent LDH release or glutathione depletion by O_2 concentration was observed. To determine if free radical metabolites were involved in HCFC-141b cytotoxicity, the effect of the spin-trapping agent PBN was investigated using both assays. PBN (30 mM) completely prevented HCFC-141b-

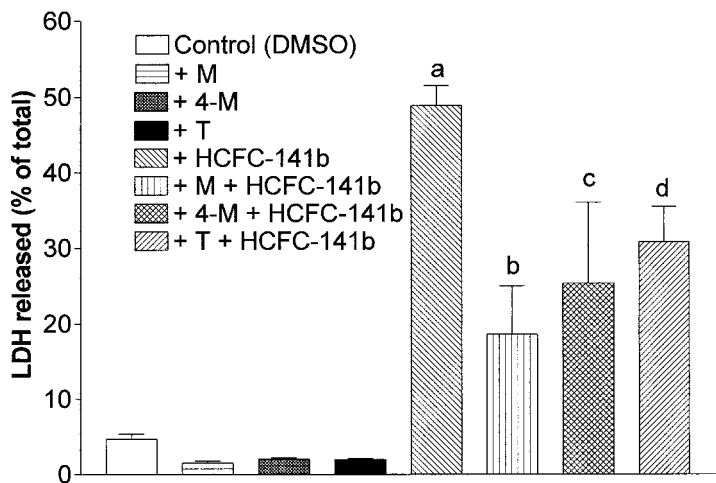


Figure 8. Partial prevention of LDH release by aspecific and specific P450 inhibitors in hepatocytes incubated with HCFC-141b under normoxic conditions. Incubations were as described for figure 4 but were pre-incubated for 20 min. Before pre-incubation, inhibitors were added in methanol at the following final concentrations: 100 μ M metyrapone (+M), 500 μ M 4-methylpyrazole (+4-M) and 100 μ M troleanandomycin (+T). After pre-incubation, LDH was measured as described in the Materials and methods. Values are mean $\pm$ SD from four incubations. (a) $p < 0.001$ versus control; (b) $p < 0.01$ versus '+M' and $p < 0.001$ versus '+HCFC-141b'; (c) $p < 0.01$ versus '+4-M' and $p < 0.01$ versus '+HCFC-141b'; (d) $p < 0.001$ versus '+T' and $p < 0.001$ versus '+HCFC-141b' (Student's *t*-test).

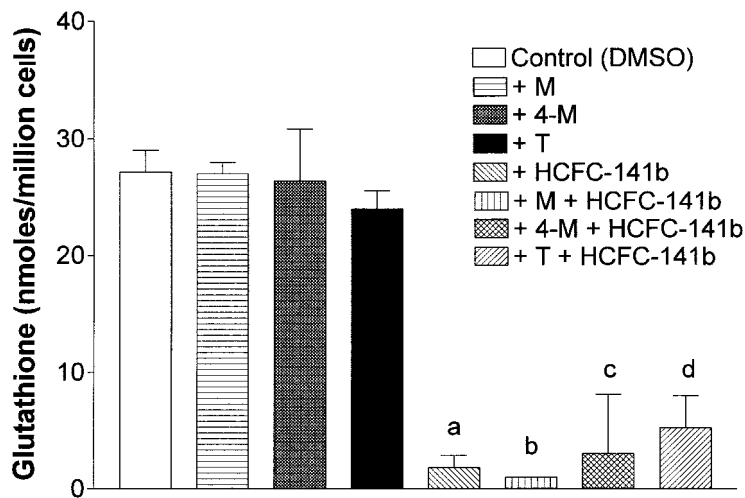


Figure 9. Lack of prevention of glutathione depletion by non-specific and specific P450 inhibitors in hepatocytes incubated with HCFC-141b under normoxic conditions. Determinations were carried out on the same incubations used for LDH assay (figure 8) and were as described in the Materials and methods. Values are mean $\pm$ SD from three or four incubations. (a) $p < 0.001$ versus control; (b) $p < 0.001$ versus '+M' and not significant versus '+HCFC-141b'; (c) $p < 0.001$ versus '+4M' and not significant versus '+HCFC-141b'; (d) $p < 0.001$ versus '+T' and not significant versus '+HCFC-141b' (Student's *t*-test).

dependent LDH release under both normoxic and hypoxic conditions (figure 6). On the contrary, in the same incubations, no protective effect of PBN on intracellular glutathione depletion was observed and, interestingly, PBN itself caused a slight glutathione depletion (figure 7). A slight decrease of LDH release was also observed

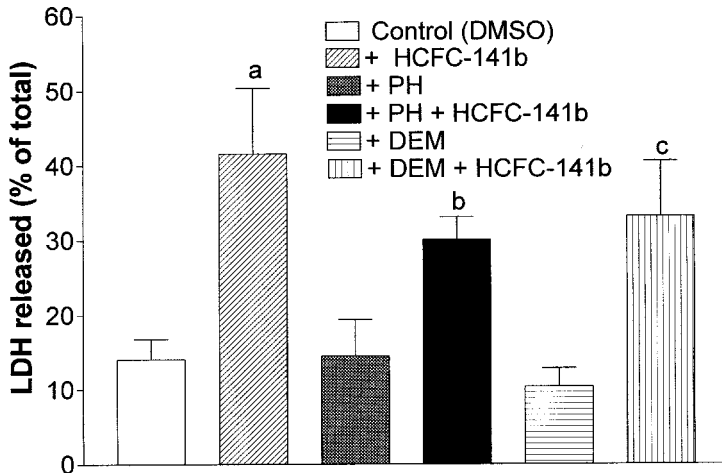


Figure 10. Protective effect of phorone (PH) and diethylmaleate (DEM) on LDH release from hepatocytes incubated with HCFC-141b under normoxic conditions. Incubations were as for figure 8. Before pre-incubation, PH (250 μ M) and DEM (500 μ M) were added in methanol. Values are mean $\pm$ SD from four incubations. (a) $p < 0.001$ versus control; (b) $p < 0.01$ versus '+PH' and $p < 0.05$ versus '+HCFC-141b'; (c) $p < 0.01$ versus '+DEM' and not significant versus '+HCFC-141b' (Student's *t*-test).

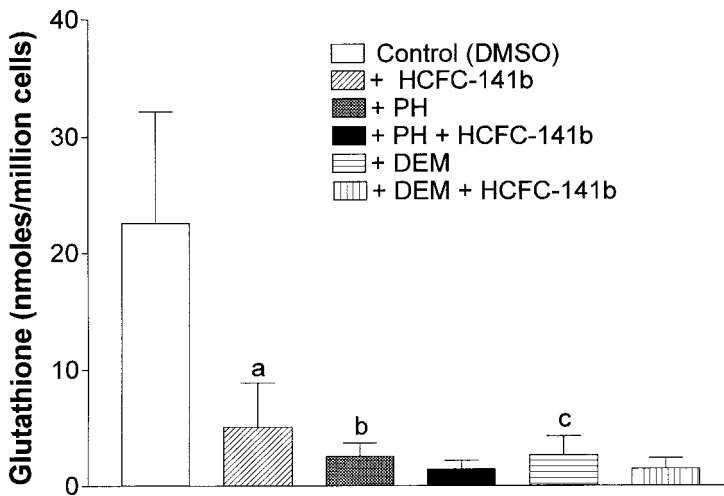


Figure 11. Glutathione depletion by HCFC-141b, phorone (PH) and diethylmaleate (DEM) in hepatocytes incubated under normoxic conditions. Determinations were carried out on the same incubations used for LDH assay (figure 10) and were as described in the Materials and methods. Values are mean $\pm$ SD from three or four incubations. (a) $p < 0.02$ versus control and not significant versus '+PH+HCFC-141b' or '+DEM+HCFC-141b'; (b) $p < 0.01$ versus control and not significant versus '+PH+HCFC-141b'; (c) $p < 0.01$ versus control and not significant versus '+DEM+HCFC-141b' (Student's *t*-test).

in the presence of PBN alone, reaching statistical significance in normoxic but not hypoxic incubations (figure 6).

To clarify the P450 isoform(s) involved in the cytotoxicity of the compound, the effects of various P450 inhibitors were studied. Under normoxic conditions, HCFC-141b-dependent LDH release was partially prevented by the broad-spectrum P450 inhibitor metyrapone (100 μ M), the P4502E1 specific inhibitor 4-methylpyrazole

(500 μM) and the P4503A specific inhibitor troleandomycin (100 μM). Interestingly, in the presence of each inhibitor a slight, but statistically significant, decrease in LDH release was also observed in the absence of HCFC-141b (control incubations) (figure 8). In contrast to the LDH data, no protective effect was produced by metyrapone, 4-methylpyrazole or troleandomycin against HCFC-141b-dependent glutathione depletion (figure 9). Finally, the role played by glutathione in the mechanism of HCFC-141b toxicity was studied further in normoxic hepatocyte incubations using 2,6-dimethyl-2,5-heptadien-4-one (phorone, PH) and diethylmaleate (DEM), two specific GSH depletors. Interestingly, both PH and DEM provided a partial protection against HCFC-141b-dependent cytotoxicity (figure 10) while they depleted cellular glutathione in the same incubations almost completely, both in presence and in absence of HCFC-141b (figure 11).

Discussion

Despite its extensive applications and use, little data on the biological effects and the metabolism of HCFC-141b *in vivo* and *in vitro* are available in the literature. Acute and subchronic inhalation exposure to HCFC-141b is responsible for various adverse effects in rats, rabbits, guinea pigs and dogs. In a 90-day study in rat, a dose-dependent reduction in body weight at 20000 ppm in both sexes and at 2000 and 8000 ppm in males, a decreased responsiveness at 20000 ppm in rat and an increase in serum cholesterol and triglycerides at 20000 ppm in both sexes and in triglycerides at 8000 in males were observed (Brock *et al.* 1995). Moreover, a positive cardiac sensitization response to an intravenous epinephrine challenge was observed in dog at 5000 ppm (Brock *et al.* 1995). Repeated inhalation exposure of rat and guinea pig to 8240–10 300 ppm HCFC-141b for 4 weeks induced changes in liver and kidney function tests and histopathological effects in the respiratory tract (ECETOC 1994). Finally, in a 13-week inhalation study in rat, signs of central nervous system depression were recorded at 20000 ppm (Dekant 1996). No information, however, on effects in humans is available and very little is known about the mechanism of bioactivation, the reactive metabolites and the activating enzyme(s) involved in HCFC-141b toxicity.

HCFC-141b is metabolized in rats to 2,2-dichloro-2-fluoroethanol (Loizou and Anders 1993). Analysis by NMR spectroscopy of urine of human subjects exposed to 1000 ppm HCFC-141b showed resonance spectra attributable to 2,2-dichloro-2-fluoroethyl glucuronide (major metabolite) and dichlorofluoroacetic acid (minor metabolite) (Tong *et al.* 1998). Previous work in the authors' laboratory has reported that the anaerobic incubation of PYR- or phenobarbital (PB)-induced rat liver microsomes with HCFC-141b in the presence of NADPH, results in a dose-dependent loss of P450 activity and content and, to a lower extent, in the inactivation of its prosthetic haem group (Tolando *et al.* 1996). These effects were accompanied by a time- and dose-dependent depletion of exogenous glutathione and were attributed to the formation of HCFC-141b reactive metabolites by rat liver microsomal P450. In presence of the spin-trapping agent PBN, a protective effect against the loss of P450 content and activity as well as the depletion of exogenous glutathione was also observed. This suggested that a primary role in P450 inactivation was played by free radical intermediates produced by the reductive metabolism of HCFC-141b. These species are known to be responsible for the *in vivo* and *in vitro* toxicity of numerous polyhalogenated chemicals, such as carbon

tetrachloride (Rush *et al.* 1986, Manno *et al.* 1988), halothane (Tomasi *et al.* 1983, Manno *et al.* 1991) and 1,1,1-trichloroethane (Albano *et al.* 1985) and they are thought to be involved in the pathogenesis of many diseases. Free radical addition reactions may result in two different mechanisms of toxicity *in vitro*: covalent binding to various macromolecules – lipids, proteins and DNA may all be free radical targets – or initiation of lipid peroxidation. The present results suggest that both these mechanisms apply to HCFC-141b. Indeed, under strictly anaerobic conditions, HCFC-141b was reductively metabolized by pyridine-induced rat liver microsomes to free radicals, as indicated by the formation of typical ESR spectra in the presence of the spin-trapping agent PBN. It is conceivable, therefore, that free radical intermediates of HCFC-141b may also be involved in the modification of microsomal membrane lipids and contribute, together with the haem loss, to the inactivation of microsomal P450 observed in Tolando *et al.* (1996).

Lipid peroxidation is known to be the mechanism by which a number of foreign compounds such as CCl₄ and halothane, produce structural tissue damage (Kappus 1991). A concentration-dependent formation of conjugated dienes, indicating lipid peroxidation, was detected, in the present study, during anaerobic incubation of pyridine-induced rat liver microsomes with increasing concentration of HCFC-141b. Moreover, PBN, GSH and vitamin C partially inhibited the formation of conjugated dienes. Therefore, the production of free radical metabolites and the formation of conjugated dienes are probably also involved in the cytotoxicity of HCFC-141b. Indeed, HCFC-141b was also responsible for a concentration-dependent increase in LDH release from the hepatocytes, under both normoxic and hypoxic conditions. The almost complete prevention of LDH release by the free radical trapper PBN provides additional indirect evidence that free radicals are responsible for the cytotoxicity of HCFC-141b. Similar results had also been obtained in isolated liver cells from PB-induced rats incubated with the hepatotoxic anaesthetic halothane in the presence of PBN under hypoxic conditions (Tomasi *et al.* 1983). The fact that HCFC-141b was found to be cytotoxic under normoxic conditions, suggests that activation to free radicals may occur even under physiological O₂ levels. Interestingly, similar results were obtained in our laboratory in the presence of another halogenated ethane, HCFC-123 (Manno *et al.* 1999), thus suggesting a common mechanism of bioactivation of different HCFC.

The protection against HCFC-141b-dependent LDH release by metyrapone, 4-methylpyrazole and troleandomycin observed in the present study suggests that P450, and in particular the CYP2E1 and CYP3A isoforms, contribute to HCFC-141b bioactivation in rats. However, since protection was only partial, other P450 isoforms or other enzymes not belonging to the P450 superfamily may also be involved. The literature reports that diallyl-sulphide, a specific inhibitor of CYP2E1, inhibits HCFC-141b metabolism *in vivo*, as indicated by a decreased uptake of HCFC-141b and a lower urinary excretion of 2,2-dichloro-2-fluoroethanol in the diallyl-sulphide-treated rat (Loizou and Anders 1993). In addition, bio-transformation of HCFC-141b to 2,2-dichloro-2-ethanol *in vitro* was enhanced when rats were treated with pyridine, an inducer of P4502E1 (Loizou and Anders 1993). Moreover, previous work has shown that during anaerobic incubation with HCFC-141b of liver microsomes from PYR- and PB-treated rats, the compound is activated to reactive metabolites (Tolando *et al.* 1996). Under these conditions, HCFC-141b caused molecular and functional damage of P450, as indicated by the concentration-dependent loss of total P450 content and isoform-specific activities

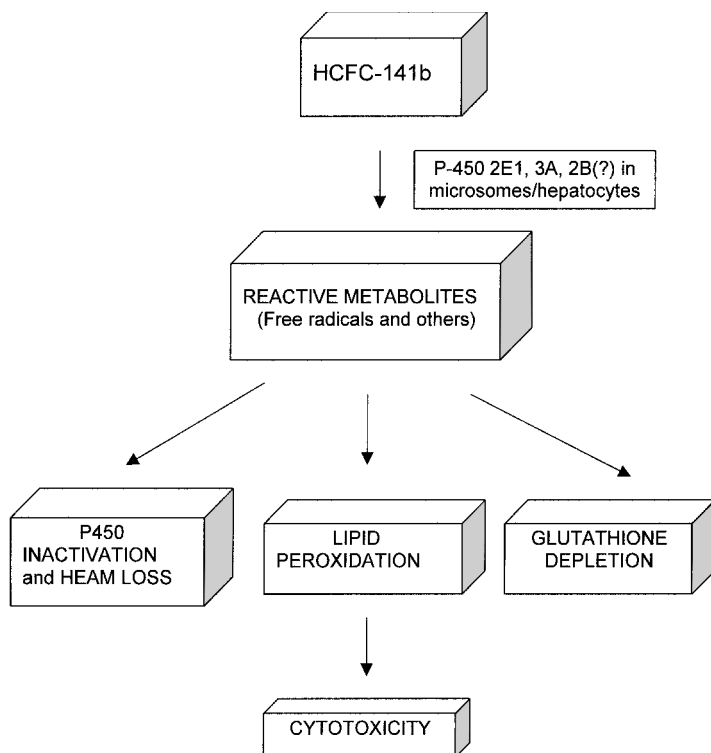


Figure 12. Proposed scheme for the bioactivation of HCFC-141b by rat liver microsomes and hepatocytes, resulting in hepatotoxicity, P450 inactivation and glutathione depletion.

observed in both PYR and PB microsomes. These results indicate overall that CYP2E1 and, probably, also CYP2B and CYP3A are both involved in the activation of HCFC-141b.

Finally, the concentration-dependent depletion of glutathione found in the present study, confirms a direct interaction of HCFC-141b reactive metabolites with intracellular glutathione, as suggested previously (Tolando *et al.* 1996). However, glutathione depletion was found to be associated with but not responsible for LDH release. Indeed, the role of glutathione in HCFC bioactivation and detoxication is complex. For example, glutathione depletion by *l*-buthionine-(*S,R*)-sulphoximine was reported to increase the hepatotoxicity of HCFC-123, suggesting a protective role of glutathione for this compound (Lind *et al.* 1995). In contrast, the nephrotoxicity of some fluoroalkenes, such as chlorotrifluoroethene, is known to be due to their biotransformation by glutathione conjugation, followed by bioactivation of the derived cysteine-*S*-conjugates by cysteine conjugate β -lyase (Anders and Dekant 1994). This latter mechanism, however, is unlikely to be of any relevance for HCFC-123 or HCFC-141b, since neither of the two compounds is nephrotoxic. Furthermore, the present study indicates that glutathione depletion may not be involved in the cytotoxicity of HCFC-141b. Indeed, when the spin-trapping agent PBN or the P450 inhibitors metyrapone, 4-methylpyrazole or troleanomycin were added to the incubation, only LDH release, but not glutathione depletion, was prevented. In addition, the glutathione depletors phorone and diethylmaleate caused a partial protection against HCFC-141b-dependent LDH release. Similar

results were also observed in our laboratory with HCFC-123 (Zanovello *et al.* 1999). Perhaps, HCFC-141b is activated to two different types of reactive species, one of which is trapped by PBN and is responsible for cytotoxicity and the other which interacts with glutathione but is not involved in hepatotoxicity.

In conclusion, a general scheme for the bioactivation of HCFC-141b to reactive species by microsomal and hepatocyte P450 resulting in cytotoxicity, P450 loss and glutathione depletion is proposed in figure 12. HCFC-141b is reductively metabolized by CYP2E1, CYP3A and, possibly, CYP2B to free radical intermediates, leading to peroxidative modification of membrane lipids. This process may contribute to the HCFC-141b-dependent inactivation of microsomal P450 observed in previous studies. Moreover, HCFC-141b is cytotoxic and depletes cellular glutathione in isolated rat hepatocytes in a concentration-dependent manner, under both normoxic and hypoxic conditions. However, cytotoxicity and GSH depletion appear to be unrelated processes, since various agents prevented cytotoxicity but not cellular glutathione depletion and, additionally, glutathione depletors did not increase cytotoxicity.

Acknowledgements

The work was partly supported by EC BIOMED-2 Contract No. BMH4-CT96-0184, INCO-DC Contract No. IC18-CT98-0341 and by the Italian MURST. The authors thank Professor A. Tomasi, Department of Biomedical Science, University of Modena, for ESR measurements and Mrs Colette Case Sperati Ruffoni for editorial assistance.

References

- ALBANO, E., TOMASI, A., CHEESEMAN, C. K., VANNINI, V. and DIANZANI, M. U., 1985, Use of isolated hepatocytes for the detection of free radical intermediates of halogenated hydrocarbons. In G. Poli, K. H. Cheeseman, M. U. Dianzani and T. F. Slater (eds), *Free Radicals in Liver Injury* (Oxford: IRL), pp. 7-16.
- ANDERS, M. W. and DEKANT W., 1994, *Conjugation-Dependent Carcinogenicity and Toxicity of Foreign Compounds* (San Diego: Academic Press).
- BROCK, W. J., TROCHIMOWICZ, H. J., MILLISCHER, R. J., FARR, C., KAWANO, T. and RUSCH, G. M., 1995, Acute and subchronic toxicity of 1,1-dichloro-1-fluoroethane (HCFC-141b). *Food and Chemical Toxicology*, **33**, 483-490.
- COOPER, D. Y., CANNON, M. D., SCHEYER, H., NOVACK, B. G. and ROSENTHAL, O., 1982, Anaerobic technique for spectrometric studies of oxidative enzymes with low oxidation-reduction potentials. In J. B. Schenkman and D. Kupfer (eds), *Hepatic Cytochrome P-450 Monooxygenase System* (Oxford: Pergamon), pp. 813-832.
- CORONGIU, F. P., POLI, G., DIANZANI, M. U., CHEESEMAN, K. H. and SLATER, T. F., 1986, Lipid peroxidation and molecular damage to polyunsaturated fatty acids in rat liver. Recognition of two classes of hydroperoxides formed under conditions *in vivo*. *Chemico-Biological Interactions*, **59**, 147-155.
- DEKANT, W., 1996, Toxicology of chlorofluorocarbon replacements. *Environmental Health Perspectives*, **104** (suppl. 1), 75-83.
- ECETOC, 1994, *Joint Assessment of Commodity Chemicals* No. 29, 1,1-Dichloro-1-fluoroethane (HCFC-141b) CAS No 1717-6 (Brussels: ECETOC).
- FERRARA, R., TOLANDO, R., KING, L. J. and MANNO, M., 1997, Cytochrome P450 inactivation during reductive metabolism of 1,1-dichloro-2,2,2-trifluoroethane (HCFC-123) by phenobarbital- and pyridine-induced rat liver microsomes. *Toxicology and Applied Pharmacology*, **143**, 420-428.
- FERRARA, R., TOLANDO, R., ZANOVELLO, A., TOMASI, A. and MANNO, M., 1999, Reductive bioactivation of HCFC-141b to free radical intermediates by rat liver microsomal cytochrome P-450. *Toxicologist*, **48**, 221.
- GRIFFITH, O. W., 1995, Glutathione and glutathione disulfide. In H. U. Bergmeyer (ed.), *Methods of Enzymatic Analysis* (Berlin: Chemie), pp. 521-529.

- HARRIS, J. W., POHL L. R., MARTIN, J. L. and ANDERS, M. W., 1991, Tissue acylation by the chlorofluorocarbon substitute 2,2-dichloro-1,1,1-trifluoroethane. *Proceedings of the National Academy of Sciences, USA*, **88**, 1407-1410.
- HALLIWELL, B. and GUTTERIDGE, J. M. C., 1989, Lipid peroxidation: a radical chain reaction. In B. Halliwell and J. M. C. Gutteridge (eds), *Free Radicals in Biology and Medicine* (Oxford: Clarendon), pp. 188-276.
- HUWYLER, J., AESCHLIMANN, D., CHRISTEN, U. and GUT, J., 1992, The kidney as a novel target tissue for protein adduct formation associated metabolism of halotane and the candidate chlorofluorocarbon replacement 2,2-dichloro-1,1,1-trifluoroethane. *European Journal of Biochemistry*, **207**, 229-238.
- JARABEK, A. M., FISCHER, J. W., RUBENSTEIN, R., LIPSCOMB, J. C., WILLIAMS, R. J., VINEGAR, A. and McDUGAL, J. N., 1994, Mechanistic insights aid the search for CFC substitutes: risk assessment of HCFC-123 as an example. *Risk Analysis*, **14**, 231-250.
- KAMATH, S. A., KUMMEROW, F. A. and NARAYAN, K. A., 1971, A simple procedure for the isolation of rat liver microsomes. *FEBS Letters*, **17**, 90-92.
- KAPPUS, H., 1991, Biochemical mechanisms of chemical-induced lipid peroxidation. In C. Vigo-Pelfrey (ed.), *Membrane Lipid Oxidation* (Boca Raton: CRC Press), pp. 103-119.
- LIND, R. C. and GANDOLFI, A. J., 1993, Concentration-dependent inhibition of halotane biotransformation in the guinea pig. *Drug Metabolism and Disposition*, **21**, 386-389.
- LIND, R. C., GANDOLFI, A. J. and HALL, P. M., 1995, Biotransformation and hepatotoxicity of HCFC-123 in the guinea pig: potentiation of hepatic injury by prior glutathione depletion. *Toxicology and Applied Pharmacology*, **134**, 175-181.
- LOIZOU, G. D. and ANDERS, M. W., 1993, Gas-uptake pharmacokinetics and biotransformation of 1,1-dichloro-1-fluoroethane (HCFC-141b). *Drug Metabolism and Disposition*, **21**, 634-639.
- LONGSTRETH, J., 1988, Cutaneous malignant melanoma and ultraviolet radiation. A review. *Cancer Metastasis Reviews*, **7**, 321-333.
- MANNO, M., CAZZARO, S. and REZZADORE, M., 1991, The mechanism of the suicidal reductive inactivation of microsomal cytochrome P-450 by halothane. *Archives of Toxicology*, **65**, 191-198.
- MANNO, M., DE MATTEIS, F. and KING, L. J., 1988, The mechanism of the suicidal, reductive inactivation of microsomal cytochrome P-450 by carbon tetrachloride. *Biochemical Pharmacology*, **37**, 1981-1990.
- MANNO, M., FERRARA, R., ZANOVELLO, A., BORTOLATO, S. and TOLANDO, R., 1999, Cytotoxicity of HCFC-123 in isolated rat hepatocytes. *Toxicologist*, **48**, 94.
- MANNO, M., ZANOVELLO, A., FERRARA, R. and TOLANDO, T., 1998, Dose- and time-dependent cytotoxicity of HCFC-123 and HCFC-141b on isolated rat hepatocytes under aerobic and hypoxic conditions. *Toxicology Letters*, **95** (suppl. 1), 162-163.
- MOLINA, M. J. and ROWLAND, F. S., 1974, Stratospheric sink for chlorofluoromethanes: chlorine atom-catalysed destruction of ozone. *Nature*, **249**, 810-815.
- RUSH, R. J., KLAUNIG, J. E., SCHULTZ, N. E., ASKARI, A. B., LACHER, D. A., PEPEIRA, M. A. and GOLDBLATT, P. J., 1986, Mechanism of chloroform and carbon tetrachloride toxicity in primary cultured mouse hepatocytes. *Environmental Health Perspectives*, **69**, 301-305.
- TAYLOR, H. R., WEST, S. K., ROSENTHAL, F. S., MUNOZ, B., NEWLAND, H. S., ABBEY, H. and EMMETT, E. A., 1988, Effect of ultraviolet radiation on cataract formation. *New England Journal of Medicine*, **319**, 1429-1433.
- TOLANDO, R., FERRARA, R., ELDIRIRI, N. I., ALBORES, A., KING L. J. and MANNO, M., 1996, Reductive activation of 1,1-dichloro-1-fluoroethane (HCFC-141b) by phenobarbital- and pyridine-induced rat liver microsomal cytochrome P450. *Xenobiotica*, **26**, 425-435.
- TOMASI, A., BILLING, S., GARNER, A., SLATER, T. F. and ALBANO, E., 1983, The metabolism of halothane by hepatocytes: a comparison between free radical spin-trapping and lipid peroxidation in relation to cell damage. *Chemico-Biological Interactions*, **46**, 353-368.
- TONG, Z., UTELL, M. J., MORROW, P. E., RUSCH, G. M. and ANDERS, M. W., 1998, Metabolism of 1,1-dichloro-1-fluoroethane (HCFC-141b) in human volunteers. *Drug Metabolism and Disposition*, **26**, 711-713.
- WILLS, E. D., 1987, Evaluation of lipid peroxidation in lipids and biological membranes. In K. Snell and B. Mullock (eds), *Biochemical Toxicology* (Oxford: IRL) pp. 127-152.
- WILLIAMS, G. M., BERMUDEZ E. and SCAMUZZINO D., 1977, Rat hepatocyte primary cell cultures. *In Vitro*, **13**, 809-817.
- ZANOVELLO, A., FERRARA, R., TOMASI, A., WHITE, I. N. H. and MANNO, M., 1999, Effect of P450 inhibitors on the bioactivation and cytotoxicity of HCFC-123 and HCFC-141b in isolated rat hepatocytes. Proceedings of the International Conference on *In Vitro* Cytotoxicity Mechanisms, 25-27 January, Rome, Abstract book, p. 68.

Metabolism of bisphenol A in isolated rat hepatocytes and oestrogenic activity of a hydroxylated metabolite in MCF-7 human breast cancer cells

Y. NAKAGAWA†* and T. SUZUKI‡

† Department of Toxicology and ‡ Tama Branch, Tokyo Metropolitan Research Laboratory of Public Health, 3-24-1, Hyakunin-cho, Shinjuku-ku, Tokyo 169-0073, Japan

Received 26 October 2000

1. The metabolites of bisphenol A (BPA; 2,2-bis(4-hydroxyphenyl)propane) in freshly isolated rat hepatocytes and the oestrogenic activities of BPA and its metabolites, particularly 3-hydroxybisphenol A (3-OH-BPA), in MCF-7 cells and competitive binding assays have been studied, respectively.

2. During a 2-h incubation, almost all of the BPA (0.25 mM) added to the hepatocyte suspensions was rapidly converted to a major conjugate, monoglucuronide (approximately 75% of total metabolites), and two minor conjugates, which were tentatively identified as monosulphates of BPA and a hydroxylated intermediate, 3-OH-BPA, as determined by mass spectroscopy coupled with HPLC or GC/MS. On the other hand, free 3-OH-BPA was identified as a trace metabolite, whose level was approximately 1 or 2 μM at 1 h in hepatocyte suspensions treated with 0.25 or 0.5 mM BPA, respectively.

3. In another experiment, 3-OH-BPA as well as BPA displaced competitively 17β -oestradiol bound to the recombinant human oestrogen receptor α in a concentration dependent-manner: IC_{50} of diethylstilbestrol, BPA and 3-OH-BPA were approximately 2.5×10^{-8} , 10^{-5} and 5×10^{-5} M, respectively. Further, BPA and 3-OH-BPA at intermediate concentrations (10^{-7} - 10^{-6} M) caused proliferation of MCF-7 human breast cancer cells, whereas the effect of BPA was more potent than that of 3-OH-BPA. At higher concentrations, both BPA ($> 10^{-4}$ M) and 3-OH-BPA ($> 10^{-5}$ M) were cytotoxic.

4. Based on the proliferative potency in MCF-7 cells and the IC_{50} for the competitive binding, the oestrogenic activity of 3-OH-BPA was less than that of BPA. These results indicate that BPA itself rather than its metabolite acts as a xeno-oestrogen and that 3-OH-BPA is cytotoxic, possibly acting via reactive semiquinone and/or quinone metabolites, rather than a xeno-oestrogenic mechanism, in MCF-7 cells.

Introduction

Bisphenol A (BPA; 2,2-bis(4-hydroxyphenyl)propane) is an important monomer used in the manufacture of a multitude of chemical products, including epoxy resins and polycarbonate. However, it is known that trace amounts of BPA are eluted from polycarbonate plasticware and from resins used in food-packaging materials for industrial processing and consumer use (Krishnan *et al.* 1993, Brotons *et al.* 1995, Yamamoto and Yasuhara 1999). Because of its widespread usage, the potential toxicity of BPA has been investigated *in vivo* and *in vitro* to assess various toxicological properties. In rat and mouse, oral administration of

* Author for correspondence: e-mail: yoshio@tokyo-eiken.go.jp

BPA may be associated with adverse reproductive effects (Bond *et al.* 1980, Morrissey *et al.* 1987) or with an increased risk of cancer of the haemopoietic system (National Toxicology Program 1982). In addition, high incidences of multinucleated hepatocellular giant cells, and degeneration and/or regeneration of renal tubules were observed in the male and female mouse treated with BPA (National Toxicology Program 1982). However, the mechanisms by which BPA elicits these adverse effects are not clear.

The predominant metabolic pathway for BPA in rat is conjugation of a phenolic hydroxyl group to glucuronide (Knaak and Sullivan 1966). Atkinson and Roy (1995a) suggested that some of the BPA added to rat hepatic microsomal suspensions is converted to 3-hydroxybisphenol A (3-OH-BPA) by cytochrome P450-dependent mixed-function oxidases and that consequently the intermediate is oxidized to bisphenol-*o*-quinone. Although bisphenol-*o*-quinone reacts with DNA and induces the formation of DNA adducts *in vivo* and *in vitro* (Atkinson and Roy 1995a, b), several *in vivo* and *in vitro* genotoxicity studies have obtained negative results (Ashby and Tennant 1988, BIBRA 1989, Ivett *et al.* 1989). In a previous study we demonstrated that in suspensions of rat hepatocytes, BPA caused a dose- and time-dependent decrease in intracellular levels of ATP which was accompanied by a loss of cell viability and that BPA at a low toxic level (0.25 mM) was converted to metabolites (Nakagawa and Tayama 2000).

In chemical structure, bisphenolic compounds such as BPA are similar to the synthetic oestrogens diethylstilbestrol and hexestrol. Recently, considerable attention has focused on BPA as well as other phenolic compounds as endocrine-disrupting chemicals having weak oestrogenic activity *in vivo* (Nagel *et al.* 1997, vom Saal *et al.* 1998) and *in vitro* (Gaido *et al.* 1997) bioassays. In the present study, we investigated (1) the identification of metabolites formed in isolated rat hepatocytes and (2) oestrogenic effects of a hydroxylated metabolite of BPA, 3-OH-BPA, on MCF-7 human breast cancer cells and the interaction with the recombinant human oestrogen receptor α .

Materials and methods

Materials

The chemical compounds used were from the following companies: BPA (purity > 97%) from Tokyo Kasei Co. (Tokyo, Japan); 17 β -oestradiol (E2), 4,4'-(1,2-diethyl-1,2-ethenediyl)bisphenol-(diethylstilbestrol; DES), β -glucuronidase, sulphatase (type VI, β -glucuronidase-free *Aerobacter aerogenes*), N-(2-hydroxyethyl)-piperazine-N-(2-ethanesulphonic acid) (HEPES) and bovine serum albumin from Sigma Chemical Co. (St Louis, MO, USA); 3-OH-BPA (purity > 98%), N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and collagenase from Wako Pure Chemicals (Osaka, Japan). All other chemicals were of the highest purity commercially available.

Isolation and incubation of hepatocytes

Male F344/DuCrj (240–260 g) rats were obtained from Charles River Japan, Inc. (Yokohama, Japan) and were housed in wire-bottom cages. The rats were allowed food (CE-2, Clea Japan, Inc., Tokyo) and water *ad libitum* before the hepatocytes were prepared. The hepatocytes were isolated by collagenase perfusion of the liver, as described by Moldéus *et al.* (1978). Hepatocyte viability was assessed by Trypan blue exclusion, and initial cell viability in each experiment was > 85%. Hepatocytes (10⁶ cells ml⁻¹) were suspended in Krebs-Henseleit buffer, pH 7.4, containing 12.5 mM HEPES and 0.1% albumin. All incubations were performed in rotating round-bottomed flasks at 37°C under a constant flow of humidified carbogen (95% O₂/5% CO₂). Reactions were started by the addition of BPA dissolved in DMSO (final concentration < 1%). Corresponding control groups received an equivalent volume of DMSO. Aliquots of incubation mixture were taken at intervals to monitor cell death and the concentrations of BPA and its metabolites.

Determination of BPA metabolites by HPLC

An equal volume of chilled methanol was added to the cell suspension and the mixture was filtered through a membrane cartridge (pore size of cellulose membrane, 0.45 μm ; 13 mm i.d. $\times$ 20 mm; Millex-SLHV, Millipore Co., Bedford, MA, USA). The eluate (20 μl) was injected onto an analytical Capcell pak C₁₈ column (4.6 mm i.d. $\times$ 250 mm, 5- μm particle size; Shiseido Co., Tokyo, Japan) or Richrosorb RP-18 column (10 mm i.d. $\times$ 250 mm, 7- μm particle size; Merck KgaA, Darmstadt, Germany) equipped with a UV absorbance detector (260 nm). The mobile phase was methanol/0.1 M ammonium dihydrogen phosphate (50:50 v/v, pH 5.3), and the flow rate was 1.0 ml min⁻¹. BPA and 3-OH-BPA were identified by co-chromatography or by comparison of their HPLC retention times with both authentic compounds. The recoveries for BPA and 3-OH-BPA were checked by the addition of known amounts of authentic compounds to hepatocyte suspensions, and were > 85%. Glucuronide and sulphate conjugates derived from BPA in the cell suspensions were enzymatically hydrolyzed with β -glucuronidase or sulphatase and the hydrolytic products were identified and/or determined by HPLC. Briefly, aliquots of cell suspensions were treated with a cell disrupter (Sonifier Branson Sonic Power Co.; Danbury, CT, USA) in iced water for 20 s and then filtered through an ultrafiltration cartridge (limit of elution; molecular weight < 5000; Ultrafree-MC centrifugal filter unit; Millipore Co.). The eluate adjusted to pH 4.5 with sodium acetate buffer was incubated with β -glucuronidase (about 500 U) or sulphatase (about 1.5 U) for 3.0 h at 37°C and then the mixture (40 μl) was injected into the HPLC system.

Identification of BPA metabolites by mass spectroscopy coupled with HPLC or GC

Each fraction (peaks a-c in figure 1) obtained separately by HPLC using the Richrosorb RP-18 column described above was concentrated by solid-phase extraction using C₁₈ Sep-Pak cartridges (Waters Co., Milford, MA, USA), and was also subjected to negative electrospray ionization/mass spectroscopy (HPLC/-ESI/MS) analysis. Negative-ion mass spectra of BPA metabolites were obtained using a LC/MS system, consisting of a Waters mass spectrometer ZMD and a Waters pump 2690 (Waters Co.). The mobile phase was 10% methanol in water and the flow rate was 0.2 ml min⁻¹. The MS spectrum was scanned from 100 to 1000 daltons in 1.0 s and produced with a core voltage of 40 V.

To confirm the chemical structure of sulphate conjugates (peaks b and c), both conjugates were enzymatically hydrolysed with sulphatase to yield free BPA or its intermediate and the hydrolytic

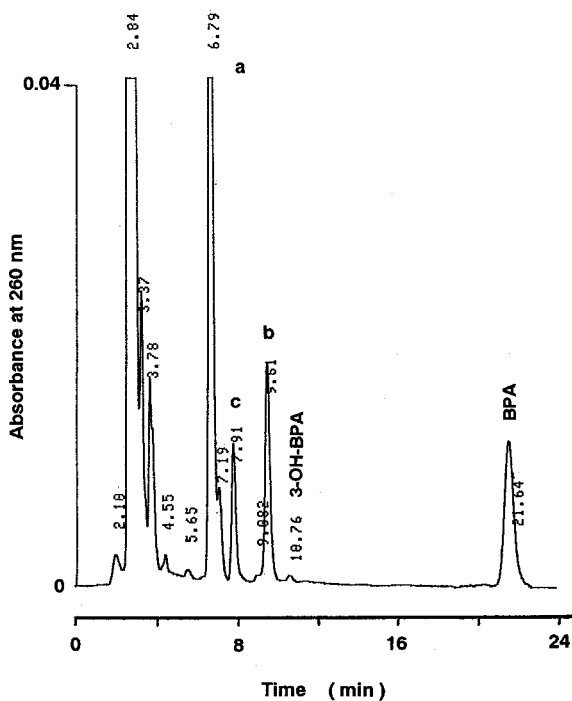


Figure 1. Typical HPLC elution profile of metabolites. Rat hepatocytes (10^6 cells ml⁻¹) were treated with 0.25 mM BPA for 1.5 h. Peaks are BPA-glucuronide (a), BPA-sulphate (b), sulphate derived from BPA metabolite (c), 3-OH-BPA (retention time 10.7 min) and BPA (retention time 21.6 min).

products were subjected to GC/MS after the trimethylsilylation. Briefly, the eluate (50 µl) in each peak was evaporated to dryness with an evaporator, and the dry residue dissolved in 400 µl sodium acetate buffer (pH 4.5) was incubated with sulphatase (1.5 U) at 37°C. The hydrolysed buffer solution was extracted with dichloromethane (3 ml) and the dichloromethane phase was evaporated to dryness. The residue was dried in a vacuum desiccator and trimethylsilylated with BSTFA at 100°C for 15 min. Mass spectrum of trimethylsilylated derivative was obtained on GC/MS by use of an HP-5890 series II (Hewlett-Packard gas chromatography, Palo Alto, CA, USA) equipped with an HP-5MS capillary column (0.25 mm i.d. × 30 m, film thickness 0.25 µm, J&W Scientific, Folsom, CA, USA) and an Automass II mass spectrometer (JEOL, Tokyo) in positive electron ionization mode (70 eV, 300 µA). The carrier gas was helium, and the column head pressure was 80 kPa. Initial oven temperature was 50°C for 1 min and subsequently increased by 10°C min⁻¹ to 300°C.

Competitive binding assay

Competitive binding between 17β-oestradiol and various compounds was determined using an oestrogen-R(α) competitor screening kit (Wako Pure Chemicals Co., Osaka, Japan); the kit consists of recombinant human oestrogen receptor α (ERα) coated on the bottom of 96-well multiwell plates and fluorescein-labelled 17β-oestradiol as the competitor for the assay. DES and other compounds dissolved in DMSO were added to a reaction solution containing fluorescein-labelled 17β-oestradiol, and the mixture (100 µl) was added into each well. After 2 h of incubation at room temperature, the mixture, which contained free compounds or the 17β-oestradiol unbound to ERα, in wells was aspirated and exchanged with the assay solution (100 µl). The concentration of fluorescein-labelled 17β-oestradiol bound to ERα on the bottom was measured in a CytoFluor 4000 fluorescence plate reader (PerSeptive Biosystems, Inc., Framingham, MA, USA) with filters set for 485-nm excitation and 535-nm emission. The results are expressed as percentages of the fluorescence values for the reaction solution without samples.

MCF-7 cell proliferation assay

MCF-7 cells (cultured human breast cancer cells) were purchased from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured in phenol red-free RPMI-1640 medium, supplemented with 5% foetal calf serum (FCS), 15 mM HEPES, 50 U ml⁻¹ penicillin, 50 µg ml⁻¹ streptomycin and 10 ng ml⁻¹ insulin at 37°C with 5% CO₂ in air at saturating humidity, and cells were routinely passed at approximately 80% confluence. Prior to initiating experiments, cells were seeded and attached in 96-well multiwell plates at 4 × 10³/well in 0.3 ml RPMI-1640 medium, supplemented with 5% oestrogen-free FCS. After 24 h, the medium was replaced with the same volume of the above medium containing 17β-oestradiol (1 nM) as a positive control, BPA or 3-OH-BPA: the concentrations of BPA and 3-OH-BPA used in this study ranged from 1 nM to 500 µM. Oestrogen-free FCS was prepared using the dextran-charcoal procedure (Soto *et al.* 1995). The cells were cultured for 5 days and cell numbers in each well were determined using a cell proliferation assay kit (cck-8; Dojindo Laboratories Co., Kumamoto, Japan): thirty microliters of WST-8 solution, 5 mM 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)-2H-tetrazolium monosodium salt and 0.2 mM 1-methoxy phenazinium methylsulphate dissolved in 150 mM KCl, were added to wells containing 0.3 ml medium with cells. After incubation for 60–90 min at 37°C in a humidified 5% CO₂ atmosphere, the absorbance of the well was read at 450 nm (reference wavelength at 650 nm) by a microculture plate reader (model 450, Bio-Rad Laboratories, New York, NY, USA). The assay, a modified tetrazolium colorimetric assay, is based on metabolic reduction of WST-8 to its corresponding formazan and the colouration obtained is directly proportional to the cell number. The colouration was found to be linear for up to 120 min after addition of the dye-containing medium, with cell numbers > 3 × 10⁴.

Statistical analysis

Statistical analysis was performed by one-way analysis of variance, followed by Dunnett's multiple comparison test. Statistical significance was assumed at *p* < 0.05.

Results

Metabolites of BPA produced in isolated rat hepatocytes

As shown by the HPLC elution profiles (figure 1), a large portion of BPA (retention time 21.6 min) at the initial concentration of 0.25 mM was effectively metabolized in hepatocytes to several metabolites during a 2 h-incubation. The metabolite eluted in peak a, a major metabolite of BPA, was enzymatically

hydrolysed with β -glucuronidase to yield free BPA, indicating that the metabolite was a glucuronide of BPA. Furthermore, the conjugate was identified as a BPA-monoglucuronide by LC/-ESI/MS; the -ESI/MS spectrum showed a base peak at m/z 403, corresponding to the $M-H^-$ and an phosphoric acid adduct (+ 98 amu) at m/z 501 (figure 2). The MS spectrum of peak a also showed a fragment ion at m/z 227 (BPA- H^-), which corresponds to a loss of 177 (glucuronyl moiety; 177 amu), $M-C_6H_9O_6^-$. The fragment ions at m/z 113 and 175 in BPA-glucuronide (peak a)

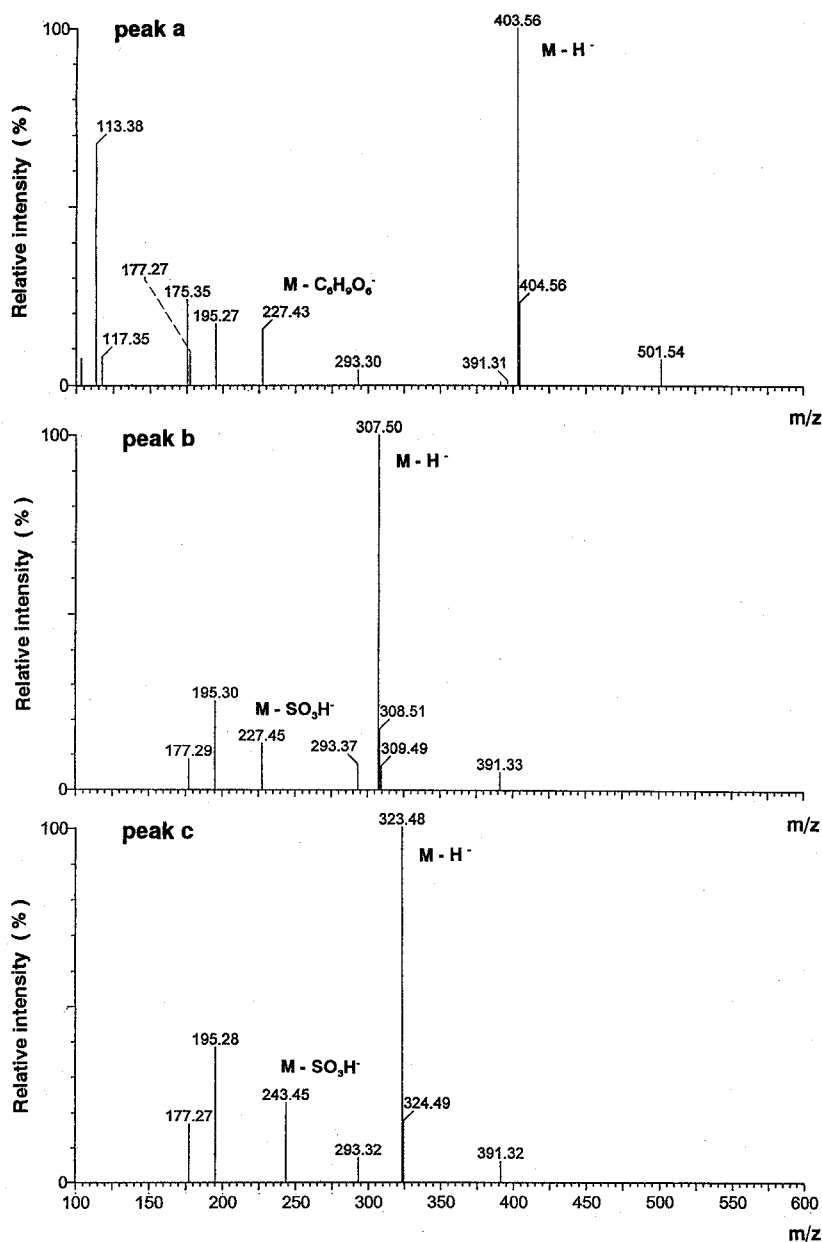


Figure 2. Typical-ESI mass spectra of peaks a-c in figure 1.

may be derived from glucuronyl moiety. The -ESI/MS spectrum of peak a was consistent with the known spectrum of the monoglucuronide conjugate of BPA (Pottenger *et al.* 2000). Because both metabolites that eluted in peaks b (retention time 9.6 min) and c (7.9 min) were enzymatically hydrolysed with sulphatase, these metabolites were suggested sulphate conjugates derived from BPA. Further, metabolites of peaks b and c were tentatively identified as monosulphate conjugates derived from BPA and its intermediate by -ESI/MS, respectively: the spectrum of peak b showed a molecular ion at m/z 307, corresponding to the $M-H^-$, and a fragment ion at m/z 227 ($BPA-H^-$), which corresponds to a loss of 81 (sulphate moiety; 81 amu), $M-HO_3S^-$. On the other hand, the MS spectrum of peak c showed a base peak at m/z 323, corresponding to the $M-H^-$ and suggesting incorporation of an oxygen atom into the BPA molecule and a fragment ion at m/z 243, which results in a loss of 81 (sulphate moiety), $M-HO_3S^-$. Since the amount of material eluted at retention time about 11 min increased by the treatment of sulphatase, the compound found in peak c is likely the sulphate conjugate of 3-OH-BPA.

In order to confirm the chemical structure of sulphate conjugates (peaks b and c), free BPA and its intermediate obtained by the enzymatic hydrolysis were trimethylsilylated with BSTFA and identified by GC/MS. The mass spectra of trimethylsilylated derivatives derived from peaks b and c were identical with those of authentic BPA and 3-OH-BPA, respectively. Main ionic species (m/z /abundance) of trimethylsilylated BPA (retention time 23.1 min) were observed: 372/13 ($[M]^+$), 357/100 ($[M-CH_3]^+$), 207/32, 191/17 and 73/78 ($[TMS]^+$). On the other hand, main ion species of trimethylsilylated 3-OH-BPA (retention time 21.3 min) were observed: 460/23 ($[M]^+$), 445/69 ($[M-CH_3]^+$), 207/39, 191/21 and 73/100 ($[TMS]^+$). These results indicated that two minor conjugates were tentatively identified as monosulphates of BPA (peak b) and 3-OH-BPA (peak c).

BPA at a low-toxic level (0.25 mM) in hepatocyte suspensions was rapidly converted to a major metabolite, a monoglucuronide and some minor metabolites, whereas at a toxic level (0.5 mM), > 65% of the compound remained unchanged 2 h after the incubation (figure 3A). Cell death caused by BPA at 0.25 and 0.50 mM was

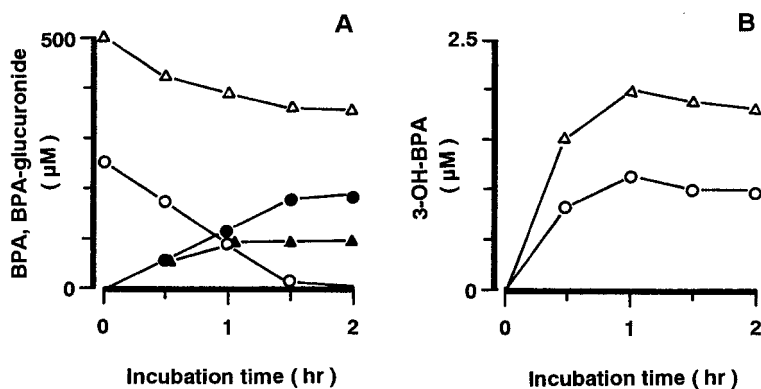


Figure 3. Changes in the levels of BPA, BPA-glucuronide and 3-OH-BPA in rat hepatocytes suspensions. (A) Hepatocytes (10^6 cells ml^{-1}) were incubated with 0.25 mM (○) and 0.50 mM (△) for 2 h. The amounts of BPA-glucuronide derived from 0.25 mM (●) and 0.50 mM (▲) BPA were measured as described in the Materials and methods. (B) Amounts of 3-OH-BPA derived from 0.25 mM (○) and 0.5 mM (△) BPA measured as described above. Results are the averages of two experiments.

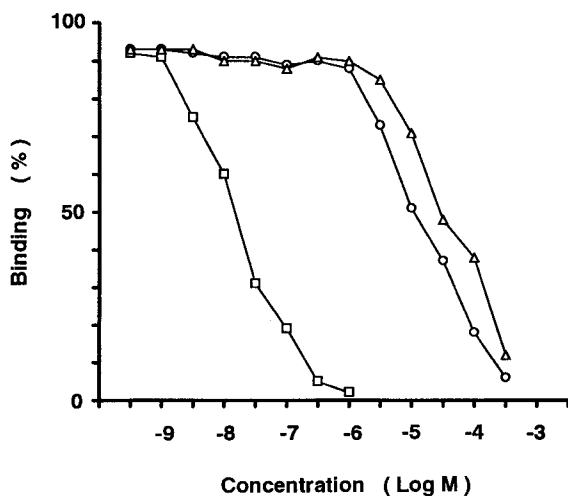


Figure 4. Competitive binding assay of DES ($\square$), BPA ($\circ$) and 3-OH-BPA (Δ) to the oestrogen receptor. The ability of these compounds for binding to $ER\alpha$ was in competition with that of fluorescein-labelled 17β -oestradiol, as described in the Materials and methods. Results are the averages of two or three determinations.

approximately 20 and 42% after a 2 h incubation, whereas cell death of untreated cells was about 14% at the end of the incubation (data not shown). Despite this, the level of 3-OH-BPA increased with time and reached an apparent steady-state at approximately $1\ \mu\text{M}$ or $2\ \mu\text{M}$ within 1 h in hepatocyte suspensions treated with 0.25 or 0.50 mM BPA (figure 3B). Because BPA itself was stable when incubated under carbogen gas for 3 h at 37°C in Krebs-Henseleit buffer without hepatocytes (data not shown), it seems that the hydroxylated BPA, 3-OH-BPA, is enzymatically derived from BPA in rat hepatocytes. Although the *ortho*-quinone metabolite was formed spontaneously by autooxidation of 3-OH-BPA in Krebs-Henseleit buffer without hepatocytes, this metabolite was not detectable in HPLC profiles.

Xeno-oestrogenic effects of BPA and 3-OH-BPA

To compare xeno-oestrogenic activity among BPA, 3-OH-BPA and DES, the ability of each compound to bind to $ER\alpha$ in competition with fluorescein-labelled 17β -oestradiol was determined (figure 4). Although BPA and 3-OH-BPA displaced the 17β -oestradiol bound to the receptor in a competitive manner, the competitive potency of both compounds was approximately three orders of magnitude lower than that of DES. IC_{50} values of DES, BPA and 3-OH-BPA in this assay were approximately 2.5×10^{-8} , 10^{-5} and 5×10^{-5} M, respectively.

Figure 5 shows the effects of BPA and 3-OH-BPA on the proliferation of MCF-7 cells. The results were compared with those from untreated control cells. Relative to control (approximately 1.2×10^4 cells/well) on day 6, BPA increased cell numbers in a dose-dependent manner from 10^{-7} to 10^{-6} M. 3-OH-BPA at 10^{-7} and 10^{-6} M also caused a slight increase in cell numbers. The concentration of 17β -oestradiol is typical of a positive control in tests evaluating the oestrogenicity of the above compounds, and the proliferation induced by oestradiol in this study is consistent with previous findings (Soto *et al.* 1995, Breinholt and Larsen,

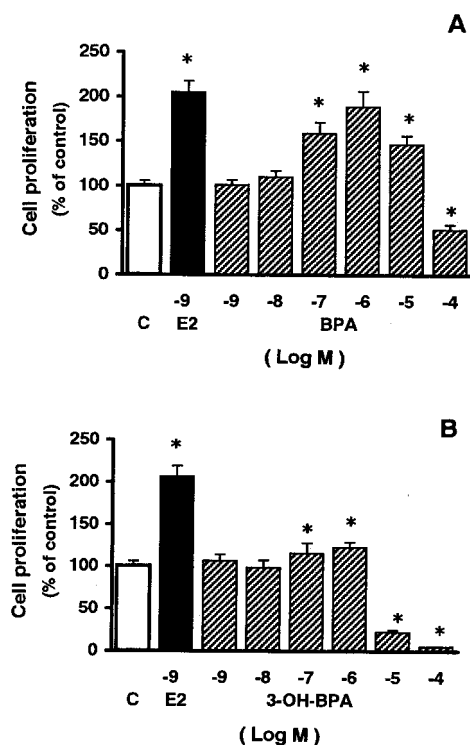


Figure 5. Effects of BPA (A) and 3-OH-BPA (B) on the growth of MCF-7 cells. The compounds were tested at concentrations ranging from 10^{-9} M (−9) to 10^{-4} M (−4) for 5 days. Results are the mean $\pm$ SE of three of four treated cells, respectively. C and E2 indicate untreated control and 17β -oestradiol (10^{-9} M)-treated cells, respectively. * Significant differences between the control group and treated groups ($p < 0.05$).

1998). BPA and 3-OH-BPA at concentrations of $> 10^{-4}$ and 10^{-5} M caused a considerable decrease in cell numbers relative to control. This indicates that both compounds at higher concentrations were cytotoxic in MCF-7 cells.

Discussion

The results obtained in the present study show that in suspensions of isolated rat hepatocytes, BPA was converted to a major conjugate, a monoglucuronide and two minor metabolites which were tentatively identified as monosulphates of BPA and 3-OH-BPA. Glucuronidation and sulphation are major detoxification pathways for metabolism and excretion of phenols in mammals (Kasper and Henton 1980). BPA at a low-toxic level (0.25 mM) was effectively converted to these conjugates in rat hepatocytes by 1.5 h (figure 3). Because the cytotoxicity of BPA was enhanced by the presence of salicylamide, a substrate for enzymatic-mediated glucuronide and/or sulphate conjugation, in BPA-induced cytotoxicity (Nakagawa and Tayama 2000), one can conclude that glucuronidation of BPA is important to the detoxification of the parent compound. Yokota *et al.* (1999) reported that BPA was effectively glucuronidated by an isoform of UDP-glucuronosyltransferase, UGT2B1, in rat microsomes. In *in vivo* experiments, it has been reported that approximately 20–30% of the BPA administered orally to rat was excreted in urine,

primarily as glucuronide through a first-pass metabolism by intestine and/or liver (Pottenger *et al.* 2000). The amount of BPA-sulphate excreted in urine was approximately 2-3% of total urinary metabolites (Pottenger *et al.* 2000). On the other hand, Knaak and Sullivan (1966) reported that BPA was converted to a hydroxylated metabolite in rat after oral administration. Atkinson and Roy (1995a) speculated that some BPA was converted by microsomal cytochrome P450 monooxygenases to 3-OH-BPA, which is bound to DNA and/or macromolecules through the formation of bisphenol-*o*-semiquinone and/or bisphenol-*o*-quinone. The rate of oxygen consumption by BPA or 3-OH-BPA in Krebs-Henseleit buffer without rat hepatocytes was measured polarographically with a Clark-type oxygen electrode (Model 5300, Yellow Springs Instruments Co., Yellow Spring, OH, USA) at 30°C (data not shown). Although BPA was essentially stable in Krebs-Henseleit buffer without hepatocytes, 3-OH-BPA elicited an increase in oxygen consumption, which indicates that autoxidation through the formation of superoxide anion radicals occurs readily (Nakagawa and Moore 1992). Whereas bisphenol-*o*-quinone was not detectable in HPLC profiles of this study, free unconjugated 3-OH-BPA was present in small amounts and reached an apparent steady-state level, approximately 1 or 2 µM, by 1 h in hepatocyte suspensions treated with 0.25 or 0.50 mM BPA (figure 3). These results indicate that although some BPA at a low-toxic dose level is enzymatically converted to reactive 3-OH-BPA, almost all of the compound is effectively conjugated by glucuronidation and/or sulphation, phase II detoxification pathways, in rat hepatocytes.

In chemical structure, bisphenolic compounds are similar to the synthetic oestrogens, DES and hexestrol. Recently, the xeno-oestrogenicity of BPA and other phenolic compounds have been demonstrated in a number of *in vitro* and *in vivo* assays (Nagel *et al.* 1997, Gaido *et al.* 1997, vom Saal *et al.* 1998). Because 3-OH-BPA as well as BPA elicits a concentration-dependent displacement of 17β-oestradiol bound to ERα, 3-OH-BPA seems to possess an intrinsic oestrogenic activity (figure 4). Based on relative IC₅₀, it follows that the order of oestrogenic potency was DES ≫ BPA > 3-OH-BPA. Although the oestrogenic potencies of 3-OH-BPA and BPA were similar in the cell-free assay, 3-OH-BPA in MCF-7 cells was weakly oestrogenic and quite toxic compared with BPA (figure 5). Thus, this result suggests that the oestrogenic and/or toxic effects of compounds are dependent on their own chemical reactivity and/or stability as well as the affinity to target sites in these bioassay systems. It is well established that hydrophobicity, as demonstrated by its close correlation with the partition coefficient of xenobiotics, is often associated with biological action, which is expressed as the structure-toxicity or -activity relationship (Hansch and Dunn 1972, Lyon *et al.* 1981, Law *et al.* 1986). The *n*-octanol/water partition coefficients (log *p*), a physical property used extensively to describe a chemical's lipophilic or hydrophobic properties, of DES, BPA and 3-OH-BPA are approximately 5.07, 3.64 and 3.16, respectively (Tang-Liu *et al.* 1992, Hansch *et al.* 1995, Meylan and Howard 1995). The introduction of a hydroxyl group at the 3-position of an aromatic ring of BPA decreases its hydrophobicity, which may be associated with the oestrogenic potencies of both compounds. Both the oestrogenic and cytotoxic effects of alkyl esters of 4-hydroxybenzoic acid (parabens) are related to the 4-hydroxyl group and their potency depends on the relative length of alkyl-side chains and is associated with the partition coefficients, an index of hydrophobicity (Nakagawa and Moldéus 1998, Routledge *et al.* 1998, Nakagawa and Moore 1999). Thus, it seems that the

incorporation of a hydroxyl group into the 3-position of aromatic ring is not related to the oestrogenic potency of its molecule. In addition, it has been reported that 4-hydroxylated biphenyl is more oestrogenic than 2- or 3-hydroxylated biphenyl in MCF-7 cells (Soto *et al.* 1995). On the other hand, because a monoglucuronide of BPA or sulphates of alkylphenols did not interact with the oestrogen receptors (Certa *et al.* 1996, Matthews and Zacharewski 1999) and did not have oestrogenic activity in MCF-7 cells which were transfected with chimeric oestrogen receptors, it is unlikely that these conjugates would have the capability to interact with the oestrogen receptor. These results indicate that BPA itself rather than its metabolites acts as a xeno-oestrogen and that 3-OH-BPA is cytotoxic, presumably acting via a reactive semi-quinone and/or quinone intermediates, rather than a xeno-oestrogenic mechanism, in MCF-7 cells.

In conclusion, the present study has shown that BPA added to isolated rat hepatocytes is converted enzymatically to a monoglucuronide and two minor conjugates, which have tentatively been identified as monosulphates. 3-OH-BPA was identified as a trace metabolite of BPA. Both 3-OH-BPA and BPA displaced 17β -oestradiol bound to ER α in a competitive- and concentration dependent-manner, and at intermediate concentrations caused proliferation of MCF-7 human breast cancer cells. Based on the relative IC₅₀ values and the proliferative effect of MCF-7 cells, it follows that the order of oestrogenic potency was BPA > 3-OH-BPA.

References

- ASHBY, J. and TENNANT, K. W., 1988, Chemical structure, *Salmonella* mutagenicity and extent of carcinogenicity as indicators of genotoxic carcinogens. *Mutation Research*, **204**, 17-115.
- ATKINSON, A. and ROY, D., 1995a, *In vitro* conversion of environmental estrogenic chemical bisphenol to DNA binding metabolite(s). *Biochemical and Biophysical Research Communications*, **210**, 424-433.
- ATKINSON, A. and ROY, D., 1995b, *In vivo* DNA adduct formation by bisphenol A. *Environmental and Molecular Mutagenesis*, **26**, 60-66.
- BIBRA, 1989, *Toxicology Profile Bisphenol A* (Carshalton: The British Industrial Biological Research Association).
- BOND, G. P., MCGINNIS, P. M., CHEEVER, K. L., HARRIS, S. J., PLATNICK, H. B. and NEIMEIER, R. W., 1980, Reproductive effects of bisphenol A. *Proceedings of the Society of Toxicology*, **19**, A23.
- BREINHOLT, V. and LARSEN, J. C., 1998, Detection of weak estrogenic flavonoids using a recombinant yeast strain and a modified MCF-7 cell proliferation. *Chemical Research in Toxicology*, **11**, 622-629.
- BROTOS, J. A., OLEA-SERRANO, M. F., VILLALOBOS, M., PEDRAZA, V. and OLEA, N., 1995, Xenoestrogens released from lacquer coatings in food cans. *Environmental Health Perspectives*, **103**, 608-612.
- CERTA, H., FEDTKE, N., WIEGAND, H. J., MÜLLER, A. M. F. and BOLT, H. M., 1996, Toxicokinetics of *p*-tert-octylphenol in male Wistar rats. *Archives of Toxicology*, **71**, 112-122.
- GAIDO, K. W., LEONARD, L. S., LOVELL, S., GOULD, J. C., BABAI, D., PORTIER, C. J. and McDONELL, D. P., 1997, Evaluation of chemicals with endocrine modulating activity in yeast-based steroid hormone receptor gene transcription assay. *Toxicology and Applied Pharmacology*, **143**, 205-212.
- HANSCH, C. and DUNN, W. J., 1972, Linear relationships between lipophilic character and biological activity of drug. *Journal of Pharmaceutical Science*, **61**, 1-19.
- HANSCH, C., HOEKMAN, D., LEO, A., ZHANG, L. and LI, P., 1995, The expanding role of quantitative structure-activity relationship (QSAR) in toxicology. *Toxicology Letters*, **79**, 45-53.
- IVETT, J. L., BROWN, B. M., ANDERSON, B. C., RESNICK, M. A. and ZEIGER, E., 1989, Chromosomal aberration and sister chromatid exchange tests in Chinese hamster ovary cells *in vitro* results with 15 chemicals. *Environmental and Molecular Mutagenesis*, **14**, 165-187.
- KASPER, C. B. and HENTON, D., 1980, Glucuronidation. In W. B. Jakoby (ed.), *Enzymatic Basis of Detoxification*, (New York: Academic Press), pp. 3-36.
- KNAAK, J. B. and SULLIVAN, L. J., 1966, Metabolism of bisphenol A in the rat. *Toxicology and Applied Pharmacology*, **8**, 175-184.

- KRISHNAN, A. V., STATHIS, P., PERMUTH, S. F., TOKES, L. and FELDMAN, D., 1993, Bisphenol-A: An estrogenic substance is related from polycarbonate flasks during autoclaving. *Endocrinology*, **132**, 2279-2286.
- LAW, P., CAMPBELL, S. D., LEPOCK, J. R. and KRUUV, J., 1986, Effects of butylated hydroxytoluene on membrane lipid fluidity and freeze-thaw survival in mammalian cells. *Cryobiology*, **23**, 317-322.
- LYON, R. C., MCCOMB, J. A., SCHREURS, J. and GOLDSTEIN, D. B., 1981, A relationship between alcohol intoxication and the disordering of brain membranes by a series of short-chain alcohols. *Journal of Pharmacology and Experimental Therapeutics*, **218**, 669-675.
- MATTHEWS, J. B. and ZACHAREWSKI, T. R., 1999, Comparison of the interactions of bisphenol and bisphenol A glucuronide with estrogen receptor α and β . In The 2nd Conference of the Japanese Society of Endocrine Disruptor Research, Kobe, p. 60.
- MEYLAN, W. M. and HOWARD, P. H., 1995, Atom/fragment contribution method for estimating octanol-water partition coefficients. *Journal of Pharmaceutical Science*, **84**, 83-92.
- MOLDÉUS, P., HOGBERG, J. and ORRENIUS, S., 1978, Isolation and use of liver cells. *Methods in Enzymology*, vol. 52, (New York: Academic Press), pp. 60-71.
- MORRISSEY, R. E., GEORGE, J. D., PRICE, C. T., TYL, R. W., MARR, M. C. and KIMMEL, C. A., 1987, The developmental toxicity of bisphenol A in rats and mice. *Fundamental and Applied Toxicology*, **8**, 571-582.
- NAGEL, S. C., VOM SAAL, F. S., THAYER, K. A., DHAR, M. G., BOECHLER, M. and WELSHONS, W. V., 1997, Relative binding affinity-serum modified access (RBA-SMA) assay predicts the relative *in vivo* bioactivity of the xenoestrogens bisphenol A and octylphenol. *Environmental Health Perspectives*, **105**, 70-76.
- NAKAGAWA, Y. and MOLDEUS, P., 1998, Mechanism of *p*-hydroxy benzoate ester-induced mitochondrial dysfunction and cytotoxicity in isolated rat. *Biochemical Pharmacology*, **55**, 1907-1914.
- NAKAGAWA, Y. and MOORE, G., 1992, Cytotoxic effects of phenyl-hydroquinone and some hydroquinones on isolated rat hepatocytes. *Biochemical Pharmacology*, **44**, 1069-1065.
- NAKAGAWA, Y. and MOORE, G., 1999, Role of mitochondrial membrane permeability transition in *p*-hydroxy benzoate ester-induced cytotoxicity in rat hepatocytes. *Biochemical Pharmacology*, **58**, 811-816.
- NAKAGAWA, Y. and TAYAMA, S., 2000, Metabolism and cytotoxicity of bisphenol A and other bisphenols in isolated rat hepatocytes. *Archives of Toxicology*, **74**, 99-105.
- NATIONAL TOXICOLOGY PROGRAM, 1982, Carcinogenesis bioassay of bisphenol A in F344 rats and B6C3F mice. *National Toxicology Program Technical Report*, **215**, pp. 1-116.
- POTTINGER, L. H., DOMORADZKI, J. Y., MARKHAM, D. A., HANSEN, S. C., CAGEN, S. Z. and WAECHTER, J. M. JR., 2000, The relative bioavailability and metabolism of bisphenol A in rats is dependent upon the route of administration. *Toxicological Sciences*, **54**, 3-18.
- ROUTLEDGE, E. J., PARKER, J., ODUM, J., ASHBY, J. and SUMPTER, J. P., 1998, Some alkyl hydroxy benzoate preservatives (parabens) are oestrogenic. *Toxicology and Applied Pharmacology*, **153**, 12-19.
- SOTO, A. M., SONNENSCHN, C., CHUNG, K. L., FERNANDEZ, M[>]F., OLEA, N. and SERRANO, F. O., 1995, The E-screen assay as a tool to identify estrogens: an uptake on estrogenic environmental pollutant. *Environmental Health Perspectives*, **103**, 113-122.
- TANG-LIU, D. D., RICHMAN, J. B. and LIU, S. S. 1992, Lenticular uptake and distribution of xenobiotics and amino acids. *Journal of Ocular Pharmacology*, **8**, 267-277.
- VOM SAAL, F. C., COOKE, P. S., BUCHANAN, D. L., PALANZA, P., THAYER, K. A., NAGEL, S. C., PAMIGIANI, S. and WELSHONS, W.V., 1998, A physiologically based approach to the study of bisphenol A and other estrogenic chemicals on the size of reproductive organs, daily sperm production, and behavior. *Toxicological Industrial Health*, **14**, 239-260.
- YAMAMOTO, T. and YASUHARA, A., 1999, Quantities of bisphenol A leached from plastic waste samples. *Chemosphere*, **38**, 2569-2576.
- YOKOTA, H., IWANO, H., ENDO, M., KOBAYASHI, T., INOUE, H., IKUSHIRO, S. and YUASA, A., 1999, Glucuronidation of the environmental oestrogen bisphenol A by an isoform of UDP-glucuronosyltransferase, UGT2B1, in the rat liver. *Biochemical Journal*, **340**, 405-409.

Species differences in β -oxidative metabolism of a thromboxane A_2 -receptor antagonist [(+)-S-145] in rat, dog and monkey

Y. YAMAGUCHI*, A. TOUCHI, T. BABA and T. MATSUBARA

Department of ADME and Toxicology for Screening, Developmental Research Laboratories, Shionogi & Co. Ltd, 3-1-1 Futaba-cho, Toyonaka, Osaka 561-0825, Japan

Received 12 October 2000

1. The formation of β -oxidized metabolites from (+)-S-145 [(+)-(Z)-7-[(1R, 2S, 3S, 4S)-3-(benzenesulphonamide)bicyclo-[2.2.1]-hept-2-yl]-5-heptenoic acid] by liver homogenates were compared between rat, dog and monkey. Species differences were found in hepatic β -oxidation capacities. The results agree with the qualitative and quantitative differences in β -oxidized metabolite proportions among these species observed *in vivo*.

2. The activities of microsomal (+)-S-145-CoA synthesis, the initial step of the β -oxidation, were determined. Species differences in their intrinsic clearances primarily agreed with those of the β -oxidized metabolite formation.

3. (+)-S-145-CoA oxidation activities towards (+)-S-145-CoA by liver homogenates were much higher than the β -oxidized metabolite formation in all species, indicating that formed (+)-S-145-CoA was immediately β -oxidized in peroxisomes. The species differences were inconsistent with those of β -oxidized metabolite formation *in vitro*.

4. Therefore, quantitative differences of hepatic (+)-S-145 β -oxidation capacity in rat, dog and monkey were considered to be mainly due to the species difference in (+)-S-145-CoA formation.

Introduction

(+)-S-145 [(+)-(Z)-7-[(1R, 2S, 3S, 4S)-3-(benzenesulphonamide)bicyclo-[2.2.1]-hept-2-yl]-5-heptenoic acid] is a potent and selective thromboxane A_2 -receptor antagonist (Hanasaki *et al.* 1989) and its calcium salt, S-1452, has been proposed to be an effective agent for treating allergic asthma (Arimura *et al.* 1992) and nasal blockage (Yasui *et al.* 1997). After oral administration to rat, (+)-S-145 was extensively metabolized via a first-pass effect and its metabolites in plasma, bile and urine were identified by NMR and mass spectroscopy (Mizojiri *et al.* 1993). The metabolite profiles of (+)-S-145 indicated that its primary metabolic pathways are β -oxidation at the carboxyl side chain and hydroxylation at the 5- or 6-positions of the bicyclo ring (Mizojiri *et al.* 1993, Norikura *et al.* 1993a, b). Our previous investigation with an *in vitro* system from rat demonstrated that β -oxidation of (+)-S-145 was the prominent metabolic pathway and that the peroxisomal enzyme system plays a major role, as has been reported for prostaglandin analogues and some drugs (Yamada *et al.* 1987, Suzuki *et al.* 1990, Yoshida *et al.* 1990, Diczfalussy 1994, Yamaguchi *et al.* 1995). In addition, as summarized in figure 1, the formation of 5-[(1R, 2S, 3S, 4S)-3-(benzenesulphonamido)bicyclo-

* Author for correspondence; e-mail: yoshitaka.yamaguchi@shionogi.co.jp

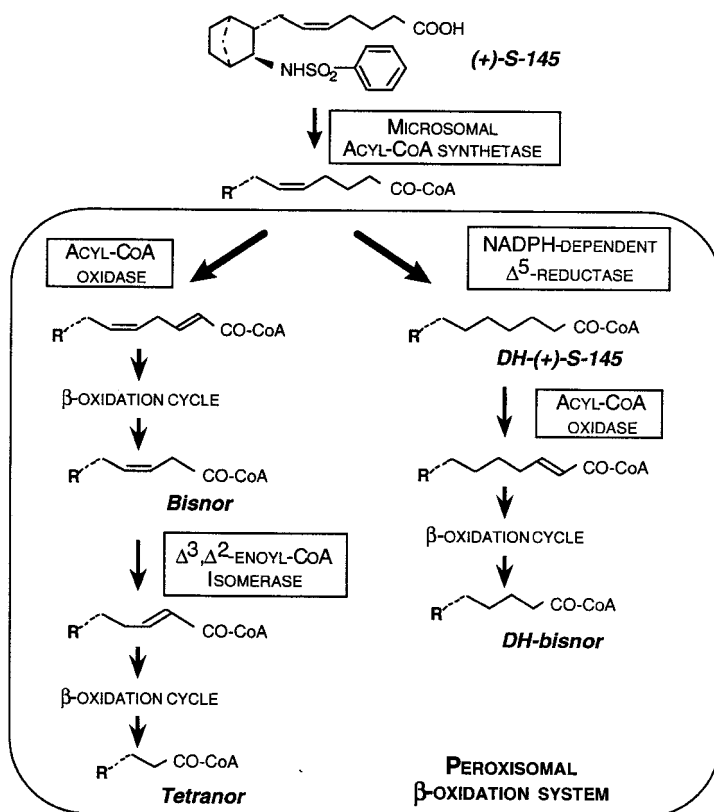


Figure 1. Two independent pathways of β -oxidation of (+)-S-145. Enzymes in boxes are proposed as being involved in the β -oxidative metabolism of (+)-S-145. The β -oxidation cycle is catalysed by 2-enoyl-CoA hydratase, 3-hydroxy acyl-CoA dehydrogenase and thiolase.

[2.2.1]hept-2-yl]pentanoic acid (DH-bisnor) occurred via an NADPH-dependent β -oxidation pathway, in contrast to formation of 3-[(1R, 2S, 3S, 4S)-3-(benzenesulphonamide)bicyclo[2.2.1]hept-2-yl]proionic acid (tetranor) and (3Z)-5-[(1R, 2S, 3S, 4S)-3-(benzenesulphonamido)bicyclo-[2.2.1]hept-2-yl]-3-pentenoic acid (bisnor) which were formed via the general β -oxidation pathway (Yamaguchi *et al.* 1995).

In vivo metabolic studies of (+)-S-145 in dog and monkey have shown that the plasma kinetics of (+)-S-145 and its β -oxidized metabolites were substantially different from those in rat. In dog, plasma levels of the unchanged drug were much higher than in rat and the β -oxidized metabolites were observed in plasma in trace amounts, while in monkey, plasma levels of (+)-S-145 were lower than those of its metabolites (Norikura *et al.* 1993a, c). As previously reported, slight sex differences (male < female) were observed in the *in vivo* formation of β -oxidized metabolites (Yamaguchi *et al.* 1996), which were much less than species differences in dog and monkey. Additionally, the composition of β -oxidized metabolites was different between these species. Although DH-bisnor was the major β -oxidized metabolite paralleling tetranor in rat, it was hardly detected in monkey plasma and bisnor was the major metabolite (Norikura *et al.* 1993a, c). In dog, these three metabolites were present at a similar level in plasma (Norikura *et al.* 1993c). Thus,

these observations seemed to suggest that species differences exist in hepatic β -oxidation capacity towards (+)-S-145 in rat, dog and monkey.

The present study compared the hepatic capacity for forming β -oxidized metabolites from (+)-S-145 among rat, dog and monkey in order to determine whether this was the reason for the species differences observed in the *in vivo* metabolic studies of (+)-S-145. Furthermore, the aim was to elucidate which particular metabolic step is responsible for the observed species differences in the β -oxidation of (+)-S-145.

Materials and methods

Chemicals

(+)-S-145 and related compounds were synthesized in the authors' laboratories (Watanabe *et al.* 1991). Radiolabelled (+)-S-145 (specific radioactivity 1.48 MBq mg⁻¹; purity 99% by HPLC with radiochemical detection) with carbon-14 at the benzene ring in the ω -side chain was synthesized from [U-¹⁴C-benzene] (Nagasaki *et al.* 1992). The CoA ester of (+)-S-145 (purity 97%) was synthesized from (+)-S-145 and CoA according to Webster *et al.* (1985). [1-¹⁴C]-palmitic acid (specific radioactivity 2.0 GBq mmol⁻¹; purity 99%) was from DuPont-New England Nuclear (Boston, MA, USA). Diazomethane was generated from *N*-nitrosodimethyl urea, which was from Maruwaka Kagaku Co., Ltd (Osaka, Japan). Palmitic acid, palmitoyl-CoA, homovanillic acid, peroxidase from horseradish and uricase from *Candida* species were from Wako Pure Chemicals (Osaka, Japan), and BSA (fatty acid free) was from Cosmo Bio Co., Ltd (Tokyo, Japan). All other chemicals were of the highest quality commercially available.

Animals and preparation of liver homogenates and microsomes

Male Sprague-Dawley rats (weighing 390–420 g) and female cynomolgus monkeys (weighing 3–4 kg) were from Japan Clea Laboratories, Inc. (Tokyo, Japan). Male beagle dogs (weighing 9–11 kg) were from Japan EDM, Inc. (Tokyo, Japan).

The livers were removed under anaesthesia with 5% sodium pentobarbital and those from dog and monkey were immediately stored at -80°C until use. Liver homogenates and microsomes were prepared according to Yamaguchi *et al.* (1995).

Protein concentration was estimated using a bichinchoninic acid protein assay kit (Pierce Chemical Co., Rockford, IL, USA) with BSA as a standard.

β -Oxidation, NADPH-dependent β -oxidation and Δ^5 -reduction by liver homogenates

Hepatic (+)-S-145 β -oxidation activities were determined according to Yamaguchi *et al.* (1995). After the pre-incubation of 50 mM (+)-S-145 and 1% (w/v) liver homogenates in the presence of 4 mM ATP and 4 mM CoA, the β -oxidation reaction was initiated by adding 2 mM NAD⁺ and the activities were calculated from formed amounts of tetranor and bisnor. This β -oxidation reaction was also performed in the presence of 2 mM NADPH and the formation rate of DH-bisnor was estimated as NADPH-dependent β -oxidation activities. Δ^5 -Reduction reaction was initiated by adding 2 mM NADPH instead of NAD⁺ and the formation rate of (+)-7-[(1R, 2S, 3S, 4S)-3-benzenesulphonamido-bicyclo-[2.2.1]hept-2-yl]-heptanoic acid (DH-(+)-S-145) was estimated as the Δ^5 -reduction activities.

Extracted metabolites from the incubation mixtures were methylated by diazomethane and determined by capillary gas chromatography as described (Okamoto *et al.* 1992a, b).

Determination of microsomal (+)-S-145-CoA synthesis activities and peroxisomal β -oxidation activities of (+)-S-145-CoA

The enzymatic synthesis of palmitoyl-CoA and (+)-S-145-CoA were determined with liver microsomes according to Krisans *et al.* (1980) and Yamaguchi *et al.* (1995) respectively.

Peroxisomal β -oxidation activities were determined by measuring acyl-CoA oxidase, the rate-limiting enzyme in peroxisomal β -oxidation system (Osmudsen *et al.* 1991). The acyl-CoA-dependent hydrogen peroxide formed by acyl-CoA oxidase was principally determined according to van Veldhoven and Mannaerts (1985). Briefly, palmitoyl-CoA or (+)-S-145-CoA (50 μM) was incubated at 37°C with 0.4% (w/v) liver homogenates in 0.5 ml 30 mM phosphate buffer (pH 7.4) containing 0.1 mM CoA, 0.15% (w/v) BSA (fatty acid free), 0.01% (v/v) Triton X-100, 12.5 mM aminotriazole, 25 units ml⁻¹ peroxidase and 0.75 mM homovanillic acid. After incubation for 5 min at 37°C , the reactions were terminated by adding 0.1 ml 1.2 M perchloric acid and centrifuged. The supernatant (50 μl) was diluted with 2 ml 0.1 M carbonate buffer (pH 10.0) and the fluorescence intensity was measured at the excitation

Table 1. Formation of β -oxidized and Δ^5 -reduced metabolites of (+)-S-145 by liver homogenates from rat, dog and monkey.

Species	Metabolite formation (nmol min ⁻¹ g ⁻¹ liver)			
	β -Oxidized metabolite ^a		NADPH-dependent β -oxidized metabolite ^a	Δ^5 -Reduced metabolite ^a
	Tetranor	Bisnor	DH-bisnor	DH-(+)-S-145
Rat (male, <i>n</i> = 4)	6.18 ± 1.18	3.26 ± 1.29	5.45 ± 1.43	6.84 ± 1.64
Dog (male, <i>n</i> = 3)	1.15 ± 0.36	2.03 ± 0.30	2.44 ± 0.46	6.77 ± 0.81
Monkey (female, <i>n</i> = 3)	0.36 ± 0.43	5.66 ± 1.57	0.62 ± 0.30	1.66 ± 0.73

^a β -Oxidation, NADPH-dependent β -oxidation and Δ^5 -reduction were performed under the conditions described in the Materials and methods.
Values are the mean ± SD of three or four animals.

wavelength of 327 nm and the emission wavelength of 420 nm. Standard mixtures contained 0.5-2 nmol uric acid and 6 munits uricase instead of acyl-CoA and liver homogenates.

Results

Formation of β -oxidized and Δ^5 -reduced metabolites from (+)-S-145 by liver homogenates

Hepatic (+)-S-145 β -oxidation capacities were compared by measuring its β -oxidized metabolites from liver homogenates prepared from rat, dog and monkey. Metabolites resulting from β -oxidation, NADPH-dependent β -oxidation and Δ^5 -reduction after incubation with liver homogenates were formed from each species but the amounts of these metabolites were very different between species (table 1). When (+)-S-145 was incubated with dog liver homogenates, both tetranor and bisnor were produced at a low level and their total amounts were ~3-fold less compared with rat. On the other hand, in monkey, bisnor was formed ~1.7-fold higher than rat, but tetranor formation was quite low. Thus, their total amounts in monkey was ~35% less than that of rat. There was no sex difference in the formation of β -oxidized metabolites by liver homogenates of dog and monkey (data not shown).

DH-bisnor was formed via NADPH-dependent β -oxidation of (+)-S-145 in parallel with tetranor and bisnor (figure 1). The highest formation of DH-bisnor was observed in rat. The amounts of DH-bisnor produced by dog liver homogenates were ~2.2-fold less than in rat and only small amounts were formed by monkey liver homogenates. The amounts of DH-(+)-S-145 that had formed after NADPH-dependent Δ^5 -reduction were also measured, this being a key step in the formation of DH-bisnor. Reduction of (+)-S-145 to DH-(+)-S-145 was considerable and similar for rat and dog, but 4-5-fold less in monkey (table 1).

Microsomal (+)-S-145-CoA synthesis and peroxisomal (+)-S-145-CoA β -oxidation

As summarized in figure 1, (+)-S-145 is initially transformed to its acyl-CoA esters by microsomal enzymes and then β -oxidized in peroxisomal enzyme systems. In order to identify the key step causing species differences in hepatic

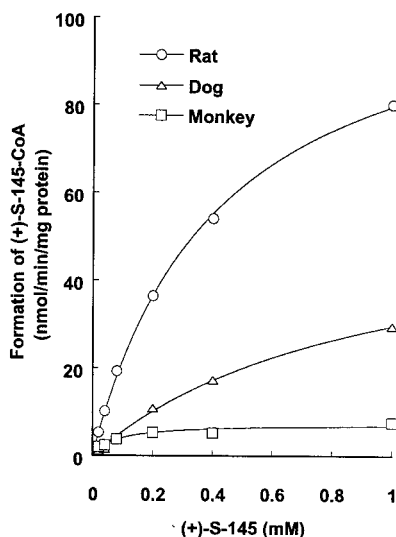


Figure 2. Dependency of substrate concentration on (+)-S-145-CoA synthesis by rat, dog and monkey liver microsomes. Each point represents the average from two animals. Data, from rat, dog and monkey, are indicated as circles, squares and triangles respectively.

Table 2. Kinetic parameters of (+)-S-145-CoA synthetase in rat, dog and monkey liver microsomes.

Species	K_m (mM)	V_{max} (nmol min ⁻¹ mg ⁻¹ protein)	V_{max}/K_m (μ l min ⁻¹ mg ⁻¹ protein)
Rat (male)	0.30	85.7	286
Dog (male)	0.66	33.5	51
Monkey (female)	0.05	6.4	132

V_{max} and K_m were determined with double reciprocal plotting from the averaged data of two animals.

β -oxidation capacity, (+)-S-145-CoA formation activities and peroxisomal β -oxidation activities of (+)-S-145-CoA in rat, dog and monkey were compared.

First, palmitoyl-CoA formation activities were determined with rat, dog and monkey liver microsomes, with activities of 87.0 ± 5.2 ($n = 4$), 59.0 ± 2.5 ($n = 3$) and 17.5 ± 0.7 ($n = 3$) nmol formed palmitoyl-CoA min⁻¹ mg⁻¹ protein respectively. (+)-S-145-CoA formation by rat, dog and monkey liver microsomes were also measured at (+)-S-145 concentrations of 0.01-1.00 mM (figure 2), and the apparent K_m , V_{max} and intrinsic clearance (V_{max}/K_m) were calculated by double reciprocal plot (table 2). Rat showed higher activities at all substrate concentrations and $V_{max} = \sim 2.6$ - and 13.4-fold higher than those of dog and monkey respectively. The lowest apparent K_m was observed in monkey and was ~ 6 - and 13-fold less than those of rat and dog respectively. Thus, intrinsic clearance (V_{max}/K_m) of (+)-S-145 formation was the highest in rat and 2.2- and 5.6-fold higher than those in monkey and dog respectively.

Next, the peroxisomal β -oxidation capacity was compared by estimating acyl-CoA oxidase activities against palmitoyl-CoA and (+)-S-145-CoA by rat, dog and monkey liver homogenates. (+)-S-145-CoA was extensively metabolized at a similar level to palmitoyl-CoA, an endogenous substrate for peroxisomal acyl-

Table 3. Palmitoyl-CoA and (+)-S-145-CoA oxidation activities by rat, dog and monkey liver homogenates.

Species	Acyl-CoA oxidation activities (nmol min ⁻¹ g ⁻¹ liver)	
	Palmitoyl-CoA	(+)-S-145-CoA
Rat (male, n = 4)	520 ± 72	457 ± 54
Dog (male, n = 3)	309 ± 18	160 ± 33
Monkey (female, n = 3)	182 ± 46	139 ± 48

Values are the mean ± SD of three or four animals.

CoA oxidase, in any species. Peroxisomal β -oxidation activities toward (+)-S-145 were 2.9- and 3.3-fold higher in rat than in dog and monkey respectively, which were similar to those for palmitoyl-CoA oxidation (table 3).

Discussion

In rat, orally administered (+)-S-145 is primarily metabolized via β -oxidation and the plasma level of the main β -oxidized metabolite was similar to that of unchanged drug (Norikura *et al.* 1993a). Metabolic studies of (+)-S-145 were also performed with dog and monkey and the plasma kinetics of (+)-S-145 and the profile of its β -oxidized metabolites were very different from those of rat. In dog, after oral administration, the plasma level of (+)-S-145 was higher than in rat and its β -oxidized metabolites were at very low levels in plasma (Norikura *et al.* 1993c). In contrast, in monkey, the level of the unchanged drug was lower than that of its metabolites in plasma. As the clearance of (+)-S-145 from plasma depended on the hepatic metabolic capacity and the β -oxidation pathway seemed to be the prominent one in all species of animals (Norikura *et al.* 1993a-c), a species difference was assumed to exist in the hepatic β -oxidation capacity between rat, dog and monkey (table 1). In dog, the lower β -oxidation capacity indicated from *in vivo* studies has been confirmed using *in vitro* experiments. Thus, both the formation and oxidation activities of (+)-S-145-CoA were lower in dog (tables 2 and 3). Also, in the metabolic studies of several HMG-CoA reductase inhibitors, the formation of β -oxidized metabolites was scarcely observed in dog (Arai *et al.* 1988, Vickers *et al.* 1990, Halpin *et al.* 1993, Boberg *et al.* 1998, Black *et al.* 1999), which might be due to the low β -oxidation capacity in dog as well as in the case of (+)-S-145.

When the calcium salt of (+)-S-145 was orally administered to monkey, the plasma concentration of (+)-S-145 was lower than that of the other animals (Norikura *et al.* 1993a, c), which led to the speculation that the formation of β -oxidized metabolites by liver homogenates would be higher in monkey than in rat and dog. However, the present study confirmed that rat possessed the highest metabolic activities of (+)-S-145 among these species. This discrepancy might be attributed to a lower first-pass effect in rat than dog and monkey, as suggested by the t_{max} of (+)-S-145 being ~3-fold less in rat than in dog and monkey after the oral administration (Norikura *et al.* 1993a, c). Further support for this conclusion comes from the fact that in the case of intravenous administration, the most rapid

disappearance of (+)-S-145 from plasma was observed in rat and the slowest in dog (Norikura *et al.* 1993a, c), in agreement with the present findings.

DH-bisnor, a β -oxidized metabolite with a saturated carboxyl side chain, is formed via NADPH-dependent β -oxidation (Yamaguchi *et al.* 1995) (figure 1). In rat and dog, DH-bisnor is a major β -oxidized metabolite of (+)-S-145, but was present at low levels in monkey plasma and urine (Norikura *et al.* 1993a, c). These observations are consistent with the present finding that DH-bisnor formation activities were negligible in monkey, as a result of the very low Δ^5 -reductase activity (table 1). As DH-bisnor and its derivatives were not detected in human clinical studies (Okamoto *et al.* 1992a, b) as in monkey, (+)-S-145 may also be slowly reduced to DH-(+)-S-145 in human.

In monkey, bisnor was the main β -oxidized metabolite, but its level was very low in rat (Norikura *et al.* 1993a, c), which was also found in the present *in vitro* β -oxidation experiments (table 1). Generally, peroxisomal β -oxidation of unsaturated fatty acids with a 3-*cis* double bond, such as bisnor, requires the involvement of Δ^3 -*cis*- Δ^2 -*trans*-enoyl-CoA isomerase instead of acyl-CoA oxidase at the first step (Osmudsen *et al.* 1991, Guzman and Geelen 1993, Hiltunen *et al.* 1993). Thus, the species difference observed in bisnor formation may be as a result of this enzyme activity being much higher in rat than in other species.

The initial step in β -oxidation pathway of fatty acids is the formation of its acyl-CoA ester (Osmudsen *et al.* 1991, Guzman and Geelen 1993, Hiltunen *et al.* 1993). In the case of (+)-S-145, this activity is localized in microsomes (Yamaguchi *et al.* 1995) and the species difference observed in their intrinsic clearances approximately agreed with that of their β -oxidized metabolite formation activities (tables 1 and 2). On the other hand, the peroxisomal enzyme system appears to play a major role in (+)-S-145 β -oxidation in any species, as (+)-S-145 β -oxidation was also independent of carnitine in dog and monkey as well as in rat (data not shown). (+)-S-145-CoA was extensively metabolized by acyl-CoA oxidase, a rate-limiting enzyme in peroxisomal β -oxidation system, at a similar level with an endogenous substrate (table 3). Although some species differences exist in peroxisomal β -oxidation activities, the activities of (+)-S-145-CoA oxidation were much higher than those of β -oxidized metabolite formation in these species. Therefore, as *in vivo* formed (+)-S-145-CoA would be immediately β -oxidized by the peroxisomal enzyme system, (+)-S-145-CoA formation in microsomes was confirmed to be a rate-limiting step in the β -oxidation pathway of (+)-S-145 rather than peroxisomal β -oxidation.

Acyl-CoA synthesis is also the initial step for amino acid conjugation (Hutt and Caldwell 1992). Taurine conjugates of (+)-S-145 and its β -oxidized metabolites are formed in rat and dog *in vivo*, but not in monkey (Mizojiri *et al.* 1993, Norikura *et al.* 1993a, c). In rat, > 50% of an orally administered dose of S-1452 was excreted as taurine-conjugated metabolites (Norikura *et al.* 1993a). Therefore, these findings revealed that some (+)-S-145-CoA formed *in vivo* would undergo taurine conjugation prior to β -oxidation and β -oxidized metabolite formation might be suppressed in rat and dog, but not in monkey. Actually, sex differences of (+)-S-145 β -oxidation (male < female) by rat liver homogenates were amplified in the presence of taurine as its taurine conjugation activities were much higher in the male rat than in the female (Yamaguchi *et al.* 1996).

The present study with the *in vitro* system proved that a species difference was present in hepatic (+)-S-145 β -oxidation capacities in rat, dog and monkey. In

human, ~80% of the administered (+)-S-145 is excreted in urine as its β -oxidized and related metabolites (Okamoto *et al.* 1992b), which indicated that clearance of (+)-S-145 from plasma would also be due to hepatic β -oxidation capacity in human. As the metabolic profiles of (+)-S-145 in plasma and urine are similar between human and monkey (Okamoto *et al.* 1992a, b, Norikura *et al.* 1993c), the characteristics of enzymes related to the metabolism of (+)-S-145 are very likely similar between human and monkey, although further studies are required to confirm this conclusion.

Acknowledgements

The authors are grateful to Dr Mitsuaki Ohtani, Dr Atsushi Kurosawa and Mr Tohru Nagasaki, Shionogi Research Laboratories, for the synthesis and supply of (+)-S-145 and related compounds. Thanks are also due to Mr Joji Okamoto and Mr Tomoyuki Ohkawa for excellent technical assistance.

References

- ARAI, M., SERIZAWA, N., TERAHARA, A., TSUJITA, Y., HORIE, M. and BABA, S., 1988, Pravastatin sodium (CS-514), a novel cholesterol-lowering agent which inhibits HMG-CoA reductase. *Sankyo Kenkyusho Nempo*, **40**, 1-38.
- ARIMURA, A., ASANUMA, F., KUROSAWA, A., HARADA, F., NAGAI, H. and KODA, A., 1992, Antiasthmatic activity of a novel thromboxane A_2 antagonist, S-1452, in guinea pig. *International Archives of Allergy and Immunology*, **98**, 239-246.
- BLACK, A. E., HAYES, R. M., ROTH, B. D., WOO, P. and WOOLF, T. F., 1999, Metabolism and excretion of atorvastatin in rats and dogs. *Drug Metabolism and Disposition*, **27**, 916-923.
- BOBERG, M., ANGERBAUER, R., KANHAI, W. K., KARL, W., KERN, A., RADTKE, M. and STENKE, W., 1998, Biotransformation of cerivastatin in mice, rats, and dogs *in vivo*. *Drug Metabolism and Disposition*, **26**, 640-652.
- DICZFALUSKY, U., 1994, β -Oxidation of eicosanoids. *Progress in Lipid Research*, **33**, 403-428.
- GUZMAN, M. and GELEN, M. J. H., 1993, Regulation of fatty acids oxidation in mammalian liver. *Biochimica et Biophysica Acta*, **1167**, 227-241.
- HALPIN, R. A., ULM, E. H., TILL, A. E., KARI, P. H., VYAS, K. P., HUNNINGKAKE, D. B. and DUGGAN, D. E., 1993, Biotransformation of lovastatin: species differences in *in vivo* metabolite profiles of mouse, rat, dog, and human. *Drug Metabolism and Disposition*, **21**, 1003-1011.
- HANASAKI, K., NAGASAKI, T. and ARITA, H., 1989, Characterization of platelet thromboxane A_2 /prostaglandin H_2 receptor by a novel thromboxane receptor antagonist, [^3H]S-145. *Biochemical Pharmacology*, **38**, 2007-2017.
- HILTUNEN, J. K., FILPPILA, S. A., HAYRINEN, H. M., KOIVURANTA, K. T. and HAKKOLA, E. H., 1993, Peroxisomal β -oxidation of poly unsaturated fatty acids. *Biochimie*, **75**, 175-182.
- HUTT, J. A. and CALDWELL, J. 1992, Amino acid conjugation. In G. J. Mulder (ed.), *Conjugation Reactions in Drug Metabolism* (London: Taylor & Francis) pp. 273-305.
- KRISANS, S. K., MORTENSEN, R. M. and LAZAROW, P. B., 1980, Acyl-CoA synthetase in rat liver peroxisomes. *Journal of Biological Chemistry*, **255**, 9599-9607.
- MIZOJIRI, K., NORIKURA, R., YOSHIMORI, T., NAKANISHI, M., IWATANI, K., TERUI, Y., KIKUCHI, J. and NAKAGAWA, Y., 1995, Identification of metabolites of a new thromboxane A_2 receptor antagonist, S-1452, in rats. *Xenobiotic Metabolism and Disposition*, **8**, 67-81 [in Japanese].
- NAGASAKI, T., WATANABE, F., KATSUYAMA, Y., HAMADA, Y., OHTANI, M. and NARISADA, M., 1992, Synthesis of [^{14}C]- and [^3H]-labeled (+)-[1R-[1a,2a(Z),3b,4a]]-7-[3-[(phenylsulfonyl)amino]-bicyclo-[2.2.1]-hept-2-yl]-5-heptenoic acid, ((+)-S-145) and its calcium salt (S-1452). *Journal of Labelled Compounds and Radiopharmaceuticals*, **31**, 23-38.
- NORIKURA, R., TANAKA, H., YOSHIMORI, T., FUTAGUCHI, S., OKABE, H., NAKANISHI, M., IWAMOTO, Y. and MIZOJIRI, K., 1993b, Studies on the metabolic fate of a new thromboxane A_2 receptor antagonist, S-1452 (II): distribution and excretion after single and repeated oral administration to rats. *Xenobiotic Metabolism and Disposition*, **8**, 97-105 [in Japanese].
- NORIKURA, R., YOSHIMORI, T., MURAKAMI, T., NAKANISHI, M. and MIZOJIRI, K., 1993a, Studies on the metabolic fate of a new thromboxane A_2 receptor antagonist, S-1452 (I): absorption, distribution, metabolism and excretion after single administration to rats. *Xenobiotic Metabolism and Disposition*, **8**, 83-96 [in Japanese].

- NORIKURA, R., YOSHIMORI, T., MURAKAMI, T., NAKANISHI, M. and MIZOJIRI, K., 1993c, Studies on the metabolic fate of a new thromboxane A_2 receptor antagonist, S-1452 (IV): absorption, distribution, metabolism and excretion in dogs and monkeys. *Xenobiotic Metabolism and Disposition*, **8**, 113-125 [in Japanese].
- OKAMOTO, J., HIRAI, E., KITAGAWA, T. and MATSUBARA, T., 1992a, Capillary gas chromatographic method for the determination of the thromboxane A_2 receptor antagonist S-1452 and its metabolites in human plasma. *Journal of Chromatography*, **583**, 43-52.
- OKAMOTO, J., HIRAI, E., SUZUKI, Y., FUJIMOTO, K., KITAGAWA, T. and MATSUBARA, T., 1992b, Capillary gas chromatographic method for the determination of the thromboxane A_2 receptor antagonist S-1452 and its metabolites in human urine. *Journal of Chromatography*, **583**, 53-62.
- OSMUDSEN, H., BREMER, J. and PEDERSEN, J. I., 1991, Metabolic aspects of peroxisomal β -oxidation. *Biochimica et Biophysica Acta*, **1085**, 141-158.
- SUZUKI, H., MORI, K., YAMADA, J. and SUGA, T., 1990, Contribution of peroxisomal β -oxidation system to the chain-shortening of *N*-(α -methylbenzyl) azelaamic acid in rat liver. *Biochemical Pharmacology*, **39**, 1975-1981.
- VAN VELDHOVEN, P. and MANNAERTS, G. P., 1985, Comparison of the activities of some peroxisomal and extarperoxisomal lipid-metabolizing enzymes in liver and extrahepatic tissues of the rat. *Biochemical Journal*, **227**, 737-741.
- VICKERS, S., DUNCAN, C. A., VYAS, K. P., KARI, P. H., ARISON, B., PRAKASH, S. R., RAMJIT, H. G., PITZENBERGER, S. M., STOKKER, G. and DUGGAN, D. E., 1990, *In vitro* and *in vivo* biotransformation of simvastatin, an inhibitor of HMG CoA reductase. *Drug Metabolism and Disposition*, **18**, 476-483.
- WATANABE, F., MATSUURA, T., SHIRAHASE, K. and OHTANI, M., 1991, Synthesis of metabolites of S-1452, an orally active thromboxane A_2 receptor antagonist. *Chemical Pharmaceutical Bulletin*, **39**, 2842-2848.
- WEBSTER, L. T. and KILLENBERG, P. G., 1985, Coenzyme A thioesters of benzoic, hydroxybenzoic, phenylacetic, and bile acids. *Methods in Enzymology*, **77**, 430-436.
- YAMADA, J., OGAWA, S., HORIE, S., WATANABE, T. and SUGA, T., 1987, Participation of peroxisomes in the metabolism of xenobiotic acyl compounds: comparison between peroxisomal and mitochondrial β -oxidation of *w*-phenyl fatty acids in rat liver. *Biochimica et Biophysica Acta*, **921**, 292-301.
- YAMAGUCHI, Y., BABA, T., TOUCHI, A. and MATSUBARA, T., 1995, *In vitro* studies to elucidate the metabolic pathway of (+)-S-145, a thromboxane A_2 receptor antagonist, in rats: evidence for two independent pathways in peroxisomal β -oxidation. *Drug Metabolism and Disposition*, **23**, 1195-1201.
- YAMAGUCHI, Y., NORIKURA, R., NAKANISHI, M., TOUCHI, A., YOSHIMORI, T., MURAKAMI, T., BABA, T., MIZOJIRI, K. and MATSUBARA, T., 1996, Sex differences in the metabolism of (+)-S-145, a novel thromboxane A_2 receptor antagonist in rat. *Xenobiotica*, **26**, 613-626.
- YASUI, K., ASANUMA, F., FURUE, Y. and ARIMURA, A., 1997, Involvement of thromboxane A_2 in antigen-induced nasal blockage in guinea pigs. *International Archives of Allergy and Immunology*, **112**, 400-405.
- YOSHIDA, Y., YAMADA, J., WATANABE, T., SUGA, T. and TAKAYAMA, H., 1990, Participation of the peroxisomal β -oxidation system in the chain-shortening of PCA16, a metabolite of the cytosine arabinoside prodrug, YNK01, in rat liver. *Biochemical Pharmacology*, **39**, 1505-1512.

Inhibition of human CYP1A2 activity *in vitro* by methylxanthines: potent competitive inhibition by 8-phenyltheophylline

S. MURRAY*, A. O. A. ODUPITAN, B. P. MURRAY,
A. R. BOOBIS and R. J. EDWARDS

Section on Clinical Pharmacology, Imperial College School of Medicine, Du Cane Road,
London W12 0NN, UK

Received 11 December 2000

1. Humans are exposed *in vivo* to methylxanthines by dietary ingestion, as well as from their use as therapeutic agents. The inhibitory effect of a series of these compounds on high-affinity phenacetin *O*-deethylase activity in the human liver microsomal fraction, a measure of CYP1A2 activity, has been evaluated.

2. Paracetamol, the product of phenacetin *O*-deethylase activity, was analysed by gas chromatography/negative-ion mass spectrometry using a novel *bis*trifluoromethylbenzoyl/trimethylsilyl derivative, and incubation conditions for assessing high-affinity phenacetin *O*-deethylase activity were examined and optimized.

3. 1-Methylxanthine, caffeine, theophylline, 8-methylxanthine, pentoxifylline and 3-isobutyl-1-methylxanthine caused moderate inhibition with $IC_{50} = 260, 140, 120, 100, 62$ and $36 \mu M$ respectively.

4. 8-Phenyltheophylline was a potent competitive inhibitor of high-affinity phenacetin *O*-deethylase activity with an $IC_{50} = 0.7 \mu M$ and $K_i = 0.11 \mu M$.

5. The specificity of inhibition by 8-phenyltheophylline was assessed by measuring its effect on debrisoquine 4-hydroxylase (CYP2D6), terfenadine hydroxylase (CYP3A4), chlorzoxazone 6-hydroxylase (CYP2E1) and tolbutamide 4-hydroxylase (CYP2C9) activities in human liver microsomal fraction. No inhibition of any of these activities was observed.

6. The potency and specificity of 8-phenyltheophylline as an inhibitor of human hepatic CYP1A2 indicate that the compound may be useful as a chemical inhibitor of this enzyme for further *in vitro* studies.

Introduction

The human P450 enzyme CYP1A2 in the liver is involved in the metabolism of a number of exogenous compounds ingested either in the diet or as therapeutic agents, as well as endogenous agents such as oestradiol (Ball *et al.* 1990). The content and hence activity of human hepatic CYP1A2 varies markedly and can be increased by induction. This occurs upon exposure to polycyclic aromatic hydrocarbons, such as those present in cigarette smoke (Sesardic *et al.* 1988) and charcoal-grilled meat (Conney *et al.* 1976), by consumption of a diet containing compounds such as indole derivatives (Michnovicz and Bradlow 1990) and by treatment with certain therapeutic agents such as omeprazole (Diaz *et al.* 1990). Human CYP1A2 is of considerable toxicological importance as it is responsible for the mutagenic activation of a number of procarcinogens (Butler *et al.* 1989, Boobis *et al.* 1994), as well as the production of cytotoxic metabolites of drugs such as

* Author for correspondence: e-mail: s.murray@ic.ac.uk

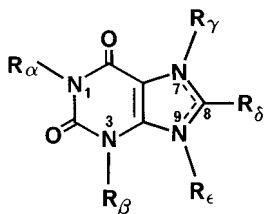


Figure 1. Chemical structure of a substituted xanthine. For methylxanthines, at least one of the five R substituents is a methyl group.

paracetamol (Raucy *et al.* 1989). Intra- and interindividual variation in activity of CYP1A2 therefore may play a major role in determining an individual's susceptibility to the toxic effects of compounds activated by this enzyme.

Methylxanthines (figure 1) are a group of compounds to which humans are widely exposed. Some members, such as caffeine and theobromine, are ingested in the diet while others, such as theophylline, dyphylline and pentoxifylline, are used therapeutically. Exposure to other methylxanthines occurs when they are formed *in vivo* as primary metabolites of methylxanthines that have been consumed. While CYP1A2 has been shown to be of major importance in the elimination, through *N*-demethylation, of caffeine and theophylline (Butler *et al.* 1989, Fuhr *et al.* 1992), another methylxanthine, furafylline, has been found to be a specific and extremely potent inhibitor of this enzyme in man (Sesardic *et al.* 1990a). The possibility of modulation of CYP1A2 activity by these compounds, especially those ingested in unknown amounts in the diet, is of concern for other compounds metabolized by this enzyme. Any large changes in CYP1A2 activity may have unexpected and unpredicted consequences on endogenous processes as well as on carcinogen activation and drug toxicity.

The high-affinity component of human hepatic phenacetin *O*-deethylase (POD) activity (Boobis *et al.* 1981) is known to be catalysed specifically by CYP1A2 (Sesardic *et al.* 1988) and a gas chromatographic/mass spectrometric assay is available for measuring the product of this activity, paracetamol, *in vitro* (Murray and Boobis 1986, Sesardic *et al.* 1990b). Both the assay for paracetamol and the incubation conditions for assessing POD activity have now been refined and then used to determine the inhibitory effects of a series of 16 methylxanthines on CYP1A2 activity in human liver microsomal fraction. The primary aim of this work was to identify methylxanthines that are potent inhibitors of CYP1A2 activity in man and to examine any relationship between structure and inhibitory function.

Materials and methods

Materials

Human hepatic microsomal fractions were prepared from samples of normal human liver as described by Boobis *et al.* (1990). The tissue, from the human liver bank maintained in the Section on Clinical Pharmacology at Imperial College School of Medicine, was obtained from liver transplant cut-downs, liver resections and kidney transplant donors. None of the donors were known to have been exposed to recognized inducers of P450 enzymes. Tissue was stored frozen at -70°C until isolation of the microsomal fraction and all samples were histologically normal. The use of samples for these studies had local Research Ethical Committee permission and, where appropriate, coroner's approval. Enzyme activities were assessed using a pool of six well-characterized samples of hepatic microsomal fraction. Xanthines, including 8-phenyltheophylline, were supplied by Sigma-Aldrich Ltd (Poole, UK) and

were of $\geq 99\%$ purity. Phenacetin, paracetamol, terfenadine, chlorzoxazone, tolbutamide, NADPH, bovine serum albumin (BSA) fraction V powder, dimethylsulphoxide (DMSO), diisopropylethylamine, *bis*(trimethylsilyl)trifluoroacetamide (BSTFA) and undecane were also from Sigma-Aldrich. Phenacetin was recrystallized from aqueous ethanol and diisopropylethylamine was passed through a column of basic alumina before use. Ultrafine Chemicals Ltd (Manchester, UK) supplied 6-hydroxychlorzoxazone and 4-hydroxytolbutamide. Hexafluoroacetylacetone and 3,5-*bis*trifluoromethylbenzoyl (*bis*TFMBO) chloride were from Fluorochem Ltd (Glossop, UK) while Bond-ElutTM solid phase extraction (SPE) columns (C18, 200 mg, 3 ml) were from Anachem (Luton, UK). Debrisoquine and 4-hydroxydebrisoquine were a kind gift from Roche Products Ltd (Welwyn Garden City, UK). [²H₄]-paracetamol was from MSD Isotopes (Montreal, Canada), [²H₄]-4-hydroxydebrisoquine was supplied by Dr C. O. Meese (Meese 1986) and [²H₃]-terfenadine containing < 1% terfenadine was synthesized as described by Adams *et al.* (1997). Acetonitrile, methanol and ammonium acetate were of HPLC grade, while other organic solvents were of analytical grade. Ethyl acetate was redistilled before use and, when required dry, stored over calcium hydride.

Phenacetin O-deethylase activity

The extraction and derivatization procedures that formed the basis of the published gas chromatographic/mass spectrometric assay for POD activity (Murray and Boobis 1986) were modified. To reduce usage of organic solvent and permit automation of the extraction procedure, SPE was used in place of solvent extraction and C8, C18 and phenyl phases were assessed. Recoveries of paracetamol from acidified microsomal incubation mixtures (pH 1) were < 25% with C8 columns but for C18 and phenyl columns, using the extraction procedure described below, recoveries were > 97%. Either C18 or phenyl columns were therefore suitable for use in this assay. The *bis*TFMBO derivative of paracetamol, while providing good sensitivity in a negative-ion selected ion-monitoring (SIM) assay for paracetamol, exhibited peak tailing on a non-polar DB5 GC stationary phase. This became much more pronounced with column age and limited the number of samples that could be analysed successfully at one time. Further derivatization was achieved by injecting the *bis*TFMBO derivative dissolved in BSTFA into the heated injector port of the gas chromatograph. A *bis*TFMBO/trimethylsilyl derivative of paracetamol was formed in > 90% yield that showed excellent chromatographic behaviour together with a negative-ion mass spectrum well suited to SIM work. The latter contained only two ions, a molecular ion of m/z 463 and a smaller fragment ion (25% of the intensity of the molecular ion) of m/z 391. When monitoring ions of m/z 463, an amount of *bis*TFMBO/trimethylsilyl derivative corresponding to 66 fmol (10 pg) paracetamol could be detected with a signal-to-noise ratio of 15:1.

Enzyme incubations. For IC₅₀ measurements, incubation mixtures consisting of human hepatic microsomal fraction (pool of six individual fractions, 0.2 mg protein), 1.2 mM NADPH and BSA (0.5 mg) in 0.1 M sodium phosphate buffer (pH 7.4, 980 μ l) were warmed to 37°C in a shaking waterbath. These were pre-incubated for 5 min with DMSO (10 μ l) containing xanthines at various concentrations. The reaction was started by the addition of phenacetin dissolved in methanol (400 μ M, 10 μ l) to a final concentration of 4 μ M (the final incubation volume was 1 ml) and the samples were then incubated for a further 10 min at 37°C. For experiments designed to optimize enzyme activity and for kinetic measurements, conditions varied from those above as detailed in the figure legends and the Results. In all cases, the reaction was stopped by the addition of 43% phosphoric acid (50 μ l), [²H₄]-paracetamol in methanol (1 μ g ml⁻¹, 50 μ l, 323 pmol) was added to each incubation mixture and the samples were placed on ice.

Extraction and derivatization. Samples were applied to Bond Elut C18 SPE columns (200 mg sorbent mass, 3 ml reservoir volume) that had been preconditioned with methanol (2 $\times$ 2 ml) and water (2 $\times$ 2 ml). The columns were washed with water (2 $\times$ 2 ml), after which paracetamol and [²H₄]-paracetamol were eluted with methanol (2 ml). The methanol eluates were evaporated to dryness under nitrogen and to each vial *bis*TFMBO chloride in dry ethyl acetate (1/100 v/v, 50 μ l) and diisopropylethylamine in dry ethyl acetate (1/100 v/v, 50 μ l) were added. The reaction mixtures were allowed to stand for 1 h at room temperature, after which reagents were removed by evaporation under nitrogen. Aqueous sodium carbonate solution (0.5 M, 300 μ l) was added to the residues, which were then extracted with ethyl acetate (750 μ l). The organic extracts were evaporated to dryness under nitrogen and BSTFA (50 μ l) added to each vial. Aliquots (2 μ l) were then injected into the gas chromatograph/mass spectrometer.

Gas chromatography/mass spectrometry. A Finnigan MAT 4500 gas chromatograph quadrupole mass spectrometer (ThermoQuest Corporation, San Jose, CA, USA) was used. The gas chromatograph was equipped with a 15 m $\times$ 0.25 mm i.d. DB5 J&W fused-silica capillary column, with helium as carrier gas at a head pressure of 70 kPa, and a Grob-type capillary injector operated in the splitless mode and maintained at 270°C. The gas chromatograph oven

temperature was held at 130°C for 1 min and then raised to 230°C at 20°C min⁻¹. Under these conditions, the retention times of the *bis*TFMBO/trimethylsilyl derivatives of paracetamol and [²H₄]-paracetamol were 4.8 min. The mass spectrometer was operated in the electron capture negative-ion mode with an electron energy of 100 eV. Ammonia gas was admitted to an indicated ion source pressure of 0.05 kPa and the indicated ion source temperature was maintained at 150°C. The mass spectrometer was set to monitor negative ions of *m/z* 463 and 467, the molecular ions of the paracetamol and [²H₄]-paracetamol derivatives respectively, and data acquisition and processing were performed by an INCOS data system.

Assay validation. An unextracted standard curve was prepared with standards containing paracetamol (0–2 nmol) and [²H₄]-paracetamol (323 pmol, 50 ng). With SIM of ions of *m/z* 463 (paracetamol) and 467 ([²H₄]-paracetamol), an eight-point standard curve was obtained for a peak-area ratio *m/z* 463/467 plotted against amount of paracetamol that was linear ($y = 0.0031x + 0.010$, $r^2 = 1.000$). Corresponding amounts of paracetamol were added to blank incubation mixtures that had no NADPH present and had not been incubated at 37°C. These samples were extracted and derivatized as described above and analysed by GC/MS. The peak area ratios of the extracted standards did not differ significantly from those of the unextracted standards, indicating that no paracetamol was present in the blank incubation mixtures and that there was no chromatographic interference. Consequently, it was appropriate to use unextracted standard curves for the routine analysis of samples. While the limit of detection of paracetamol in a microsomal incubation was < 5 pmol, the limit of quantification was set at 66 pmol (10 ng), which was a lower level of paracetamol than was found in any incubation. The accuracy and precision at the limit of quantification were 67.1 ± 2.8 pmol (mean $\pm$ SD, $n = 6$, CV 4.2%).

Enzyme kinetics. Enzyme activity was expressed as pmol paracetamol produced min⁻¹ mg⁻¹ microsomal protein. The kinetics of the rate of reaction were analysed assuming a two-component model (Boobis *et al.* 1981) using the formula:

$$v = (V_{\max 1}[S]/(K_{m1} + [S])) + (V_{\max 2}[S]/(K_{m2} + [S])),$$

where v is the reaction velocity (pmol min⁻¹ mg⁻¹ microsomal protein), $V_{\max 1}$ and $V_{\max 2}$ are the maximum velocities for the high- and low-affinity components respectively (pmol min⁻¹ mg⁻¹ microsomal protein), $[S]$ is the substrate concentration (μ M), and K_{m1} and K_{m2} are the substrate concentrations (μ M) for half-maximal velocities for the high- and low-affinity components respectively.

Kinetic studies were performed using eight substrate concentrations between 1 and 1000 μ M and the resultant reaction curves were fitted using the two-component model by iterative least-squares non-linear regression analysis with FigP software v.6.0a (Biosoft, Cambridge, UK). The results of the calculations were checked by initiating the calculations from a variety of starting values. In all cases the goodness of fit was within 95% of the expected limits for a correct model of the data. To determine the kinetics of inhibition of high-affinity POD activity by 8-phenyltheophylline, reaction velocities were also determined in the presence of 1, 3 and 10 μ M 8-phenyltheophylline. The contribution of the low-affinity component was determined, as described above, and was subtracted from the total reaction velocities to give the velocities for the high-affinity component. These values were then fitted by iterative non-linear regression using the single enzyme Michaelis-Menten equation:

$$v = (V'_{\max}[S]/(K'_m + [S])),$$

where K'_m and $V'_{\max}$ are apparent values for K_m and $V_{\max}$ determined in the presence of each concentration of inhibitor.

K_i at each inhibitor concentration was determined using the equation:

$$K_i = K_m I / (K'_m - K_m),$$

where I is the concentration of the inhibitor (μ M).

Debrisoquine 4-hydroxylase activity

Debrisoquine 4-hydroxylase activity in microsomal incubations was measured by modifying a gas chromatographic/mass spectrometric assay for 4-hydroxydebrisoquine (Boobis *et al.* 1983, Kobayashi *et al.* 1989).

Enzyme incubations. Incubation mixtures consisting of human hepatic microsomal fraction (pool of six individual fractions, 5 mg ml⁻¹, 100 μ l), 1.2 mM NADPH (10 mg ml⁻¹, 100 μ l) and 0.05 M sodium phosphate buffer (pH 7.4, 749 μ l) were warmed to 37°C in a shaking waterbath. These were then pre-incubated for 5 min with DMSO (1 μ l) containing 8-phenyltheophylline at various concentrations. The reaction was started by the addition of debrisoquine dissolved in

0.05 M sodium phosphate buffer (2.4 mM, 50 μ l) to a final concentration of 120 μ M (the final incubation volume was 1 ml) and the samples were then incubated for a further 20 min at 37°C. The reaction was stopped by the addition of 1 M sodium hydroxide solution (200 μ l), [^{2} H $_4$]-4-hydroxydebrisoquine in water (1 μ g ml $^{-1}$, 100 μ l, 513 pmol) was added to each incubation mixture and the samples were placed on ice.

Extraction and derivatization. Each sample was extracted with chloroform (3 $\times$ 3 ml) to remove unmetabolized debrisoquine, after which the aqueous phases were neutralized by the addition of 1 M hydrochloric acid (150 μ l). The samples were transferred to quickfit glass test tubes and saturated sodium bicarbonate solution (200 μ l) and hexafluoroacetylacetone in toluene (1/20 v/v, 1.1 ml) were added. The test tubes were fitted with short air condensers and 4-hydroxydebrisoquine and [^{2} H $_4$]-4-hydroxydebrisoquine in the samples were then converted into their 3,5-bistrifluoromethylpyrimidinyl derivatives by heating the tubes in a boiling water bath at 100°C for 1 h. After cooling and centrifugation, the upper organic layers were transferred to glass vials and evaporated to dryness under nitrogen. The hydroxyl groups present in the bistrifluoromethylpyrimidinyl derivatives of 4-hydroxydebrisoquine and [^{2} H $_4$]-4-hydroxydebrisoquine were then trimethylsilylated prior to analysis to improve gas chromatographic behaviour. BSTFA (50 μ l) was added to each vial, the vials were securely capped and allowed to stand at room temperature overnight. Just prior to analysis by gas chromatography/mass spectrometry, vial contents were evaporated to dryness under nitrogen and the residues were reconstituted in undecane (50 μ l). Aliquots (2 μ l) were then injected into the gas chromatograph/mass spectrometer.

Gas chromatography/mass spectrometry. Gas chromatography/mass spectrometry conditions were as described above for the measurement of POD activity, except for the gas chromatograph temperature program and SIM parameters. The gas chromatograph oven temperature was held at 160°C for 1 min and then raised to 240°C at 20°C min $^{-1}$. Under these conditions, the retention times of the 3,5-bistrifluoromethylpyrimidinyl/trimethylsilyl derivatives of 4-hydroxydebrisoquine and [^{2} H $_4$]-4-hydroxydebrisoquine were 3 min. The mass spectrometer operated in the electron capture negative-ion mode was set to monitor negative ions of m/z 435 and 439, which were the molecular ions of the 4-hydroxydebrisoquine and [^{2} H $_4$]-4-hydroxydebrisoquine derivatives respectively (Murray and Waddell 1982).

Assay validation. Standard curves were constructed using aqueous solutions of 4-hydroxydebrisoquine. These were linear over the range 0–500 pmol and, because they did not differ significantly from standard curves where 4-hydroxydebrisoquine had been extracted from blank microsomal incubations, were used for the analysis of samples. While the limit of detection of 4-hydroxydebrisoquine in a microsomal incubation was < 10 pmol, the limit of quantification was set at 50 pmol, which was a lower level of 4-hydroxydebrisoquine than was found in any incubation. The accuracy and precision at the limit of quantification were 56.7 ± 4.8 pmol (mean $\pm$ SD, $n = 6$, CV 8.5%). Enzyme activity was expressed as pmol 4-hydroxydebrisoquine produced min $^{-1}$ mg $^{-1}$ microsomal protein.

Terfenadine hydroxylase activity

Terfenadine hydroxylase activity was assessed by measuring loss of the substrate terfenadine rather than the generation of a metabolite. Incubation conditions were similar to those described by Yun *et al.* (1993), while an assay based on a liquid chromatography/mass spectrometry method for the measurement of the compound in plasma (Adams *et al.* 1997) was used.

Enzyme incubations. Incubation mixtures consisting of human hepatic microsomal fraction (pool of six individual fractions, 5 mg ml $^{-1}$, 75 μ l), 1.2 mM NADPH (10 mg ml $^{-1}$, 50 μ l) and 0.1 M sodium phosphate buffer (pH 7.4, 360 μ l) were warmed to 37°C in a shaking waterbath. These were then pre-incubated for 5 min with DMSO (5 μ l) containing 8-phenyltheophylline at various concentrations. The reaction was started by the addition of terfenadine dissolved in methanol (100 μ M, 10 μ l) to a final concentration of 2 μ M (the final incubation volume was 500 μ l) and the samples were then incubated for a further 60 min at 37°C. The reaction was stopped by the addition of ice-cold methanol (500 μ l) and the samples were placed on ice.

Sample preparation. Samples were centrifuged (12 000g, 10 min) to remove precipitated protein and aliquots (50 μ l) of the clear supernatants transferred to glass vials. [^{3} H $_3$]-terfenadine in methanol (400 ng ml $^{-1}$, 50 μ l, 42 pmol) was added to each vial and solvent then removed by evaporation under nitrogen. The residues were reconstituted in acetonitrile/methanol/100 mM ammonium acetate buffer, pH 4 (20/20/60 v/v/v, 15 μ l) and aliquots (10 μ l) were injected into the liquid chromatograph/mass spectrometer.

Liquid chromatography/mass spectrometry. The HPLC system used consisted of a Waters 616 liquid chromatography pump and 600S flow controller (Waters, Watford, UK), together with a Rheodyne model 8125 injector (Rheodyne, Cotati, CA, USA) fitted with a 20- μ l sample loop. Chromatography was carried out on a Spherisorb S5CN column of dimensions 25 cm $\times$ 1 mm i.d. (Hichrom, Theale, UK), connected to a Phenomenex (Macclesfield, UK) cyano guard cartridge (3 cm $\times$ 1 mm i.d.). A mobile phase of acetonitrile/methanol/100 mM ammonium acetate buffer, pH 4 (30/30/40 v/v/v) was used and, under isocratic conditions and at a flow rate of 50 μ l min⁻¹, the retention times of terfenadine and [²H₃]-terfenadine were 7 min. The HPLC column was connected directly to the electrospray interface of a VG Quattro II triple quadrupole mass spectrometer (VG Biotech, Altrincham, UK). This instrument, supplied with nitrogen as both drying and nebulizing gas, was operated in the positive-ion electrospray mode with a source temperature of 70°C, a cone voltage of 35 V and a capillary voltage of 3.5 kV. The mass spectrometer was used with Q1 acting as mass filter and was set to monitor positive ions of m/z 472 and 475, the protonated molecular ions of terfenadine and [²H₃]-terfenadine respectively. Data acquisition and processing were performed with Masslynx software.

Assay validation. Standard curves were constructed using methanolic solutions of terfenadine. These were linear over the range 0–50 pmol and, because they did not differ significantly from standard curves where the compound had been added to blank microsomal incubations, were used for the analysis of samples. While the limit of detection of terfenadine was < 1 pmol, the limit of quantification was set at 5 pmol, which was a lower level of terfenadine than was found in a 50- μ l aliquot of any sample. The accuracy and precision of the assay at the limit of quantification were 5.07 ± 0.07 pmol (mean $\pm$ SD, $n=6$, CV 1.4%). Enzyme activity was expressed as pmol terfenadine consumed min⁻¹ mg⁻¹ microsomal protein.

Chlorzoxazone 6-hydroxylase activity

An assay for chlorzoxazone 6-hydroxylase activity, based on a method described by Peter *et al.* (1990), was used.

Enzyme incubations. Incubation mixtures consisting of human hepatic microsomal fraction (pool of six individual fractions, 5 mg ml⁻¹, 100 μ l), 1.2 mM NADPH (20 mg ml⁻¹, 50 μ l) and 0.1 M sodium phosphate buffer (pH 7.4, 839 μ l) were warmed to 37°C in a shaking waterbath. These were then pre-incubated for 5 min with DMSO (1 μ l) containing 8-phenyltheophylline at various concentrations. The reaction was started by the addition of chlorzoxazone dissolved in 60 mM sodium hydroxide (5 mM, 10 μ l) to a final concentration of 50 μ M (the final incubation volume was 1 ml) and the samples were then incubated for a further 20 min at 37°C. The reaction was stopped by the addition of 43% phosphoric acid (50 μ l) and the samples were placed on ice.

Extraction and HPLC analysis. Samples were applied to Bond Elut C18 SPE columns (200 mg sorbent mass, 3 ml reservoir volume) that had been preconditioned with methanol (2 $\times$ 2 ml) and water (2 $\times$ 2 ml). The columns were washed with water (2 $\times$ 2 ml), after which 6-hydroxy-chlorzoxazone was eluted with methanol (2 ml). The methanol eluates were evaporated to dryness under nitrogen and the residues reconstituted in 0.5% phosphoric acid/acetonitrile (70/30 v/v, 100 μ l). Aliquots (20 μ l) were injected onto a Spherisorb 5 μ m ODS2 HPLC column of dimensions 25 cm $\times$ 4.6 mm i.d. (Jones Chromatography, Hengoed, UK). A mobile phase of 0.5% phosphoric acid/acetonitrile (70/30 v/v) was used and, under isocratic conditions and at a flow rate of 1.5 ml min⁻¹, the retention time of 6-hydroxychlorzoxazone was 3.3 min. The UV detector was set at 287 nm and a Hewlett Packard 1100 Chromatography Workstation was used for data acquisition and processing.

Assay validation. 6-Hydroxychlorzoxazone was isolated from microsomal incubations not with a solvent extraction procedure but with the use of C18 SPE columns. These gave a recovery of > 98% and the metabolite was quantified by reference to the absolute response of standard solutions of 6-hydroxychlorzoxazone analysed by HPLC. Extracted standard curves covering the range 0–3.5 nmol were linear and, while the limit of detection of 6-hydroxychlorzoxazone was 50 pmol, the limit of quantification was set at 250 pmol. The accuracy and precision of the assay at the limit of quantification were 253 ± 4.3 pmol (mean $\pm$ SD, $n=6$, CV 1.7%). Enzyme activity was expressed as pmol 6-hydroxychlorzoxazone produced min⁻¹ mg⁻¹ microsomal protein.

Tolbutamide 4-hydroxylase activity

The assay for tolbutamide 4-hydroxylase activity was based on that of Miners *et al.* (1988).

Enzyme incubations. Incubation mixtures consisting of human hepatic microsomal fraction (pool of six individual fractions, 5 mg ml^{-1} , $100 \mu\text{l}$), 1.2 mM NADPH (20 mg ml^{-1} , $50 \mu\text{l}$) and 0.1 M sodium phosphate buffer (pH 7.4, $830 \mu\text{l}$) were warmed to 37°C in a shaking waterbath. These were then pre-incubated for 5 min with DMSO ($10 \mu\text{l}$) containing 8-phenyltheophylline at various concentrations. The reaction was started by the addition of tolbutamide dissolved in methanol (12.5 mM , $10 \mu\text{l}$) to a final concentration of $125 \mu\text{M}$ (the final incubation volume was 1 ml) and the samples were then incubated for a further 60 min at 37°C . The reaction was stopped by the addition of 43% phosphoric acid ($50 \mu\text{l}$) and the samples were placed on ice.

Extraction and HPLC analysis. The extraction procedure and HPLC conditions were as described above for the analysis of chlorzoxazone 6-hydroxylase activity except that a mobile phase of 0.5% phosphoric acid/acetonitrile (60/40 v/v) was used. Under isocratic conditions and at a flow rate of 1.7 ml min^{-1} , the retention time of 4-hydroxytolbutamide was 2.7 min. The UV detector was set at 227 nm and a Hewlett Packard 1100 Chromatography Workstation was used for data acquisition and processing.

Assay validation. The use of C18 SPE columns gave a recovery of 4-hydroxytolbutamide from microsomal incubations of $> 98\%$ and the metabolite was quantified by reference to the absolute response of standard solutions of 4-hydroxytolbutamide analysed by HPLC. Extracted standard curves covering the range 0–1.2 nmol were linear and, while the limit of detection of 4-hydroxytolbutamide was 50 pmol , the limit of quantification was set at 200 pmol . The accuracy and precision of the assay at the limit of quantification were $197 \pm 5.1 \text{ pmol}$ (mean $\pm$ SD, $n = 6$, CV 2.6%). Enzyme activity was expressed as $\text{pmol 4-hydroxytolbutamide produced min}^{-1} \text{ mg}^{-1}$ microsomal protein.

Results

Assay for POD activity

The reagents and conditions used previously for microsomal incubation (Murray and Boobis 1986, Sesardic *et al.* 1990b) were examined in order to optimize POD activity. Examination of the effect of phosphate buffer pH on enzyme activity indicated that maximum POD activity occurred at pH 7.8 (figure 2a). However activity at pH 7.4 was 85% of the maximum and this pH was chosen as it represented the physiological value. Addition of Tris-HCl buffer, pH 7.4, to the reaction mixture was found to exert an inhibitory effect on enzyme activity that increased linearly with Tris concentration (figure 2b). This could be due to inhibition by Tris itself or a consequence of increasing ionic strength; the exact cause was not investigated further. Consequently phosphate buffer, pH 7.4, alone, which was already present from the preparation of the hepatic microsomal fraction (Boobis *et al.* 1990), was used as the incubation buffer. Magnesium chloride, which was previously incorporated as a cofactor when using an NADPH-regenerating system, was found to have little effect on enzyme activity (figure 2c) and was therefore omitted. The addition of BSA was found to enhance enzyme activity (figure 2d). A minimum of $250 \mu\text{g ml}^{-1}$ BSA was required for maximum stimulation and so an amount of BSA twice that was subsequently used. The effects of methanol and DMSO on enzyme activity were assessed, as an organic solvent was required to dissolve the methylxanthines added to microsomal incubations for inhibition studies. Methanol ($10 \mu\text{l}$) inhibited POD activity by $9.3 \pm 2.3\%$ (mean $\pm$ SD, $n = 6$) whereas the same volume of DMSO inhibited enzyme activity by $16.0 \pm 1.8\%$ (mean $\pm$ SD, $n = 6$). Although methanol caused less inhibition, preliminary experiments indicated that the solubility of some methylxanthines in methanol was limited. Consequently DMSO was used for these studies. Using the modified incubation conditions described above and a final concentration of phenacetin of $4 \mu\text{M}$, POD activity was linear for an incubation of 20 min at 37°C

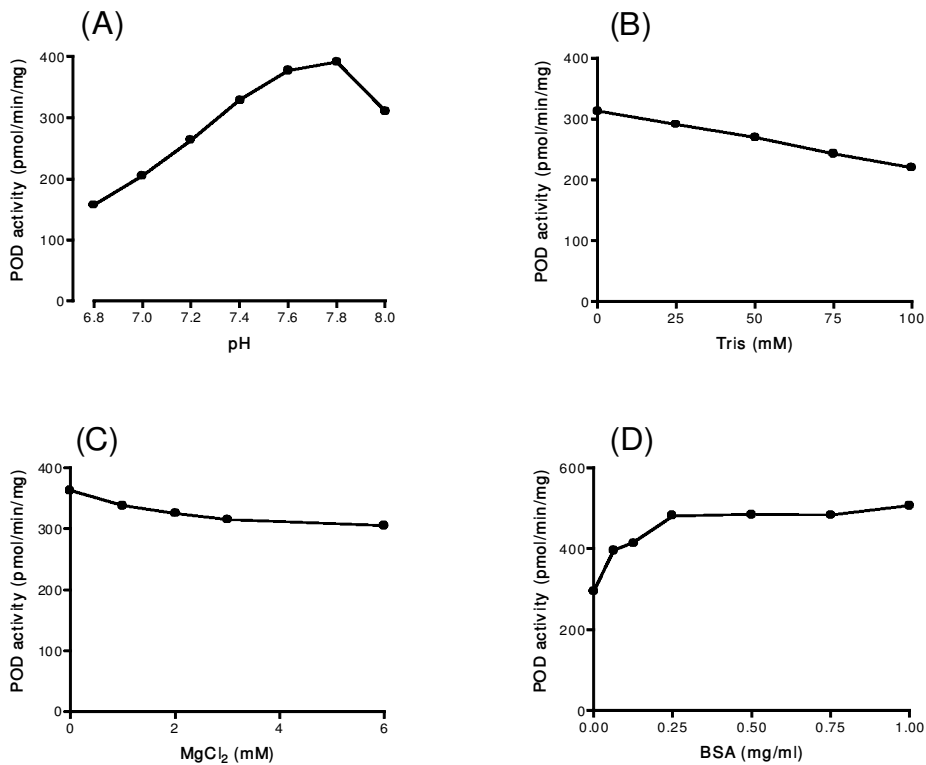


Figure 2. Effect of various conditions on human liver microsomal high-affinity phenacetin *O*-deethylase (POD) activity. Each of the reaction mixtures (1 ml) contained 0.1 M sodium phosphate buffer, pH 7.4 (except for experiment A where the pH was altered), 0.2 mg human liver microsomal protein, 1.2 mM NADPH and 4 μ M phenacetin, which were incubated at 37°C for 20 min. The product, paracetamol, was extracted from the reaction mixture, quantified and enzyme activity calculated as described in the Materials and methods. The conditions examined were (A) variation in the pH of the 0.1 M phosphate buffer, (B) addition of Tris buffer, pH 7.4, (C) addition of magnesium chloride and (D) addition of BSA. The data shown are from single experiments representative of two experiments with similar results, with each datum point being the mean of duplicate determinations.

(figure 3). The reaction was also reasonably linear with respect to the amount of microsomal protein used in the incubation (figure 4a). However, it was noted that levels < 100 μ g consistently yielded a lower than expected activity. Although the deviation from the regression line was not great, calculation of specific activity from such measurements produced disproportionately low values (figure 4b). The effect was most pronounced in the absence of BSA. However, supplementation of the reaction mixture with rat kidney microsomal protein, which had no detectable POD activity, largely restored activity to the expected level (figure 4b).

Inhibition of POD activity

The effects of 16 methylxanthines on high-affinity POD activity were assessed using the assay described above. For each compound, inhibition of POD activity

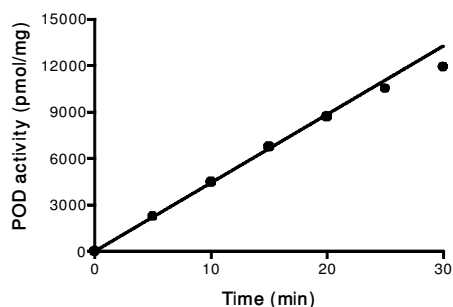


Figure 3. Relationship between human liver microsomal high-affinity phenacetin *O*-deethylase (POD) activity and reaction time. Each of the reaction mixtures (1 ml) contained 0.1 M sodium phosphate buffer, pH 7.4, 0.2 mg human liver microsomal protein, 1.2 mM NADPH, 0.5 mg BSA and 4 μ M phenacetin, and were incubated for between 0 and 30 min at 37°C. The product, paracetamol, was extracted from the reaction mixture and quantified as described in the Materials and methods. Enzyme activity is expressed as pmol product mg^{-1} microsomal protein. A regression line was fitted to the points representing incubations times between 0 and 20 min: $y = 442x$, $r^2 = 1.00$. The data shown are from a single experiment representative of two experiments with similar results, with each datum point being the mean of duplicate determinations.

was measured in duplicate at six different methylxanthine concentrations and IC_{50} (table 1) were obtained by linear regression analysis of POD activity versus log methylxanthine concentration. Nine of the methylxanthines were very poor inhibitors with $\text{IC}_{50} > 400 \mu\text{M}$ whereas isobutylmethylxanthine, pentoxifylline, 8-methylxanthine, theophylline, caffeine and 1-methylxanthine caused more potent inhibition with IC_{50} in the range 36–260 μM . Amongst the series of methylxanthines studied, 8-phenyltheophylline was the most potent inhibitor of high-affinity POD activity with an $\text{IC}_{50} = 0.7 \mu\text{M}$.

In order to elucidate the mechanism of inhibition of POD activity by 8-phenyltheophylline, the kinetics of inhibition by this compound were explored. POD activity was measured over a range of phenacetin concentrations (0–1000 μM) with no inhibitor and with 1, 3 and 10 μM concentrations of 8-phenyltheophylline present (figure 5). The calculated K_m and $V_{\max}$ for the high-affinity component of the uninhibited reaction and for each concentration of methylxanthine are shown in table 2. $V_{\max}$ for the four curves were very similar, indicating that 8-phenyltheophylline was acting as a competitive inhibitor with a $K_i = 0.11 \mu\text{M}$. This experiment was repeated twice more on separate occasions and the same results were obtained.

Inhibition of other enzyme activities

There was no effect of 8-phenyltheophylline on debrisoquine 4-hydroxylase, chlorzoxazone 6-hydroxylase, tolbutamide 4-hydroxylase or terfenadine hydroxylase activities at concentrations of up to 10 μM (table 3). However the vehicle, DMSO, had an inhibitory effect on all of the enzyme activities except for terfenadine hydroxylase, and this effect was greatest for debrisoquine 4-hydroxylase and chlorzoxazone 6-hydroxylase (table 3).

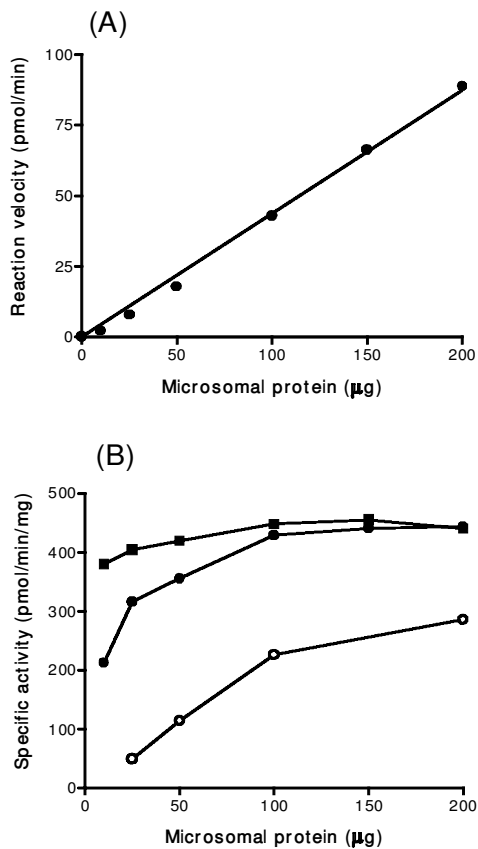


Figure 4. Relationship between human liver microsomal high-affinity phenacetin *O*-deethylase (POD) activity and microsomal protein. (A) Each of the reaction mixtures (1 ml) contained 0.1 M sodium phosphate buffer, pH 7.4, 0–0.2 mg human liver microsomal protein, 1.2 mM NADPH, 0.5 mg BSA and 4 μM phenacetin, and were incubated for 10 or 30 min (where microsomal protein was < 0.1 mg) at 37°C. The product, paracetamol, was extracted from the reaction mixture and quantified as described in the Materials and methods. Enzyme activity is expressed as pmol product min⁻¹. A regression line was fitted to the points: $y = 0.437x$, $r^2 = 0.998$. (B) Representation of the same data expressed as specific activities (filled circles) compared with incubations performed in the absence of BSA (open circles) and with incubations where the total amount of microsomal protein was maintained at 0.2 mg by supplementation with rat kidney microsomal protein (filled squares). The data shown are from single experiments representative of two experiments with similar results, with each datum point being the mean of duplicate determinations.

Discussion

With human P450 enzymes, it is possible to evaluate the inhibitory nature of xenobiotic compounds on enzyme activity by carrying out *in vitro* incubations, using selective substrates for individual enzymes in human liver microsomal fractions. One enzyme activity that is associated specifically with human hepatic CYP1A2 is high-affinity POD activity. Whereas there are reports indicating that POD activity in human liver microsomal fraction conforms to monophasic kinetics (e.g. Rodrigues *et al.* 1997), several groups have identified two components of activity (Boobis *et al.* 1981, Gillam and Reilly 1988) and it has been known for a decade that CYP1A2 is the major contributor to the high-affinity enzyme (Sesardic

Table 1. Inhibition of high-affinity phenacetin *O*-deethylase (POD) activity of methylxanthines.

Methylxanthine	Trivial name	IC ₅₀ (μM)
1-Methylxanthine		260
3-Methylxanthine		> 400
7-Methylxanthine		> 400
8-Methylxanthine		100
1,3-Dimethylxanthine	theophylline	120
3,7-Dimethylxanthine	theobromine	> 400
1,7-Dimethylxanthine	paraxanthine	> 400
1,3,7-Trimethylxanthine	caffeine	140
1,3,9-Trimethylxanthine	isocaffeine	> 400
1,3-Dimethyl-7-(2-hydroxyethyl)xanthine	etophylline	> 400
1,3-Dimethyl-7-(2-hydroxypropyl)xanthine	proxiphylline	> 400
1,3-Dimethyl-7-(2,3-dihydroxypropyl)xanthine	dyphylline	> 400
1,3-Dimethyl-8-chloroxanthine	8-chlorotheophylline	> 400
1,3-Dimethyl-8-phenylxanthine	8-phenyltheophylline	0.7
3,7-Dimethyl-1-(5-oxohexyl)xanthine	pentoxifylline	62
3-Isobutyl-1-methylxanthine	isobutylmethylxanthine	36
1,8-Dimethyl-3-(2'-furfuryl)xanthine	furaфylline	< 0.1 ^a

Each of the reaction mixtures (1 ml) contained 0.1 M sodium phosphate buffer, pH 7.4, 0.2 mg human liver microsomal protein, 1.2 mM NADPH, and 0.5 mg BSA and was pre-incubated for 5 min at 37°C with DMSO (10 μl) or DMSO containing xanthines at various concentrations (10 μl). Each reaction was started by the addition of phenacetin dissolved in methanol (400 μM, 10 μl) to a final concentration of 4 μM and the samples were then incubated for a further 10 min at 37°C. Each reaction was stopped by the addition of 43% phosphoric acid and the product, paracetamol, was extracted from the reaction mixture and quantified as described in the Materials and methods. The potency of each xanthine to inhibit POD activity is described as the inhibitor concentration that reduces enzyme activity to half of the maximal activity (IC₅₀) determined in the absence of inhibitor. This value was estimated by extrapolation from duplicate measurements at four or more concentrations of inhibitor that spanned the IC₅₀.

^a IC₅₀ determined by Sesardic *et al.* (1990a).

et al. 1988, 1990a). Recently it has been confirmed using heterologously expressed human P450 enzymes that at low substrate concentrations (< 50 μM), CYP1A2 is the only high-affinity POD in human liver microsomal fraction (Venkatakrishnan *et al.* 1998).

In the current study we have modified the extraction and derivatization procedures of our previously described gas chromatographic/mass spectrometric assay for paracetamol (Murray and Boobis 1986, Sesardic *et al.* 1990b), the primary product of POD activity, in order to facilitate the analysis of larger numbers of samples. Incubation conditions were also optimized and, of the factors affecting enzyme activity, the increase caused by the addition of BSA is of particular interest as previous workers have shown that aryl hydrocarbon hydroxylase activity can also be stimulated by the addition of this protein (Alvares *et al.* 1970, Nebert and Gielen 1972). It appears likely that at low microsomal protein concentrations, access of phenacetin to the active site of the enzyme is limited due to unfavourable partitioning of the substrate into lipid in the microsomal mixture. At higher microsomal protein concentrations, or when BSA is added, changes in distribution of phenacetin within the various compartments of the microsomal mixture result in more substrate being available to the enzyme. This stimulation of activity supports models of P450 in which the substrate access channel is exposed in the cytosol (Edwards *et al.* 1991). The latter is a highly proteinaceous environment, comprising numerous proteins including albumin, and therefore increasing the protein concentration in the incubation mixtures by addition of BSA gives

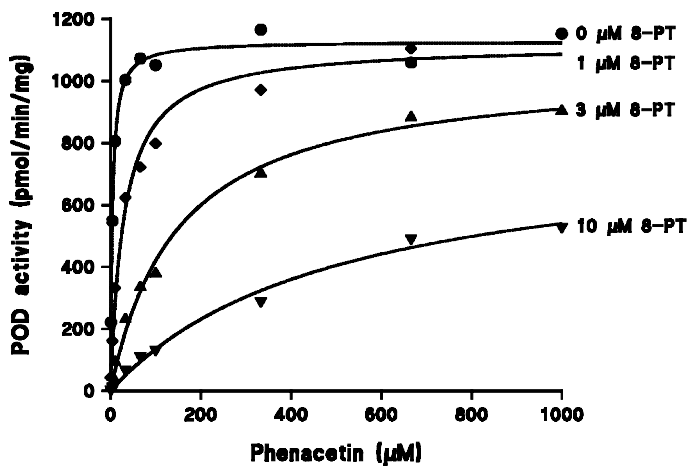


Figure 5. Inhibitory effect of 8-phenyltheophylline (8-PT) on human liver microsomal high-affinity phenacetin *O*-deethylase (POD) activity. Reaction mixtures (1 ml) contained 0.1 M sodium phosphate buffer, pH 7.4, 0.2 mg human liver microsomal protein, 1.2 mM NADPH, 0.5 mg BSA and DMSO (10 μ l) or 8-phenyltheophylline (dissolved in 10 μ l DMSO; final concentration 1-10 μ M). These were pre-incubated for 5 min at 37°C prior to addition of phenacetin (1-1000 μ M) and incubation for 10 min at 37°C. The product, paracetamol, was extracted from the reaction mixture and quantified as described in the Materials and methods. The contribution of the high-affinity component of POD activity to the reactions was calculated as described in the Materials and methods. The data shown are from a single experiment representative of three experiments with similar results, with each datum point being the mean of duplicate determinations. The kinetic parameters deduced from this experiment are summarized in table 2.

Table 2. Inhibition of high-affinity phenacetin *O*-deethylase (POD) activity by 8-phenyltheophylline.

8-Phenyltheophylline (μ M)	K_m or K'_m (μ M)	V_{max} or V'_{max} (pmol min ⁻¹ mg ⁻¹)
0	4.1	1128
1	30.0	1119
3	149	1045
10	503	811

Kinetic parameters describing the inhibition of high-affinity POD activity by 8-phenyltheophylline. K'_m and V'_{max} are apparent values for K_m and V_{max} determined in the presence of each concentration of inhibitor. The data are calculated from the experiment described in figure 5 and are derived from eight determinations, performed in duplicate, at each concentration of 8-phenyltheophylline.

probably a truer representation of the environment of the enzyme *in vivo*. It was not anticipated that binding of methylxanthines to BSA would have a significant effect on IC₅₀, since protein binding of xanthine-based drugs is generally low. This was confirmed by measuring IC₅₀ both in the presence and in the absence of BSA for the seven methylxanthines that inhibited POD activity and no significant increase in IC₅₀ for any these compounds in the presence of BSA was observed (data not shown). Incubations were performed at a substrate concentration of 4 μ M phenacetin, a value comparable with the reported K_m for this enzyme activity of 6.3 μ M (Boobis *et al.* 1981). Measuring enzyme activity at a substrate concentration close to or less than the K_m of that enzyme ensures that the degree of inhibition caused by either a competitive or non-competitive inhibitor will be independent of

Table 3. Inhibition of P450-catalyzed enzyme activities by 8-phenyltheophylline.

Enzyme activity	Substrate concentration (μM)	DMSO concentration in incubation (%)	Inhibition of activity by DMSO (%)	Metabolite formation with DMSO present (pmol min ⁻¹ mg ⁻¹)	Inhibition of metabolite formation by 8-phenyltheophylline (%)			
					0.3 μM	1 μM	3 μM	10 μM
Phenacetin <i>O</i> -deethylase	4	1	16	375	10.1	69.7	92.2	97.7
Debrisoquine 4-hydroxylase	120	0.1	46	26.5	< 5	< 5	< 5	< 5
Chlorzoxazone 6-hydroxylase	50	0.1	51	553	< 10	< 10	< 10	< 10
Tolbutamide 4-hydroxylase	125	1	45	73.4	< 10	< 10	< 10	< 10
Fall in substrate concentration with DMSO present (%)					Inhibition of substrate metabolism by 8-phenyltheophylline (%)			
					0.3 μM	1 μM	3 μM	10 μM
Terfenadine hydroxylase	2	1	< 5	85	< 5	< 5	< 5	< 5

The inhibitory potency of 8-phenyltheophylline on high-affinity phenacetin *O*-deethylase (POD) and other P450-catalysed activities was determined as described in the Materials and methods using substrate concentrations at or about the *K_m* for each enzyme activity. Data are derived from duplicate determinations in all cases. DMSO was used as a vehicle to dissolve 8-phenyltheophylline and the inhibitory effect of this solvent on each activity is indicated.

substrate concentration (Boobis 1995). Incubations also included a pre-incubation step so that mechanism-based inhibition would be detected.

The inhibitory potential of a series of 16 methylxanthines on enzyme activity was examined and only four had an $IC_{50} \leq 100 \mu M$ (table 1). By far the most potent inhibitor was 8-phenyltheophylline, which is used experimentally *in vitro* as a non-selective adenosine receptor antagonist (Peterson 1996). This compound had an $IC_{50} = 0.7 \mu M$ and was only slightly less potent an inhibitor of CYP1A2 than furafylline, which has an $IC_{50} < 0.1 \mu M$ (Sesardic *et al.* 1990a). However, while furafylline has been shown to be a mechanism-based, non-competitive inhibitor of high-affinity POD activity (Sesardic *et al.* 1990a, Kunze and Trager 1993), analysis of the kinetics of inhibition by 8-phenyltheophylline (table 2 and figure 5) showed that this compound was acting as a competitive inhibitor with a $K_i = 0.11 \mu M$.

The specificity of inhibition of CYP1A2 by 8-phenyltheophylline was assessed by examining the effect of this compound on other enzyme activities associated with specific P450s. Debrisoquine 4-hydroxylase, terfenadine hydroxylase, chlorzoxazone 6-hydroxylase and tolbutamide 4-hydroxylase activities in man are associated with CYP2D6 (Kimura *et al.* 1989), CYP3A4 (Yun *et al.* 1993), CYP2E1 (Peter *et al.* 1990) and CYP2C9 (Veronese *et al.* 1993) respectively. Existing assays for debrisoquine 4-hydroxylase, chlorzoxazone 6-hydroxylase and tolbutamide 4-hydroxylase activities *in vitro* were used with modifications. Terfenadine is metabolized to two major products in man, one by oxidative *N*-dealkylation and the other by *C*-oxidation. Both reactions are hydroxylation reactions catalysed primarily by CYP3A4 (Yun *et al.* 1993). Terfenadine hydroxylase activity was therefore assessed by determining the rate of disappearance of parent compound and a novel assay based on liquid chromatography/mass spectrometry was developed, incorporating a deuterated analogue of terfenadine as an internal standard. Enzyme activity was measured using a terfenadine concentration close to the K_m of CYP3A4 in order to detect any inhibition of activity by 8-phenyltheophylline. Under these conditions substantial substrate depletion occurred, possibly resulting in non-linear kinetics. However, assay conditions were suitable for qualitative detection of enzyme inhibition. Initial experiments showed that DMSO had a much greater inhibitory effect on three of these enzyme activities than on POD activity, with only terfenadine hydroxylase being unaffected by the presence of this solvent (table 3). The effects of organic solvents on various P450 activities in human liver microsomal fraction have been reported previously (Chauret *et al.* 1998) and our findings for POD, chlorzoxazone 6-hydroxylase and tolbutamide 4-hydroxylase activities are very similar to those reported by these workers. However, whereas our experiments described here demonstrated that debrisoquine 4-hydroxylase activity was extensively inhibited by DMSO (46% reduction in activity at 0.1% concentration), dextromethorphan *O*-demethylase activity (another measure of CYP2D6) is reported to be much less reduced (17% at 1% concentration) (Chauret *et al.* 1998). In addition, the latter group reported that testosterone 6 β -hydroxylase was extensively inhibited by DMSO (47% reduction in activity at 1% concentration) whereas our measurements of terfenadine hydroxylase, also a measure of CYP3A4 activity, were unaffected (< 5% inhibition at 1% concentration). This variability in the effect of an organic solvent does highlight a potential problem when choosing an enzyme activity to measure a specific P450, as

many potential chemical inhibitors are highly lipophilic and require water-miscible organic solvents for effective solubilization.

8-Phenyltheophylline at concentrations of up to $10\text{ }\mu\text{M}$ had no effect on debrisoquine 4-hydroxylase, tolbutamide 4-hydroxylase, chlorzoxazone 6-hydroxylase or terfenadine hydroxylase activities, indicating that at this concentration the methylxanthine does not inhibit CYP2D6, CYP2C9, CYP2E1 or CYP3A4, respectively. Thus, 8-phenyltheophylline appears to be a specific inhibitor of human hepatic CYP1A2.

The relationship between methylxanthine chemical structure and potency of inhibition of CYP1A2 was examined. Previous studies of rat CYP1A2 have indicated that 1,8-dimethyl-3-alkyl or 3-aryl trisubstituted xanthines, but not 1,3-disubstituted xanthines, are inhibitors (Segura *et al.* 1989). It has been suggested that the ability of 8-methylated xanthines to inhibit CYP1A2 activity in the rat is related to the position of that particular methyl group within the active site of the enzyme (Sanz *et al.* 1994). The relevance of these findings to human hepatic CYP1A2 is uncertain, however, as there are major species differences in the specificity of this enzyme (e.g. furafylline has been shown to be a potent non-competitive inhibitor of CYP1A2 in man whereas it is a much weaker inhibitor in the rat; Sesardic *et al.* 1990a). 8-Methylxanthine, which does contain a methyl group at C-8, was examined in this study but this compound is a monosubstituted xanthine and is only a moderate inhibitor of human hepatic CYP1A2 activity ($\text{IC}_{50}=100\text{ }\mu\text{M}$). 8-Phenyltheophylline, which is a 1,3,8-trisubstituted xanthine, was a potent inhibitor of this enzyme activity, but this compound contains a phenyl rather than a methyl group at position 8 of the xanthine nucleus. Furafylline, also a 1,3,8-trisubstituted xanthine, has been shown to be a potent inhibitor of CYP1A2 in man (Sesardic *et al.* 1990a) but similarities in structure and function with 8-phenyltheophylline appear to be limited. Furafylline contains a furfuryl group at N-3 and a methyl group at C-8 while 8-phenyltheophylline contains a methyl group at N-3 and a phenyl group at C-8. The mechanisms of inhibition also differ in that furafylline is a mechanism-based, non-competitive inhibitor and experiments presented here show that 8-phenyltheophylline is a competitive inhibitor of human hepatic CYP1A2 activity. The only other 1,3,8-trisubstituted xanthine examined in this study was 8-chlorotheophylline and, interestingly, this compound is not an inhibitor of CYP1A2 activity ($\text{IC}_{50}>400\text{ }\mu\text{M}$).

Of the seven methylxanthines with $\text{IC}_{50}<400\text{ }\mu\text{M}$, only pentoxifylline, theophylline and caffeine are ingested either as therapeutic agents or dietary components. Pentoxifylline is used as a vasodilator and a peak plasma concentration of the parent compound of up to $0.5\text{ }\mu\text{M}$ is reached after a 400 mg oral dose of the slow-release preparation (Dollery 1991). With an $\text{IC}_{50}=62\text{ }\mu\text{M}$, therapeutic doses of the drug should therefore have little effect on CYP1A2 activity *in vivo*, although there has been a report of pentoxifylline causing elevation of theophylline serum levels during combination therapy (Ellison *et al.* 1990). Theophylline is used as a bronchodilator in the treatment of asthma and chronic obstructive pulmonary disease and caffeine as a central nervous stimulant. In therapeutic use, plasma concentrations of theophylline are normally kept to a maximum of $100\text{ }\mu\text{M}$ as above this value a rapidly increasing incidence of neurotoxicity is observed (Dollery 1991). Nevertheless, with an $\text{IC}_{50}=120\text{ }\mu\text{M}$, a plasma concentration of $100\text{ }\mu\text{M}$ may cause inhibition of CYP1A2 activity *in vivo* and this could affect the bioavailability or elimination of other compounds metabolized by this enzyme. Caffeine has been

used to treat apnoea of prematurity and plasma concentrations of 40-100 μM have been reported to be therapeutically optimal (Dollery 1991). With an $\text{IC}_{50} = 140 \mu\text{M}$, plasma concentrations of caffeine at the top end of the therapeutic range may also have an inhibitory effect on CYP1A2 activity *in vivo*. However, lower plasma concentrations of caffeine and theophylline resulting from dietary ingestion are unlikely to have any effect on CYP1A2 activity.

In conclusion, we have demonstrated that 8-phenyltheophylline is a potent and specific inhibitor of CYP1A2 in man. This compound acts as a competitive inhibitor and may well be useful for *in vitro* studies to determine the contribution of CYP1A2 to P450-mediated reactions.

References

- ADAMS, D. A., MURRAY, S., CLIFFORD, C. P., RENDELL, N. B., DAVIES, D. S. and TAYLOR, G. W., 1997, Analysis of terfenadine in human plasma using microbore high-performance liquid chromatography-electrospray ionisation mass spectrometry. *Journal of Chromatography B*, **693**, 345-351.
- ALVARES, A. P., SCHILLING, G., GARBUS, A. and KUNTZMAN, R., 1970, Studies on the hydroxylation of 3,4-benzpyrene by hepatic microsomes: effect of albumin on the rate of hydroxylation of 3,4-benzpyrene. *Biochemical Pharmacology*, **19**, 1449-1455.
- BALL, S. E., FORRESTER, L. M., WOLF, C. R. and BACK, D. J., 1990, Differences in the cytochrome P450 isoenzymes involved in the 2-hydroxylation of oestradiol and 17 α -ethynylestradiol. Relative activities of rat and human liver enzymes. *Biochemical Journal*, **267**, 221-226.
- BOOBIS, A. R., 1995, Prediction of inhibitory drug-drug interactions by studies *in vitro*. In G. M. Pacifici and G. N. Fracchia (eds), *Advances in Drug Metabolism in Man* (Luxembourg: Office for Official Publications of the European Communities), pp. 513-539.
- BOOBIS, A. R., KAHN, G. C., WHYTE, C., BRODIE, M. J. and DAVIES, D. S., 1981, Biphasic *O*-deethylation of phenacetin and 7-ethoxycoumarin by human and rat liver microsomal fractions. *Biochemical Pharmacology*, **30**, 2451-2456.
- BOOBIS, A. R., LYNCH, A. M., MURRAY, S., DE LA TORRE, R., SOLANS, A., FARRE, M., SEGURA, J., GOODERHAM, N. J. and DAVIES, D. S., 1994, CYP1A2-catalyzed conversion of dietary heterocyclic amines to their proximate carcinogens is their major route of metabolism in humans. *Cancer Research*, **54**, 89-94.
- BOOBIS, A. R., MURRAY, S., KAHN, G. C., ROBERTZ, G.-M. and DAVIES, D. S., 1983, Substrate specificity of the form of cytochrome P-450 catalysing the 4-hydroxylation of debrisoquine in man. *Molecular Pharmacology*, **23**, 474-481.
- BOOBIS, A. R., SESARDIC, D. and GOODERHAM, N. J., 1990, Methods in drug metabolism. In C. Hansch, P. G. Sammes and J. B. Taylor (eds), *Comprehensive Medicinal Chemistry*, vol. 5: *Biopharmaceutics* (Oxford: Pergamon), pp. 443-478.
- BUTLER, M. A., IWASAKI, M., GUENGERICH, F. P. and KADLUBAR, F. F., 1989, Human cytochrome P-450_{PA} (P-450IA2), the phenacetin *O*-deethylase, is primarily responsible for the hepatic 3-demethylation of caffeine and *N*-oxidation of carcinogenic arylamines. *Proceedings of the National Academy of Sciences, USA*, **86**, 7696-7700.
- CHAURET, N., GAUTHIER, A. and NICOLL-GRIFFITH, D. A., 1998, Effect of common organic solvents on *in vitro* cytochrome P450 mediated metabolic activities in human liver microsomes. *Drug Metabolism and Disposition*, **26**, 1-4.
- CONNAY, A. H., PANTUCK, E. J., HSIAO, K.-C., GARLAND, W. A., ANDERSON, K. E., ALVARES, A. P. and KAPPA, A., 1976, Enhanced phenacetin metabolism in human subjects fed charcoal-broiled beef. *Clinical Pharmacology and Therapeutics*, **20**, 633-642.
- DIAZ, D., FABRE, I., DAUJAT, M., SAINT-AUBERT, B., BORIES, P., MICHEL, H. and MAUREL, P., 1990, Omeprazole is an aryl hydrocarbon like inducer of human hepatic cytochrome P450. *Gastroenterology*, **99**, 737-747.
- DOLLERY, C., 1991, Caffeine, oxpentifylline and theophylline. In *Therapeutic Drugs* (Edinburgh: Churchill Livingstone), pp. C3-C6, O50-O53, T32-T41 respectively.
- EDWARDS, R. J., MURRAY, B. P., SINGLETON, A. M. and BOOBIS, A. R., 1991, Orientation of cytochromes P450 in the endoplasmic reticulum. *Biochemistry*, **30**, 71-76.
- ELLISON, M. J., HORNER, R. D., WILLIS, S. E. and CUMMINGS, D. M., 1990, Influence of pentoxifylline on steady-state theophylline serum concentrations from sustained-release formulations. *Pharmacotherapy*, **10**, 383-386.

- FUHR, U., DOEHMER, J., BATTULA, N., WOLFEL, C., KUDLA, C., KEITA, Y. and STAIB, A. H., 1992, Biotransformation of caffeine and theophylline in mammalian cell lines genetically engineered for expression of single cytochrome P450 isoforms. *Biochemical Pharmacology*, **43**, 225-235.
- GILLAM, E. M. and REILLY, P. E., 1988, Phenacetin *O*-deethylation by human liver microsomes: kinetics and propranolol inhibition. *Xenobiotica*, **18**, 95-104.
- KIMURA, S., UMEMO, M., SKODA, R. C., MEYER, U. A. and GONZALEZ, F. J., 1989, The human debrisoquine 4-hydroxylase (CYP2D) locus: sequence and identification of the polymorphic CYP2D6 gene, a related gene, and a pseudogene. *American Journal of Human Genetics*, **45**, 889-904.
- KOBAYASHI, S., MURRAY, S., WATSON, D., SESARDIC, D., DAVIES, D. S. and BOOBIS, A. R., 1989, The specificity of inhibition of debrisoquine 4-hydroxylase activity by quinidine and quinine in the rat is the inverse of that in man. *Biochemical Pharmacology*, **38**, 2795-2799.
- KUNZE, K. L. and TRAGER, W. F., 1993, Isoform-selective mechanism-based inhibition of human cytochrome P4501A2 by furafylline. *Chemical Research in Toxicology*, **6**, 649-656.
- MEESE, C. O., 1986, Synthesis of 1,1,3,3,-(²H₄)-4-hydroxydebrisoquine sulphate. *Journal of Labelled Compounds and Radiopharmaceuticals*, **23**, 427-432.
- MICHNOVICZ, J. J. and BRADLOW, H. L., 1990, Induction of estradiol metabolism by dietary indole-3-carbinol in humans. *Journal of the National Cancer Institute*, **82**, 947-949.
- MINERS, J. O., SMITH, K. J., ROBSON, R. A., McMANUS, M. E., VERONESE, M. E. and BIRKETT, D. J., 1988, Tolbutamide hydroxylation by human liver microsomes. Kinetic characterisation and relationship to other cytochrome P450 dependent xenobiotic oxidations. *Biochemical Pharmacology*, **37**, 1137-1144.
- MURRAY, S. and BOOBIS, A. R., 1986, An assay for paracetamol, produced by the *O*-deethylation of phenacetin *in vitro*, using gas chromatography/electron capture negative-ion chemical ionization mass spectrometry. *Biomedical and Environmental Mass Spectrometry*, **13**, 91-93.
- MURRAY, S. and WADDELL, K. A., 1982, The electron capture negative-ion chemical ionisation mass spectra of 2-(4',6'-bistrifluoromethyl-2'-pyrimidinyl)tetrahydroisquinoline and 4-substituted analogues. *Biomedical Mass Spectrometry*, **9**, 466-472.
- NEBERT, D. W. and GIELEN, J. E., 1972, Genetic regulation of aryl hydrocarbon hydroxylase induction in the mouse. *Federation Proceedings*, **31**, 1315-1325.
- PETER, R., BOCKER, R., BEAUNE, P. H., IWASAKI, M., GUENGERICH, F. P. and YANG, C. S., 1990, Hydroxylation of chlorzoxazone as a specific probe for human liver cytochrome P-450IIE1. *Chemical Research in Toxicology*, **3**, 566-573.
- PETERSON, T. C., 1996, Inhibition of fibroproliferation by pentoxifylline. Activity of metabolite-1 and lack of role of adenosine receptors. *Biochemical Pharmacology*, **52**, 597-602.
- RAUCY, J. L., LASKER, J. M., LIEBER, C. S. and BLACK, M., 1989, Acetaminophen activation by human liver cytochrome P450IIE1 and P450IA2. *Archives of Biochemistry and Biophysics*, **271**, 270-283.
- RODRIGUES, A. D., SURBER, B. W., YAO, Y., WONG, S. L. and ROBERTS, E. M., 1997, [*O*-ethyl ¹⁴C]phenacetin *O*-deethylase activity in human liver microsomes. *Drug Metabolism and Disposition*, **25**, 1097-1100.
- SANZ, F., LOPEZ-DE-BRINAS, E., RODRIGUEZ, J. and MANAUT, F., 1994, Theoretical study on the metabolism of caffeine by cytochrome P450IA2 and its inhibition. *Quantitative Structure-Activity Relationships*, **13**, 281-284.
- SEGURA, J., ROBERTS, D. J. and TARRUS, E., 1989, The influence of structure on the accumulation of caffeine induced by methyl xanthine derivatives. *Journal of Pharmacy and Pharmacology*, **41**, 129-131.
- SESARDIC, D., BOOBIS, A. R., EDWARDS, R. J. and DAVIES, D. S., 1988, A form of cytochrome P450 in man, orthologous to form d in the rat, catalyses the *O*-deethylation of phenacetin and is inducible by cigarette smoking. *British Journal of Clinical Pharmacology*, **26**, 363-372.
- SESARDIC, D., BOOBIS, A. R., MURRAY, B. P., MURRAY, S., SEGURA, J., DE LA TORRE, R. and DAVIES, D. S., 1990a, Furafylline is a potent and selective inhibitor of cytochrome P450IA2 in man. *British Journal of Clinical Pharmacology*, **29**, 651-663.
- SESARDIC, D., EDWARDS, R. J., DAVIES, D. S., THOMAS, P. E., LEVIN, W. and BOOBIS, A. R., 1990b, High affinity phenacetin *O*-deethylase is catalysed specifically by cytochrome P450d (P450IA2) in the liver in the rat. *Biochemical Pharmacology*, **39**, 489-498.
- VENKATKRISHNAN, K., VON MOLTKE, L. L. and GREENBLATT, D. J., 1998, Human cytochromes P450 mediating phenacetin *O*-deethylation *in vitro*: validation of the high affinity component as an index of CYP1A2 activity. *Journal of Pharmaceutical Sciences*, **87**, 1502-1507.
- VERONESE, M. E., DOECKE, C. J., MACKENZIE, P. I., McMANUS, M. E., MINERS, J. O., REES, D. L., GASSER, R., MEYER, U. A. and BIRKETT, D. J., 1993, Site-directed mutation studies of human liver cytochrome P-450 isoenzymes in the CYP2C subfamily. *Biochemical Journal*, **289**, 533-538.
- YUN, C.-H., OKERHOLM, R. A. and GUENGERICH, F. P., 1993, Oxidation of the antihistamine drug terfenadine in human liver microsomes. Role of cytochrome P450 3A(4) in *N*-dealkylation and *C*-hydroxylation. *Drug Metabolism and Disposition*, **21**, 403-409.

Specificity of 17 β -oestradiol and benzo[a]pyrene oxidation by polymorphic human cytochrome P4501B1 variants substituted at residues 48, 119 and 432

T. SHIMADA^{†*}, J. WATANABE[‡], K. INOUE[†],
F. P. GUENGERICH[§] and E. M. J. GILLAM[¶]

[†] Osaka Prefectural Institute of Public Health, 3-69 Nakamichi 1-chome, Higashinari-ku, Osaka 537-0025, Japan

[‡] Saitama Cancer Center Research Institute, Kitaadachi-gun, Saitama 362-0806, Japan

[§] Department of Biochemistry and Center in Molecular Toxicology, Vanderbilt University School of Medicine, Nashville, TN 37232, USA

[¶] Department of Physiology and Pharmacology, University of Queensland, St Lucia, Queensland 4072, Australia

Received 11 December 2000

1. Eight human cytochrome P4501B1 (CYP1B1) allelic variants, namely Arg⁴⁸Ala¹¹⁹Leu⁴³², Arg⁴⁸Ala¹¹⁹Val⁴³², Gly⁴⁸Ala¹¹⁹Leu⁴³², Gly⁴⁸Ala¹¹⁹Val⁴³², Arg⁴⁸Ser¹¹⁹Leu⁴³², Arg⁴⁸Ser¹¹⁹Val⁴³², Gly⁴⁸Ser¹¹⁹Leu⁴³² and Gly⁴⁸Ser¹¹⁹Val⁴³² (all with Asn⁴⁵³), were expressed in *Escherichia coli* together with human NADPH-P450 reductase and their catalytic specificities towards oxidation of 17 β -oestradiol and benzo[a]pyrene were determined.

2. All of the CYP1B1 variants expressed in bacterial membranes showed Fe²⁺-CO versus Fe²⁺ difference spectra with wavelength maxima at 446 nm and they reacted with antibodies raised against recombinant human CYP1B1 in immunoblots. The ratio of expression of the reductase to CYP1B1 in these eight preparations ranged from 0.2 to 0.5.

3. CYP1B1 Arg⁴⁸ variants tended to have higher activities for 17 β -oestradiol 4-hydroxylation than Gly⁴⁸ variants, although there were no significant variations in 17 β -oestradiol 2-hydroxylation activity in these eight CYP1B1 variants. Interestingly, ratios of formation of 17 β -oestradiol 4-hydroxylation to 2-hydroxylation by these CYP1B1 variants were higher in all of the Val⁴³² forms than the corresponding Leu⁴³² forms.

4. In contrast, Leu⁴³² forms of CYP1B1 showed higher rates of oxidation of benzo[a]pyrene (to the 7,8-dihydroxy-7,8-dihydrodiol in the presence of epoxide hydrolase) than did the Val⁴³² forms.

5. These results suggest that polymorphic human CYP1B1 variants may cause some altered catalytic specificity with 17 β -oestradiol and benzo[a]pyrene and may influence susceptibilities of individuals towards endogenous and exogenous carcinogens.

Introduction

Cytochrome P450 (P450 or CYP) is a superfamily of monooxygenases that catalyses the oxidation of a variety of structurally diverse endobiotic and xenobiotic chemicals in mammals (Nebert *et al.* 1989, Nelson *et al.* 1996). The

* Author for correspondence; e-mail: shimada@iph.pref.osaka.jp or gillam@plpk.ug.edu.au

functional roles of P450s include the metabolism and regulation of endobiotic chemicals and on metabolism and detoxication of xenobiotic chemicals such as drugs, environmental chemicals and carcinogens (Gonzalez 1989, Nelson *et al.* 1993). In a few instances P450 enzymes participate in the activation of chemicals to reactive intermediates that cause tissue necrosis and cell transformation by interacting with and irreversibly binding to intracellular macromolecules such as DNA, RNA and proteins (Pelkonen and Nebert 1982, Guengerich and Shimada 1991, 1998). Among multiple forms of P450 enzymes identified to date, CYP1B1 is thought to be one of the most important for understanding the basis of chemical carcinogenesis in humans, since it catalyses the activation of diverse procarcinogens such as polycyclic aromatic hydrocarbons, heterocyclic- and aryl-amines and nitroarenes (Shimada *et al.* 1996, Agarwal *et al.* 1997, Crofts *et al.* 1997, Luch *et al.* 1998). CYP1B1 has also been reported to catalyse 17 β -oestradiol 4-hydroxylation; 4-hydroxyoestradiol has been suggested to be involved in the development of breast cancer in humans (Spink *et al.* 1992, Liehr and Rocci 1996, Spink *et al.* 1997).

It has been reported that there are at least four genetic polymorphisms in the human *CYP1B1* gene, with changes at amino acid residues 48, 119, 432 and 453 (Bailey *et al.* 1998, Bejjani *et al.* 1998, Stoilov *et al.* 1998, Inoue *et al.* 1999, Watanabe *et al.* 2000). Polymorphisms at residues 48 and 119 of CYP1B1 have been shown to be genetically linked, with Arg⁴⁸ linked to Ala¹¹⁹ and Gly⁴⁸ to Ser¹¹⁹ (Watanabe *et al.* 2000). It is of interest to determine whether alterations in amino acids in CYP1B1 cause changes in catalytic specificities towards drugs, toxic chemicals and carcinogens in order to understand the basis of different susceptibilities of individuals towards these xenobiotic chemicals and also oestrogens. It has been shown that alterations in amino acid residues 119 and 432 cause changes in the substrate specificity of CYP1B1 towards 17 β -oestradiol, oestrone, testosterone and progesterone using the CYP1B1 variants Arg⁴⁸Ala¹¹⁹Leu⁴³²Asn⁴⁵³ (CYP1B1*1)², Arg⁴⁸Ala¹¹⁹Val⁴³²Asn⁴⁵³ (CYP1B1*3)², Arg⁴⁸Ser¹¹⁹Leu⁴³²Asn⁴⁵³ and Arg⁴⁸Ser¹¹⁹Val⁴³²Asn⁴⁵³ (Shimada *et al.* 1999b). Of particular interest is the observation that among the four CYP1B1 variants examined, the Val⁴³² forms were higher in the ratio of formation of 4-hydroxyoestradiol to 2-hydroxyoestradiol than the Leu⁴³² forms of the enzyme and the same trend was noted in the ratio of oestrone 4-hydroxylation to oestrone 2-hydroxylation catalysed by CYP1B1 variants. In the previous studies, however, we did not determine if the replacement of Arg⁴⁸ with Gly⁴⁸ changed the catalytic properties of polymorphic human CYP1B1.

In the present study, the effects of changes in amino acid residues at positions 48, 119 and 432 of CYP1B1 were further investigated on its catalytic specificity towards 17 β -oestradiol and benzo[*a*]pyrene. Eight CYP1B1 allelic variants, namely Arg⁴⁸Ala¹¹⁹Leu⁴³²Asn⁴⁵³ (1B1/RALN; CYP1B1*1)², Arg⁴⁸Ala¹¹⁹Val⁴³²Asn⁴⁵³ (1B1/RAVN; CYP1B1*3)², Gly⁴⁸Ala¹¹⁹Leu⁴³²Asn⁴⁵³ (1B1/GALN), Gly⁴⁸Ala¹¹⁹Val⁴³²Asn⁴⁵³ (1B1/GAVN), Arg⁴⁸Ser¹¹⁹Leu⁴³²Asn⁴⁵³ (1B1/RSLN), Arg⁴⁸Ser¹¹⁹Val⁴³²Asn⁴⁵³ (1B1/RSVN), Gly⁴⁸Ser¹¹⁹Leu⁴³²Asn⁴⁵³ (1B1/GSLN; CYP1B1*2)² and Gly⁴⁸Ser¹¹⁹Val⁴³²Asn⁴⁵³ (1B1/GSVN; CYP1B1*6)² were expressed in *Escherichia coli* together with hNPR. Among these CYP1B1 variants, 1B1/RALN is considered 'wild type' (most common) and four forms (1B1/GALN, 1B1/GAVN, 1B1/RSLN, 1B1/RSVN) are not apparently natural.

Materials and methods

Chemicals.

B[a]P and L- α -dilauroyl-*sn*-glycero-3-phosphocholine were from Sigma Chemical Co. (St Louis, MO, USA). B[a]P-7,8-diols [(+)- and (–)] were from the National Cancer Institute Chemical Carcinogen Repository Midwest Research Institute (Kansas City, MO, USA). Other chemicals and reagents used were from sources described previously or were of the highest quality commercially available (Shimada *et al.* 1994, Shimada *et al.* 1999a, b).

Construction of different types of CYP1B1 cDNAs expression plasmids

Variant sequences were introduced into the monocistronic CYP1B1 expression plasmid pCW'/1B1 (construct #3) described (Shimada *et al.* 1998a), which encodes Arg at position 48, Ala at position 119, Val at position 432 and Asn at position 453. The 1B1/RALN (CYP1B1*1)², 1B1/RAVN (CYP1B1*3)², 1B1/RSLN and 1B1/RSVN variants were constructed in bicistronic format as described (Shimada *et al.* 1999b). The Gly⁴⁸ variants were constructed by mutating the codon specifying Arg⁴⁸ to that for Gly by PCR-directed mutagenesis of the 5' coding sequence of CYP1B1 between the *Nde*I site centered on the start codon and the internal *Apa*I site. The mutated PCR product was then ligated into *Nde*I and *Eco*RI sites of the bicistronic expression vector, together with *Apa*I-*Eco*RI fragments containing each of the four different variant 3' coding sequences.

Bacterial 'bicistronic' CYP1A1 and CYP1A2 were prepared as described by Parikh *et al.* (1997).

Expression

Recombinant CYP1B1 variants, and CYP1A1 and CYP1A2, were co-expressed in *E. coli* with human hNPR and bacterial membranes were harvested and characterized as described (Parikh *et al.* 1997, Shimada *et al.* 1998a,b). Briefly, about 0.1 μ g DNA of the plasmids was introduced into DH5 α competent cells (40 μ l stock bacterial suspension) by a heat shock procedure. A single colony was picked and the bacteria were grown in LB medium containing 100 μ g ampicillin/ml and 0.1% glucose (w/v) at 37°C for 24 h. Following dilution of the bacteria with 100 vols TB medium containing 1.0 mM thiamine, 0.25 ml of a trace element solution/liter (Gillam *et al.* 1993), 100 μ g ampicillin ml⁻¹, 1.0 mM isopropyl-1-thio- β -D-galactopyranoside and 0.5 mM δ -aminolevulinic acid, the cells were grown for 24 h at 30°C with shaking at 150 rpm (using triple-baffle flasks) in a Bio-Shaker incubator (type BR-300LF, TAITEC Co, Tokyo, Japan). Finally, the bacterial membranes corresponding to ~300 ml culture media were prepared and suspended in 5 ml 10 mM Tris-Cl buffer (pH 7.4) containing 1 mM EDTA and 20% glycerol (v/v).

Other enzymes and antibody

Rat liver epoxide hydrolase was prepared as described (Guengerich *et al.* 1979, Johnson *et al.* 1997). Human epoxide hydrolase was isolated from liver sample HL-96, using modifications of previously described techniques (Johnson *et al.* 1997). Rabbit liver NADPH-P450 reductase were purified as described (Yasukochi and Masters 1976, Taniguchi *et al.* 1979). Recombinant CYP1B1 expressed in microsomes of *Trichoplusia ni* cells and anti-CYP1B1 (WB-1B1) were from Gentest Co. (Woburn, MA, USA).

Assay methods

17 β -Oestradiol 4- and 2-hydroxylation activities were determined using HPLC as described with a slight modification (Yamazaki *et al.* 1998, Shimada *et al.* 1999b). Briefly, the standard incubation mixture consisted of recombinant P450 (5–10 pmol), 1.0 mM ascorbic acid and oestradiol (80 μ M) in a final volume of 0.25 ml 100 mM potassium phosphate buffer (pH 7.4) containing an NADPH-generating system consisting of 0.5 mM NADP⁺, 5 mM glucose 6-phosphate and 0.5 unit glucose 6-phosphate dehydrogenase ml⁻¹ (Shimada *et al.* 1998a, b). For the reconstitution studies, purified CYP1B1 enzymes were mixed with 2-fold molar excess of rabbit liver NADPH-P450 reductase, 0.01% sodium cholate and 5 μ g L- α -dilauroyl-*sn*-glycero-3-phosphocholine ml⁻¹. Incubations were carried out at 37°C for 30 min and terminated by adding 1.5 ml each of dichloromethane and 0.3 M NaCl. The organic phase was recovered and taken to dryness under a nitrogen stream at 37°C. The residue was dissolved in a mixture of 30% acetonitrile (v/v) in 1.0% acetic acid (v/v). Product formation was determined by HPLC with a C₁₈ (5 μ m) analytical column (4.6 $\times$ 150 mm, Kanto Chemical, Tokyo, Japan). Elution was with a mixture of 30% acetonitrile (v/v) in 1.0% acetic acid (v/v) at flow rate of 1.0 ml min⁻¹ and detection was by UV absorbance at 280 nm (Yamazaki *et al.* 1998).

Standard reaction mixtures for the metabolism of B[a]P included P450 (10 pmol) in bacterial membranes, rat or human epoxide hydrolase (40 pmol) and L- α -dilauroyl-*sn*-glycero-3-phosphocholine (5 μ g), 20 μ M B[a]P dissolved in MeOH (final concentration, 0.8%), and an NADPH-generating system in 50 mM potassium phosphate buffer (pH 7.4) in a final volume of 0.25 ml. Incubations were conducted

at 37 °C for 10 min and terminated by the addition of 500µl cold acetone. The formation of B[a]P-7,8-diol was determined using HPLC as described by Shimada *et al.* (1999a).

Other assays

P450 and protein contents were estimated by the methods of Omura and Sato (1964) and Lowry *et al.* (1951). CYP1B1 in bacterial membranes was detected by coupled sodium dodecyl sulphate-polyacrylamide gel electrophoresis/immunochemical development ('Western-blotting') (Guengerich *et al.* 1982). NADPH-cytochrome *c* reductase activities were determined as described (Williams and Kamin 1962, Noshiro and Omura 1978).

Statistical analysis

Kinetic parameters for the oxidation of 17β-oestradiol by recombinant human P450 enzymes were estimated by non-linear regression analysis using the KaleidaGraph program (Synergy Software, Reading, PA, USA). Statistical analysis was performed by Student's *t*-test.

Results

Expression of CYP1B1 variants in E. coli

The plasmids pCW'/1B1^{RALN}/hNPR, pCW'/1B1^{RAVN}/hNPR, pCW'/1B1^{GALN}/hNPR, pCW'/1B1^{GAVN}/hNPR, pCW'/1B1^{RSLN}/hNPR, pCW'/1B1^{RSVN}/hNPR, pCW'/1B1^{GSLN}/hNPR and pCW'/1B1^{GSVN}/hNPR were introduced into *E. coli* and the bacterial membranes were isolated. CO-difference spectra showed that all of the constructs produced CYP1B1 holo-proteins, 1B1/RALN (CYP1B1*1)², 1B1/RAVN (CYP1B1*3)², 1B1/GALN, 1B1/GAVN, 1B1/RSLN, 1B1/RSVN, 1B1/GSLN (CYP1B1*2)² and 1B1/GSVN (CYP1B1*6)², respectively, in the membrane fraction. Yields of P450, as determined by the original method (Omura and Sato 1964), ranged between 40 and 220 nmol l⁻¹ medium, (table 1) and all of the CYP1B1 preparations showed wavelength maxima at 446 nm for the reduced CO complex (data not shown). NADPH-P450 reductase expression in these bacterial membranes ranged from 20 to 75 nmol l⁻¹ culture and the P450/reductase ratio was determined to be 2.0-4.5 (table 1). Both P450 and reductase yields were lower with the GALN variant and to a lesser extent with the GAVN variant, compared with the other forms.

Table 1. Levels of P450 and the reductase in membranes of *E. coli* in which human CYP1B1 and hNPR cDNAs have been introduced.

CYP1B1 variant	Suggested name*	P450 (nmol ⁻¹)	Reductase (nmol ⁻¹)	P450/Reductase* (molar ratio)
1B1/RALN	CYP1B1*1	180	40	4.5
1B1/RAVN	CYP1B1*3	190	50	3.8
1B1/RSLN		220	64	3.4
1B1/RSVN		130	45	2.9
1B1/GALN		38	18	1.9
1B1/GAVN		90	38	2.1
1B1/GSLN	CYP1B1*2	150	71	2.1
1B1/GSVN	CYP1B1*6	150	75	2.0

*Suggested by Oscarson *et al.* (<http://www.imm.ki.se/CYPalle/es>).
P450 content and NADPH-cytochrome *c* reductase activity were determined as described in the Materials and methods. The amounts of NADPH-P450 reductase were calculated by assuming that 1 nmol P450 reductase corresponds to 3 µmol cytochrome *c* reduced min⁻¹.
*Molar ratio of P450 to reductase in bacterial membranes.

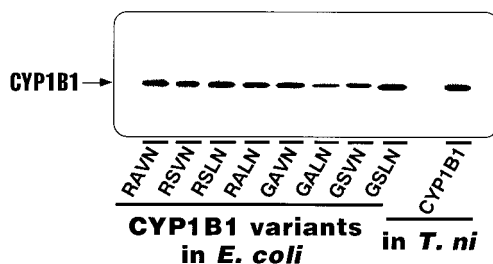


Figure 1. Immunoblotting of CYP1B1 variants of CYP1B1/RAVN, CYP1B1/RSVN, CYP1B1/RSLN, CYP1B1/RALN, CYP1B1/GAVN, CYP1B1/GALN, CYP1B1/GSVN and CYP1B1/GSLN in *E. coli* membranes in combination with hNPR. Sample buffers containing 0.8 pmol of each of the CYP1B1 variants (in membranes) were applied to the gel. Recombinant CYP1B1 expressed in *Trichoplusia ni* cells was also analysed for comparison.

Western immunoblotting analysis showed that all of the bacterial membranes contained protein that showed immunoreactivity with anti-human CYP1B1 antibodies (figure 1). The GALN variant showed markedly lower apoprotein yield. The bands detected from the CYP1B1 variants comigrated with CYP1B1 expressed in microsomes of *Trichoplusia ni* cells (Gentest Co., Woburn, MA, USA).

17 β -Oestradiol 4- and 2-hydroxylation by CYP1B1 variants

Recombinant CYP1A2 showed higher activities of 2-hydroxylation than 4-hydroxylation of 17 β -oestradiol (figure 2A) and formation of both products was abolished by inclusion of α -naphthoflavone in the incubation mixture (figure 2B). In contrast, CYP1B1 produced more 4-hydroxylated product than 2-hydroxylated product (figure 2C); the formation of these hydroxylated metabolites was also completely inhibited by α -naphthoflavone (figure 2D). The minor product (U) in incubations with CYP1A2 and CYP1B1 was not changed by α -naphthoflavone, indicating that this peak may originate from the action of bacterial membranes. When purified CYP1B1 was reconstituted with purified NADPH-P450 reductase in the L- α -dilauroyl-*sn*-glycero-3-phosphocholine vesicles, this minor product was not formed (figure 2E).

Dependence of 17 β -oestradiol 4- and 2-hydroxylation activities on substrate concentrations was determined with eight forms of recombinant CYP1B1 (figure 3). Arg⁴⁸ forms of CYP1B1 tended to have higher 17 β -oestradiol 4-hydroxylation activities than the corresponding Gly⁴⁸ forms. There were no significant variations in 17 β -oestradiol 2-hydroxylation activities among these eight CYP1B1 variants. Interestingly, ratios of formation of 4-hydroxylation to 2-hydroxylation of 17 β -oestradiol by these CYP1B1 proteins were higher in the Val⁴³² forms than those catalysed by the Leu⁴³² variants (figures 3a-d). The high ratios of 4-hydroxylation to 2-hydroxylation were found to be due to the higher 4-hydroxylation activities in CYP1B1/RAVN and CYP1B1/GSVN variants than those of CYP1B1/RALN and GSLN (figure 3A and D).

In order to examine the differences in the formation of 4-hydroxylation and 2-hydroxylation of 17 β -oestradiol by recombinant CYP1B1 variants, activities were determined under standard incubation conditions at a substrate concentration of 80 μ M in three separate determinations in each case (figure 4). Also compared were 17 β -oestradiol hydroxylation activities of recombinant CYP1A1 and CYP1A2.

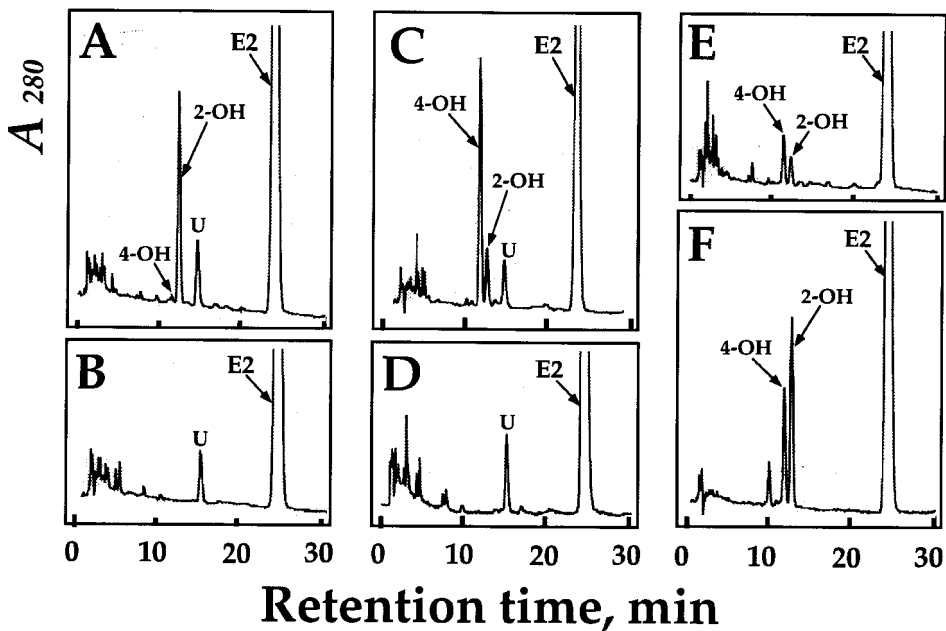


Figure 2. HPLC analysis of 17β-oestradiol hydroxylation by recombinant human CYP1A2 and CYP1B1/RAVN in membranes of *E. coli* co-expressing human NADPH-P450 reductase and by the reconstituted system containing purified CYP1B1/RAVN and human NADPH-P450 reductase. 17β-Oestradiol was incubated with CYP1A2 (A, B) and CYP1B1/RAVN (C, D) in the bicistronic system in the absence (A, C) and presence (B, D) of 10 μM α-naphthoflavone, and the products formed were analysed by HPLC. 17β-Oestradiol was also incubated with the reconstituted system containing CYP1B1/RAVN and NADPH-P450 reductase, and the products were analysed (E). Authentic standards were analysed using HPLC (F). 4-OH, 4-hydroxyoestradiol; 2-OH, 2-hydroxyoestradiol; E2, 17β-oestradiol; U, unknown peak.

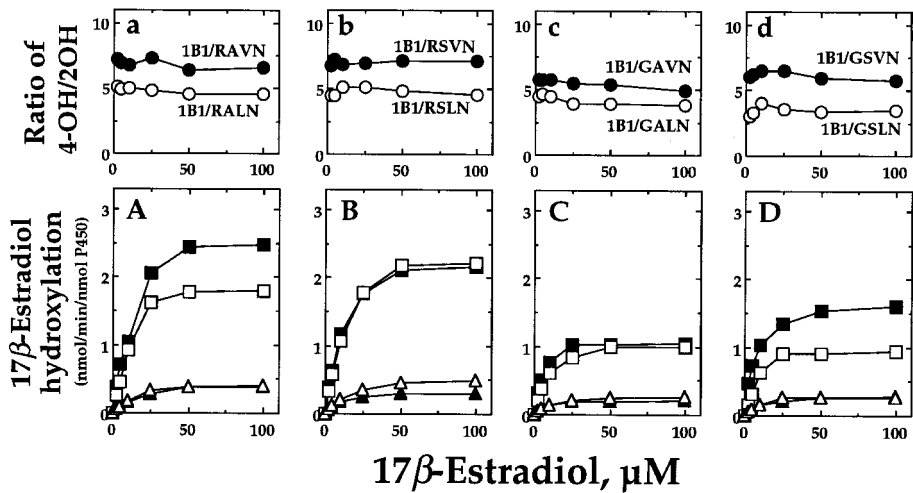


Figure 3. Dependence of substrate concentrations on 17β-oestradiol 4-hydroxylation (□, ■) and 2-hydroxylation (△, ▲) activities by CYP1B1 variants of 1B1/RALN and 1B1/RAVN (A), 1B1/RSLN and 1B1/RSVN (B), 1B1/GALN and 1B1/GAVN (C) and 1B1/GSLN and 1B1/GSVN (D). The ratios of 17β-oestradiol 4- to 2-hydroxylation activities are shown at the upper panels in which the results of 1B1/RALN and 1B1/RAVN (a), 1B1/RSLN and 1B1 RSVN (b), 1B1/GALN and 1B1/GAVN (c) and 1B1 GSLN and 1B1/GSVN (d) were compared. The experimental data shown are from single determinations.

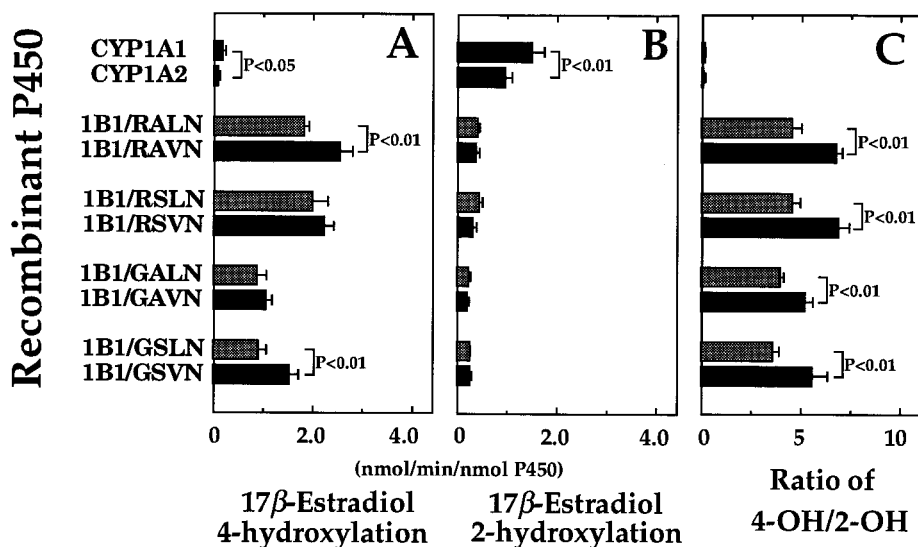


Figure 4. 17 β -Oestradiol 4- (A) and 2- (B) hydroxylation activities catalysed by CYP1B1 variants, CYP1A1 and CYP1A2 at a substrate concentration of 80 μ M. The ratios of 4-hydroxylation versus 2-hydroxylation of 17 β -oestradiol are also shown in (C). Each bar is the mean of three determinations $\pm$ SD.

The ratios of 4- to 2-hydroxylation by CYP1A1 and 1A2 were very low compared with the CYP1B1 variants. Again, Val⁴³² forms of CYP1B1 showed statistically higher ratios of 4-hydroxylation versus 2-hydroxylation than Leu⁴³² forms in these four cases (figure 4C). Formation of the 4-hydroxylated product was significantly higher in 1B1/RAVN and 1B1/GSVN than 1B1/RALN and 1B1/GSLN, respectively.

Oxidation of B[a]P by CYP1B1 variants in the presence of purified rat and human epoxide hydrolase

It has been shown that CYP1B1 is one of the important enzymes that catalyse oxidation of B[a]P to its proximate carcinogenic form, B[a]P-7,8-diol, in the presence of liver epoxide hydrolase (Shimada *et al.* 1999a). In the present study, we first examined the dependence on incubation time of the formation of B[a]P-7,8-diol by CYP1B1/GSLN in the presence of rat liver epoxide hydrolase as described in the Materials and methods (figure 5). At least four peaks were found in the HPLC chromatogram and peak d has been shown to be B[a]P-7,8-diol (figure 5A) (Shimada *et al.* 1999a). The other three metabolites were not identified; however, peak c may be B[a]P-4,5-diol (Shimada *et al.* 1999a). Using (+)B[a]P-7,8-diol as a standard, time-course analysis showed that all of the four metabolites including B[a]P-7,8-diol increased non-linearly (figure 5B). These results are consistent with the formation of B[a]P-diol derivatives by two enzymes, CYP1B1 and epoxide hydrolase, confounding attempts to derive kinetic parameters of oxidation of B[a]P by CYP1B1.

We examined the metabolism of B[a]P to B[a]P-7,8-diol by CYP1B1 variants in the presence of rat liver epoxide hydrolase (with 20 μ M B[a]P) by incubating at 37°C for 10 min (figure 6). CYP1B1/GALN and 1B1/GSLN had the highest activities for B[a]P oxidation followed by 1B1/RALN and 1B1/RSLN. All of the

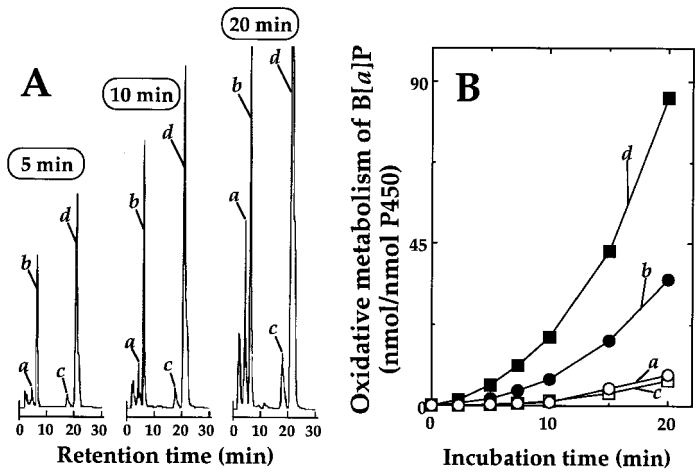


Figure 5. Dependence on incubation time on the formation of B[a]P-7,8-diol from B[a]P by recombinant CYP1B1 variants and rat liver epoxide hydrolase. (A) HPLC elution profiles from incubations of 5, 10 and 20 min. (B) Time-course profiles of the metabolism of B[a]P to B[a]P-7,8-diol (d) and unidentified metabolites (a-c) by recombinant CYP1B1/GSVN and rat epoxide hydrolase. Formation of metabolites a-c as well as d were tentatively calculated using (+)B[a]P-7,8-diol as a standard.

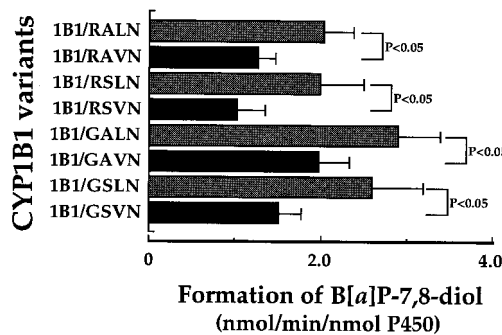


Figure 6. Formation of B[a]P-7,8-diol from B[a]P by recombinant CYP1B1 variants and rat liver epoxide hydrolase. Each bar is the mean $\pm$ SD of three determinations.

Val⁴³² forms had lower activities for B[a]P oxidation than the cognate Leu⁴³² forms.

Discussion

In the present study, we prepared membranes of *E. coli* in which eight possible combinations of CYP1B1 cDNAs were introduced together with hNPR cDNA, thus producing CYP1B1 variants 1B1/RALN (CYP1B1*1)², 1B1/RAVN (CYP1B1*1)², 1B1/GALN, 1B1/GAVN, 1B1/RSLN, 1B1/RSVN, 1B1/GSLN (CYP1B1*2)² and 1B1/GSVN (CYP1B1*6)² together with active NADPH-P450 reductase protein. Four of these CYP1B1 variants (1B1/GALN, 1B1/GAVN, 1B1/RSLN, 1B1/RSVN) do not occur naturally because polymorphisms in residues 48 and 119 of CYP1B1 have been shown to be genetically linked: Arg⁴⁸ to Ala¹¹⁹ and

Gly⁴⁸ to Ser¹¹⁹ (Watanabe *et al.* 2000). All of the eight CYP1B1 variants were found to show Fe²⁺·CO versus Fe²⁺ difference spectra with wavelength maxima at 446 nm (Shimada *et al.* 1998a) and to react with antibodies raised against recombinant human CYP1B1 (figure 1). These results suggest that these CYP1B1 variants expressed in *E. coli* are catalytically active without causing a major problem with protein folding. Both P450 and NADPH-P450 reductase expression in *E. coli* were lower in 1B1/GALN construct than other 1B1 variants on analysis with spectral and immunochemical analysis. The reasons of lower expression of 1B1/GALN variant in the bacteria are unknown at present, although this P450 form is not apparently natural (see above).

Also determined were NADPH-cytochrome c (P450) reductase activities in membranes of *E. coli* expressing P450 and hNPR. It was found that the molar ratio of P450 and NADPH-P450 reductase in the membranes ranged between 2.0 and 4.5 (table 1). These ratios are very similar to those reported by Parikh *et al.* (1997), i.e. that P450/reductase ratios ranged between 1 and 4 in membranes of *E. coli* in which CYP1A1, CYP1A2, CYP2C9, CYP2D6, CYP2E1 and CYP3A4 cDNAs were expressed together with the cDNA for hNPR cDNA. It has been reported that NADPH-P450 reductase produced in the bacterial (*E. coli*) membranes is more active in interacting with CYP1B1 proteins in the bicistronic system than the reductase added to artificial phospholipid vesicles or bacterial membranes (Shimada *et al.* 2000). In these experiments, purified CYP1B1 enzymes (under optimal reconstitution conditions) catalysed 7-ethoxycoumarin *O*-deethylation at rates one-third to one-quarter of those catalysed by membranes of *E. coli* co-expressing CYP1B1 and the reductase proteins (full catalytic activities in reconstituted systems were achieved with a 2-fold molar excess of NADPH-P450 reductase to CYP1B1). In the membranes containing bicistronic constructs, there was no additional stimulation of 7-ethoxycoumarin *O*-deethylation by added NADPH-P450 reductase, despite the fact that the molar ratio of expression levels of reductase to CYP1B1 was < 0.2 in the membranes. These results support the use of these membrane preparations co-expressing CYP1B1 and the reductase to compare catalytic activities of CYP1B1 variants.

Human CYP1B1 is known to be more active in catalysing 17 β -oestradiol 4-hydroxylation than 2-hydroxylation and the former product has been implicated as one of the factors causing breast cancer in humans (Liehr and Rocci 1996, Kabat *et al.* 1997). We previously reported that the ratio of 4-hydroxy- to 2-hydroxy-oestradiol formation is higher for the Val⁴³² forms of CYP1B1 than the Leu⁴³² forms when 1B1/RALN, 1B1/RAVN, 1B1/RSLN and 1B1/RSVN were used as catalysts (Shimada *et al.* 1999b). In the current study we further examined 17 β -oestradiol hydroxylation activities with eight CYP1B1 variants and found again that Val⁴³² forms of CYP1B1 consistently yielded higher 4- to 2-hydroxylation ratios for 17 β -oestradiol than the Leu forms. Differences in catalytic activities in these CYP1B1 variants are unlikely to be due to variable expression of NADPH-P450 reductase in bacterial membranes expressing these CYP1B1 variants, because we have reported that supplementation of bacterial membranes with additional reductase (added from a preparation of bacterial membranes) failed to affect 7-ethoxycoumarin *O*-deethylation activities (Shimada *et al.* 2000). CYP1B1/RAVN and CYP1B1/GSVN were particularly active in catalysing 17 β -oestradiol 4-hydroxylation activity, with $V_{\max}$ to K_m ratios of 4-hydroxylation to 2-hydroxylation of 7.3 and 7.1, respectively (table 2). These results are of interest since

Table 2. Comparison of 17β-oestradiol hydroxylation activities by recombinant CYP1B1 variants reported in different laboratories.

CYP1B1 variants	17β-Oestradiol hydroxylation						V_{4-OH}/K_{4-OH} V_{2-OH}/K_{2-OH}
	4-Hydroxylation			2-Hydroxylation			
	K_m	V_{max}	V_{max}/K_m	K_m	V_{max}	V_{max}/K_m	
Present study							
RALN	14	2.2	0.16	16	0.49	0.031	5.3
RAVN	15	3.0	0.20	18	0.47	0.027	7.3
RSLN	15	2.7	0.18	17	0.58	0.034	5.3
RSVN	13	2.6	0.21	12	0.35	0.030	7.0
GALN	9.1	1.1	0.12	12	0.30	0.025	4.8
GAVN	5.8	1.2	0.21	7.3	0.23	0.032	6.6
GSLN	9.1	1.1	0.12	9.7	0.31	0.032	3.8
GSVN	6.7	1.7	0.25	8.1	0.29	0.036	7.1
Hanna <i>et al.</i> (2000)							
RALN	11	3.7	0.34	21	2.2	0.11	3.2
RAVN	40	4.4	0.11	34	1.9	0.056	2.0
RAVS	17	4.5	0.27	39	2.8	0.072	3.7
RSVN	10	3.8	0.38	18	2.3	0.13	3.0
GAVN	19	6.0	0.32	29	3.2	0.11	2.9
GSLS	15	4.4	0.29	29	2.5	0.086	3.4
Li <i>et al.</i> (2000)							
RALN	13	1.6	0.13	11	0.31	0.025	5.2
RALS	11	1.5	0.14	12	0.32	0.030	4.6
RAVN	3.8	1.7	0.45	3.5	0.37	0.097	4.6
RAVS	3.7	1.8	0.49	3.9	0.39	0.11	4.6
RSVN	3.3	1.5	0.46	3.5	0.36	0.11	4.2
GAVN	3.7	1.6	0.43	4.2	0.40	0.11	4.0
GSVN	3.1	1.6	0.52	3.4	0.30	0.097	5.3
McLellan <i>et al.</i> (2000)							
RALN	3.5	0.94	0.23	11	0.22	0.021	14
GSLN	3.3	0.80	0.27	10	0.17	0.017	13

K_m (μM); V_{max} (nmol min⁻¹ nmol⁻¹ P450); V_{max}/K_m (μM⁻¹ min⁻¹).

Liehr and Rocci (1996) reported that microsomes of fibroadenoma and adenocarcinoma in human breast tissues catalyse 17β-oestradiol 4-hydroxylation more actively than the 2-hydroxylation (ratio of 4- to 2-hydroxylation ~3.6 in both cases); microsomes from normal tissues preferentially catalyse the 2-hydroxylation pathway. The latter authors also reported that microsomes from human uterine myoma tissues have high 17β-oestradiol 4-hydroxylation activities (Liehr *et al.* 1995).

Recently, several laboratories have reported on similar studies on the effects of CYP1B1 polymorphisms on 17β-oestradiol hydroxylation activities (Hanna *et al.* 2000, Li *et al.* 2000, McLellan *et al.* 2000). Kinetic analyses for 17β-oestradiol 4- and 2-hydroxylation activities by CYP1B1 variants can be compared in some, but not all, cases for these four laboratories, including the present paper (table 2). It should be mentioned, however, that different expression systems have been used in these experiments. Hanna *et al.* (2000) used a purified CYP1B1 expressed in bacteria using a different N-terminal oligo-histidine tag and GC/MS analysis of the products. Li *et al.* (2000) utilized HPLC analyses with a bacterial membrane system somewhat different to our own. McLellan *et al.* (2000) used a yeast microsomal system (for expression) and HPLC analysis. We have segregated the four studies in table 2 because internal comparisons are considered more reliable

than comparisons of parameters obtained with different systems. The parameter of most interest is the ratio $(V_{4\text{-OH}}/K_{4\text{-OH}})/(V_{2\text{-OH}}/K_{2\text{-OH}})$, although the influences on this parameter will be discussed.

The results of McLellan *et al.* (2000) are difficult to compare because only the CYP1B1/RALN and GSLN variants were compared. These results are characterized by $(V_{4\text{-OH}}/K_{4\text{-OH}})/(V_{2\text{-OH}}/K_{2\text{-OH}})$ ratios of 13-14, which are unusually high compared with the others and to the rest of the literature. V_{max} for 2-hydroxylation are low and K_m for 2-hydroxylation are 3-fold greater than for 4-hydroxylation. Li *et al.* (2000) used a catalytic system most similar to our own. These results are characterized by a striking lack of differences among any of the allelic variants. The only differences of any apparent significance are the higher K_m values seen for both hydroxylations when the Leu⁴³² alleles are compared with the Val counterparts. The study of Hanna *et al.* (2000), to which one of our laboratories contributed to, yielded somewhat high V_{max} for both hydroxylations. K_m were also higher and the V_{max}/K_m ratios are similar to ours for 4-hydroxylation. The ratio $(V_{4\text{-OH}}/K_{4\text{-OH}})/(V_{2\text{-OH}}/K_{2\text{-OH}})$ increased when RALN is compared with RAVN, in contrast to the tendency for a decreased ratio seen in the present study, whenever Leu is substituted for Val at 432. This was the only comparable L438V substitution. Another point about the present study is the decrease in K_m whenever Gly was substituted for Arg at position 48.

To summarize the 17 β -oestradiol work, we have described a consistent but modest effect of substituting Leu for Val at 432. In only one case (RAVN versus RALN) has such a comparison been made by others. In the two other laboratories that did make this comparison, one saw an effect opposite to ours when using different system. The patterns seen here are statistically significant (figure 4) and have been highly reproducible in repeated assays for over a year. The significance of the 17 β -oestradiol hydroxylation changes remains to be further evaluated.

B[a]P has been shown to be one of the potent carcinogens requiring metabolic activation by both P450 and epoxide hydrolase to form active metabolites that cause several types of cancers in lung and mammary glands in experimental animals and possibly in humans (Pelkonen and Nebert 1982, Guengerich and Shimada 1991). The first step in the activation of B[a]P is the formation of B[a]P-7,8-oxide by P450, followed by formation of B[a]P-7,8-diol by microsomal epoxide hydrolase (Gelboin 1980). We have previously shown that recombinant human CYP1B1 is more active than CYP1A1 in catalysing oxidation of B[a]P to B[a]P-7,8-diol when human or rat liver epoxide hydrolase is present in the reaction mixture (Shimada *et al.* 1999a). The dihydrodiol metabolites thus produced have been reported to be activated by CYP1B1 and also by CYP1A1, to DNA-damaging products, presumably the reactive diol-epoxides of B[a]P, detected as *umu* gene expression in the *Salmonella typhimurium* NM2009 tester strain (Shimada *et al.* 1996, 1999b). These results collectively indicated that CYP1B1 (as well as CYP1A1) is the major enzyme in the activation of B[a]P to form proximate carcinogen B[a]P-7,8-epoxide and ultimate carcinogen B[a]P-7,8-diol-epoxide in humans. Recent studies using the AhR- and CYP1b1-knock out mouse suggested that B[a]P and 7-12-dimethylbenz[a]anthracene are actually activated by CYP1A1 and CYP1B1 that cause tumours *in vivo* (Buters *et al.* 1999, Shimizu *et al.* 2000).

In the present study, it was found that the Leu⁴³² types of CYP1B1 variants are usually more active in the metabolism of B[a]P to B[a]P-7,8-diol (in the presence of epoxide hydrolase) than the Val⁴³² types. As mentioned above, 17 β -oestradiol 4-

hydroxylation activities tended to be higher with Val⁴³² than with Leu⁴³² CYP1B1 variants. These differences in catalytic specificities of these CYP1B1 variants may be related to the differences in the protein structures of CYP1B1 and further work will be required to address and understand these variations and their biological significance.

Acknowledgements

The authors thank Dr M. Oscarson for his suggestion on the nomenclature of CYP1B1 variants. The work was supported in part by grants from the Ministry of Education, Science and Culture of Japan and the Ministry of Health and Welfare of Japan (T. S.), by US Public Health Service Grants R35 CA44353 and P30 ES00267 (F. P. G.) and by the Kathleen Cuninghame Foundation for Breast Cancer Research (E. M. J. G.).

References

- AGARWAL, R., COFFING, S. L., BAIRD, W. M., KISELYOV, A. S., HARVEY, R. G. and DIPPLE, A., 1997, Metabolic activation of benzo[*g*]chrysene in the human mammary carcinoma cell line MCF-7. *Cancer Research*, **57**, 415-419.
- BAILEY, L. R., ROODI, N., DUPONT, W. D. and PARL, F. F., 1998, Association of cytochrome P4501B1 (CYP1B1) polymorphism with steroid receptor status in breast cancer. *Cancer Research*, **58**, 5038-5041.
- BEJJANI, B. A., LEWIS, R. A., TOMEY, K. F., ANDERSON, K. L., DUEKER, D. K., JABAK, M., ASTLE, W. F., OTTERUD, B., LEPPERT, M. and LUPSKI, J. R., 1998, Mutations in CYP1B1, the gene for cytochrome P4501B1, are the predominant cause of primary congenital glaucoma in Saudi Arabia. *American Journal of Human Genetics*, **62**, 325-333.
- BUTERS, J. T. M., SAKAI, S., RICHTER, T., PINEAU, T., ALEXANDER, D. L., SAVAS, U., DOEHMER, J., WARD, J. M., JEFEOATE, C. R. and GONZALEZ, F. J., 1999, Cytochrome P450 CYP1B1 determines susceptibility to 7,12-dimethylbenz[*a*]anthracene-induced lymphomas. *Proceedings of National Academy of Sciences, USA*, **96**, 1977-1982.
- CROFTS, F. G., STRICKLAND, P. T., HAYES, C. L. and SUTTER, T. R., 1997, Metabolism of 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) by human cytochrome P4501B1. *Carcinogenesis*, **18**, 1793-1798.
- GELBOIN, H. V., 1980, Benzo[*a*]pyrene metabolism, activation and carcinogenesis: role and regulation of mixed-function oxidases and related enzymes. *Physiology Reviews*, **60**, 1107-1166.
- GILLAM, E., BABA, T., KIM, B.-R., OHMORI, S. and GUENGERICH, F. P., 1993, Expression of modified human cytochrome P450 3A4 in *Escherichia coli* and purification and reconstitution of the enzyme. *Archives of Biochemistry and Biophysics*, **305**, 123-131.
- GONZALEZ, F. J., 1989, The molecular biology of cytochrome P450s. *Pharmacological Reviews*, **40**, 243-288.
- GUENGERICH, F. P. and SHIMADA, T., 1991, Oxidation of toxic and carcinogenic chemicals by human cytochrome P-450 enzymes. *Chemical Research in Toxicology*, **4**, 391-407.
- GUENGERICH, F. P. and SHIMADA, T., 1998, Activation of procarcinogens by human cytochrome P450 enzymes. *Mutation Research*, **400**, 201-213.
- GUENGERICH, F. P., WANG, P. and DAVIDSON, N. K., 1982, Estimation of isozymes of microsomal cytochrome P-450 in rats, rabbits and humans using immunochemical staining coupled with sodium dodecyl sulphate-polyacrylamide gel electrophoresis. *Biochemistry*, **21**, 1698-1706.
- GUENGERICH, F. P., WANG, P. W., MITCHELL, M. B. and MASON, P. S., 1979, Rat and human liver microsomal epoxide hydratase. Purification and evidence for the existence of multiple forms. *Journal of Biological Chemistry*, **254**, 12248-12254.
- HANNA, I. H., DAWLING, S., ROODI, N., GUENGERICH, F. P. and PARL, F. F., 2000, Cytochrome P450 1B1 (CYP1B1) pharmacogenetics: association of polymorphisms with functional differences in oestrogen hydroxylation activity. *Cancer Research*, **60**, 3440-3444.
- INOUE, K., ASAO, T. and SHIMADA, T., 1999, Ethnic-related differences in frequency distribution of genetic polymorphisms in *CYP1A1* and *CYP1B1* genes in Japanese and Caucasian populations. *Xenobiotica*, **30**, 285-295.

- JOHNSON, W. W., YAMAZAKI, H., SHIMADA, T., UENG, Y.-F. and GUENGERICH, F. P., 1997, Aflatoxin B₁ 8,9-epoxide hydrolysis in the presence of rat and human epoxide hydrolase. *Chemical Research in Toxicology*, **10**, 672-676.
- KABAT, G. C., CHANG, C. J., SPARANO, J. A., SEPKOVIC, D. W., HU, X. P., KHALIL, A., ROSENBLATT, R. and BRADLOW, H. L., 1997, Urinary oestrogen metabolites and breast cancer: a case-control study. *Cancer Epidemiology, Biomarkers and Prevention*, **6**, 505-509.
- LI, D. N., SEIDEL, A., PRITCHARD, M. P., WOLF, C. R. and FRIEDBERG, T., 2000, Polymorphisms in P450 CYP1B1 affect the conversion of oestradiol to the potentially carcinogenic metabolite 4-hydroxyoestradiol. *Pharmacogenetics*, **10**, 343-353.
- LIEHR, J. G., RICCI, M. J., JEFEOATE, C. R., HANNIGAN, E. V., HOKANSON, J. A. and ZHU, B. T., 1995, 4-Hydroxylation of oestradiol by human uterine myometrium and myoma microsomes: implications for the mechanism of uterine tumorigenesis. *Proceedings of National Academy of Sciences, USA*, **92**, 9220-9224.
- LIEHR, J. G. and ROCCI, M. J., 1996, 4-Hydroxylation of oestrogens as marker of human mammary tumors. *Proceedings of National Academy of Sciences, USA*, **93**, 3294-3296.
- LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. and RANDALL, R. J., 1951, Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry*, **193**, 265-275.
- LUCH, A., CONG, S. L., TANG, Y. M., SCHNEIDER, A., SOBALLA, V., GREIM, H., JEFEOATE, C. R., SEIDEL, A., GREENLEE, W. F., BAIRD, W. M. and DOEHMER, J., 1998, Stable expression of human cytochrome P4501B1 in V79 Chinese hamster cells and metabolically catalysed DNA adduct formation of dibenzo[a,l]pyrene. *Chemical Research in Toxicology*, **11**, 686-695.
- McLELLAN, R. A., OSCARSON, M., HIDOESTRAND, M., LEIDVIK, B., JONSSON, E., OTTER, C. and INGELMAN-SUNDBERG, M., 2000, Characterization and functional analysis of two common human cytochrome P4501B1 variants. *Archives of Biochemistry and Biophysics*, **378**, 175-181.
- NEBERT, D. W., NELSON, D. R. and FEYEREISEN, R., 1989, Evolution of the cytochrome P450 genes. *Xenobiotica*, **19**, 1149-1160.
- NELSON, D. R., KAMATAKI, T., WAXMAN, D. J., GUENGERICH, F. P., ESTABROOK, R. W., FEYEREISEN, R., GONZALEZ, F. J., COON, M. J., GUNSALUS, I. C., GOTOH, O., OKUDA, K. and NEBERT, D. W., 1993, The P450 superfamily: update on new sequences, gene mapping, accession numbers, early trivial names of enzymes and nomenclature. *DNA and Cell Biology*, **12**, 1-51.
- NELSON, D. R., KOYMANS, L., KAMATAKI, T., STEGEMAN, J. J., FEYEREISEN, R., WAXMAN, D. J., WATERMAN, M. R., GOTOH, O., COON, M. J., ESTABROOK, R. W., GUNSALUS, I. C. and NEBERT, D. W., 1996, P450 superfamily: update on new sequences, gene mapping, accession numbers and nomenclature. *Pharmacogenetics*, **6**, 1-42.
- NOSHIRO, M. and OMURA, T., 1978, Immunochemical study on the electron pathway from NADH to cytochrome P-450 of liver microsomes. *Journal of Biochemistry*, **83**, 61-77.
- OMURA, T. and SATO, R., 1964, The carbon monoxide-binding pigment of liver microsomes. I. Evidence for its hemoprotein nature. *Journal of Biological Chemistry*, **239**, 2370-2378.
- OSCARSON *et al.*, n.d. [<http://www.imm.ki.se/CYPalleles>].
- PAKIKH, A., GILLAM, E. M. J. and GUENGERICH, F. P., 1997, Drug metabolism by *Escherichia coli* expressing human cytochromes P450. *Nature Biotechnology*, **15**, 784-788.
- PELKONEN, O. and NEBERT, D. W., 1982, Metabolism of polycyclic aromatic hydrocarbons: Etiologic role in carcinogenesis. *Pharmacological Reviews*, **34**, 189-222.
- SHIMADA, T., GILLAM, E. M. J., ODA, Y., TSUMURA, F., SUTTER, T. R., GUENGERICH, F. P. and INOUE, K., 1999a, Metabolism of benzo[a]pyrene to *trans*-7,8-dihydroxy-7,8-dihydrobenzo[a]pyrene by recombinant human cytochrome P4501B1 and purified liver epoxide hydrolase. *Chemical Research in Toxicology*, **12**, 623-629.
- SHIMADA, T., HAYES, C. L., YAMAZAKI, H., AMIN, S., HECHT, S. S., GUENGERICH, F. P. and SUTTER, T. R., 1996, Activation of chemically diverse procarcinogens by human cytochrome P4501B1. *Cancer Research*, **56**, 2979-2984.
- SHIMADA, T., TSUMURA, F., GILLAM, E. M. J., GUENGERICH, F. P. and INOUE, K., 2000, Role of NADPH-P450 reductase in the *O*-deethylation of 7-ethoxycoumarin by recombinant human cytochrome P4501B1 variants in *Escherichia coli*. *Protein Expression and Purification*, **20**, 73-80.
- SHIMADA, T., WATANABE, J., KAWAJIRI, K., SUTTER, T. R., GUENGERICH, F. P., GILLAM, E. M. J. and INOUE, K., 1999b, Catalytic properties of polymorphic human cytochrome P4501B1 variants. *Carcinogenesis*, **20**, 1607-1613.
- SHIMADA, T., WUNSCH, R. M., HANNA, I. H., SUTTER, T. R., GUENGERICH, F. P. and GILLAM, E. M. J., 1998a, Recombinant human cytochrome P4501B1 expression in *Escherichia coli*. *Archives of Biochemistry and Biophysics*, **357**, 111-120.
- SHIMADA, T., YAMAZAKI, H., FOROOZESH, M., HOPKINS, N. E., ALWORTH, W. L. and GUENGERICH, F. P., 1998b, Selectivity of polycyclic inhibitors for human cytochromes P450 1A1, 1A2 and 1B1. *Chemical Research in Toxicology*, **11**, 1048-1056.

- SHIMADA, T., YAMAZAKI, H., MIMURA, M., INUI, Y. and GUENGERICH, F. P., 1994, Interindividual variations in human liver cytochrome P-450 enzymes involved in the oxidation of drugs, carcinogens and toxic chemicals: studies with liver microsomes of 30 Japanese and 30 Caucasians. *Journal of Pharmacology and Experimental Therapeutics*, **270**, 414-423.
- SHIMIZU, Y., NAKATSURU, Y., ICHINOSE, M., TAKAHASHI, Y., KUME, H., MIMURA, J., FUJII-KURIYAMA, Y. and ISHIKAWA, T., 2000b, Benzo[a]pyrene carcinogenicity is lost in mice lacking the aryl hydrocarbon receptor. *Proceedings of National Academy of Sciences, USA*, **97**, 779-782.
- SPINK, D. C., EUGSTER, H. P., LINCOLN II, D. W., SCHUETZ, J. D., SCHUETZ, E. G., JOHNSON, J. A., KAMINSKY, L. S. and GIERTHY, J. F., 1992, 17 β -Oestradiol hydroxylation catalysed by human cytochrome P450 1A1: a comparison of the activities induced by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in MCF-7 cells with those from heterologous expression of the cDNA. *Archives of Biochemistry and Biophysics*, **293**, 342-348.
- SPINK, D. C., SPINK, B. C., CAO, J. Q., GIERTHY, J. F., HAYES, C. L., LI, Y. and SUTTER, T. R., 1997, Induction of cytochrome P4501B1 and catechol oestrogen metabolism in ACHN human renal adenocarcinoma cells. *Journal of Steroid and Biochemical Molecular Biology*, **62**, 223-232.
- STOILOV, I., AKARSU, A. N., ALOZIE, I., CHILD, A., BARSOU-HOMSY, M., TURACLI, M. E., OR, M., LEWIS, R. A., OZDEMIR, N., BRICE, G., AKTAN, S. G., CHEVRETTE, L., COCA-PRADOS, M. and SARFARAZI, M., 1998, Sequence analysis and homology modeling suggest that primary congenital glaucoma on 2p21 results from mutations disrupting either the hinge region or the conserved core structures of cytochrome P4501B1. *American Journal of Human Genetics*, **62**, 573-584.
- TANIGUCHI, H., IMAI, Y., IYANAGI, T. and SATO, R., 1979, Interaction between NADPH-cytochrome P-450 reductase and cytochrome P-450 in the membrane of phosphatidylcholine vesicles. *Biochimica et Biophysica Acta*, **550**, 341-356.
- WATANABE, J., SHIMADA, T., GILLAM, E. M. J., IKUTA, T., SUEMASU, K., HIGASHI, Y., GOTOH, O. and KAWAJIRI, K., 2000, Association of *CYP1B1* genetic polymorphism with incidence to breast and lung cancer. *Pharmacogenetics*, **10**, 25-33.
- WILLIAMS, C. H., JR. and KAMIN, H., 1962, Microsomal triphosphopyridine nucleotide-cytochrome *c* reductase of liver. *Journal of Biological Chemistry*, **237**, 587-595.
- YAMAZAKI, H., SHAW, P. M., GUENGERICH, F. P. and SHIMADA, T., 1998, Roles of cytochrome P450 1A2 and 3A4 in the oxidation of oestradiol and oestrone in human liver microsomes. *Chemical Research in Toxicology*, **11**, 659-665.
- YASUKOCHI, Y. and MASTERS, B. S. S., 1976, Some properties of a detergent-solubilized NADPH-cytochrome *c* (cytochrome P-450) reductase purified by biospecific affinity chromatography. *Journal of Biological Chemistry*, **251**, 5337-5344.



Altered diltiazem metabolism in the neonatal rabbit following intra-uterine chronic exposure to diltiazem

L. J. FRAILE†, M. A. BREGANTE†*, M. A. GARCIA‡ and C. SOLANS†

Departments of †Pharmacology and Physiology and ‡Analytical Chemistry, Faculty of Veterinary Science, University of Zaragoza, E-50013 Zaragoza, Spain

Received 12 October 2000

1. Diltiazem undergoes extensive metabolism in hepatic and extrahepatic tissues. Deacetyldiltiazem (M1) and N-demethyldiltiazem (MA) are two of the main basic metabolites of diltiazem that retain pharmacological activity. This drug impairs its own metabolism after chronic administration in the adult patient. The study examines the possibility that intra-uterine exposure following chronic maternal therapy with DTZ from mid-gestation to term also impairs DTZ metabolism of its offspring.

2. DTZ was incubated in homogenates from liver, lung, brain and gut and in the whole blood of animals whose mothers were exposed to chronic treatment with diltiazem or unexposed (placebo). DTZ and its metabolites were assayed by HPLC.

3. DTZ deacetylase activity observed in liver, lung and brain homogenates from 1-, 8- and 16-day-old rabbits was significant lower in exposed animals. In gut homogenates, this age-dependent effect was not so clear. This inhibition could not be detected in any organ of 30-day-old rabbits. On the other hand, the activity observed in whole blood was not altered by intra-uterine chronic exposure to DTZ.

4. DTZ demethylase activity showed no differences in tissue homogenates and in whole blood from exposed compared with the unexposed rabbit.

5. In conclusion, the findings suggest that intra-uterine chronic exposure to DTZ has a large and prolonged effect on newborn metabolism deacetylase activity compared with the unexposed rabbit.

Introduction

Diltiazem (DTZ) is now established as a first-line therapy for the management of hypertension (Weir 1995). As a consequence, its use for the treatment of hypertension occurring during pregnancy has been postulated (Holbrook *et al.* 1988), especially in those clinical cases resistant to traditional therapy. DTZ has been shown to be extensively metabolized by at least five distinct pathways including deacetylation, N-demethylation, O-demethylation, ring hydroxylation and acid formation in hepatic and extrahepatic tissues of several animal species (Homsy *et al.* 1995, Fraile *et al.* 1996). These various reactions lead to the formation of at least 20 distinct metabolites (Sugawara *et al.* 1988), some of which - deacetyldiltiazem (M1), N-demethyldiltiazem (MA) and N-demetyldeacetyldiltiazem (M2) - retain potent pharmacological activity as antihypertensives and coronary vasodilators (Yabana *et al.* 1985). M1 and MA have been recognized as primary metabolites of DTZ and seem to be the most important basic

* Author for correspondence; e-mail: Bregante@posta.unizar.es

metabolites in mammals (Yeung *et al.* 1990, 1998), whereas M2 is assumed to be the product of a secondary reaction. In spite of some recent reports suggesting an extensive contribution of extrahepatic organs to the overall metabolism of DTZ, the *in vivo* metabolic contribution of each particular organ is yet not clear (Fraile *et al.* 1997). The formation of MA is catalysed by the NADPH-dependent CYP3A in rabbit and human (Pichard *et al.* 1990). In addition, it has been demonstrated that DTZ deacetylation is catalysed by a specific esterase without the presence of a co-enzyme being required (Leboeuf and Grech-Belanger 1987).

The antihypertensive agent diltiazem impairs hepatic drug metabolism. In fact, after chronic oral administration, DTZ blood concentration increases proportionally to the duration of the treatment (Kates 1983, Hermann and Morselli 1985) and there is accumulation of certain DTZ metabolites during dosage with the drug, as well as dose-related differences in DTZ pharmacokinetics, suggesting that DTZ metabolites may also participate in impairing its metabolism (Murray and Butler 1996). It has been suggested that inhibition of DTZ metabolism is due to interaction with enzymes from the microsomal mixed-function oxidase system and, in particular, cytochrome P4503A (Renton 1985, Hussain *et al.* 1994). Recently, Jones *et al.* (1999) suggested that DTZ inhibition of this enzyme is due to metabolite intermediate complex formation and others have also demonstrated that N-demethylated metabolites of DTZ may contribute to CYP3A4 inhibition (Sutton *et al.* 1997).

A recent paper reported that the production of M1 was reduced in lung and intestinal homogenates of animals receiving chronic administration of DTZ. This result suggests that the esterase that catalyse this enzymatic reaction could also be susceptible to inhibition because of chronic exposure to this drug (Lefebvre *et al.* 1996). Recently, it has been reported that DTZ metabolism is present in the newborn rabbit and its ability to metabolize this drug is lower, equal and in some instances higher than that observed in the young and adult animals depending on the enzymatic route studied (Fraile *et al.* 1996, 1997).

The present paper has carried out *in vitro* experiments in order to elucidate the effect of DTZ on the neonatal hepatic and extrahepatic DTZ metabolism in rabbit following chronic, maternal intra-uterine exposure. To this end, the *in vitro* metabolic activity in different tissue homogenates (liver, lung, brain, gut) and in whole blood was examined in the newborn rabbit from mothers receiving DTZ or placebo throughout pregnancy.

Material and methods

Products

Diltiazem, imipramine, glucose 6-phosphate, glucose 6-phosphate dehydrogenase, NAD⁺, and NADP⁺ were from Sigma Chemical Co. (St Louis, MO, USA). Deacetyldiltiazem (M1) and demethyldiltiazem (MA) were a gift from Dr R. Latini, Mario Negri Institute, Milan. Other chemicals were from commercial sources and were of reagent or HPLC grade.

Animals, housing and drug administration

Pregnant New Zealand White rabbits, on day 10 of gestation, weighing 3.5–4.0 kg, were from the Animal Experimentation Service of the University of Zaragoza. They were housed in rooms with a controlled temperature (20°C) and light cycle (12/12 h). Does were maintained on water and standard laboratory chow *ad libitum* throughout the study.

All experiments were approved by the Institutional Animal Care and Use Committee of the University of Zaragoza and this research was also adhered to the principles of Laboratory Animal Care (NIH publication #85-23, revised 1985).

DTZ was administered to four pregnant rabbits (from gestation day 15 to delivery) by using a mini-osmotic pump (Model 2002, Alzet Co., Palo Alto, CA, USA). The pump was inserted in a subcutaneous pocket in the back. The concentration of DTZ solution used to fill the pump reservoir was calculated according to the equation: $C_d = K_0/Q$, where C_d is the concentration of DTZ in the vehicle (saline solution), K_0 is the mass delivery rate and Q is the volume delivery rate of the vehicle solution from the pump ($0.5 \mu\text{l h}^{-1}$). K_0 was calculated according to the equation: $K_0 = C_{ss} \cdot \text{CL}$, where C_{ss} is the desired concentration at steady-state ($10\text{--}20 \text{ ng ml}^{-1}$), and CL ($40 \text{ ml min}^{-1} \text{ kg}^{-1}$) is the total body clearance (Bregante *et al.* 2000). DTZ concentration was determined at 0, 5, 8 and 15 days after pump implantation in venous samples (2 ml) drawn from the marginal ear vein to confirm the presence of the drug in maternal blood. Four additional pregnant rabbits were administered with normal saline (0.9% NaCl) using the same pumps as controls.

One week prior to delivery, does were placed in individual whelping cages, which contained a nest box. The day of whelping was considered post-partum day 0 (30–32 days of gestation). Periodical controls to confirm the growth of the offspring and the health status of the does were carried out. Newborn suckling rabbits were housed beside their dams. After weaning (post-partum day 28), young rabbits were housed individually and maintained on water and standard laboratory chow *ad libitum*.

Homogenate preparation

Spontaneously delivered 1- ($n = 12$), 8- ($n = 12$), 16- ($n = 4$) and 30-day-old ($n = 4$) rabbits from chronic DTZ-treated and untreated does were used. Pups were obtained from eight litters (four treated, four untreated) of at least eight members. Members of each litter were selected to be included in each age group. Immediately before tissue collection, arterial blood samples (5 ml) were drawn from all animals by cardiac puncture under light ether anaesthesia, for *in vitro* studies. Heparin ($10 \mu\text{l } 150 \text{ IU ml}^{-1}$ solution) had been previously added to the tubes to prevent blood clotting. To obtain a suitable volume of neonatal blood, samples from three littermates were pooled and used as single specimens ($n = 4$). Thereafter, rabbits were anaesthetized by intraperitoneal administration of sodium pentobarbital (50 mg kg^{-1}), the abdomen opened and the portal vein cannulated. Ice-cold normal saline was perfused through the catheter at a flow rate of $3\text{--}5 \text{ ml min}^{-1} \text{ g}^{-1}$ liver. In order to minimize the blood content in organs, animals were bled via the abdominal cava vein. When the colour of the liver became light tan, the liver, small intestine (jejunum), lungs and brain were excized. Faeces remaining in the small intestine were removed by ice-cold normal saline perfusion. Weighed tissue samples (in the range of 3 g) were minced with scissors and homogenized with ice-cold 1.15% (w/v) KCl-0.01 M phosphate buffer (pH 7.4) in a Potter-Elvehjem to yield 20% (w/v) homogenates. Each homogenate was centrifuged first at $1000g$ for 10 min, and then at $9000g$ for 20 min at 4°C . The supernatant was collected and stored frozen at -80°C until used. Previous experiments (data not shown) have demonstrated that this storage procedure does not modify significantly the diltiazem deacetylase and demethylase activities for, at least, 3 months in each organ and age studied. The protein content of the supernatant was determined by the method Lowry *et al.* (1951), using bovine serum albumin as standard.

DTZ demethylase and deacetylase activity in homogenates and blood

Previous experiments (data not shown) have demonstrated that both assays were linear with regard to incubation time and protein concentration. Furthermore, pH 7.4 was considered optimal for the enzymatic activities studied and that 1 mM DTZ was a saturating concentration for both activities. These previous experiments to optimize the enzyme assays were carried out as described (Leboeuf and Grech-Belanger 1987).

Demethylation of DTZ by tissue homogenates was assessed in the presence of an NADPH-regenerating system. In a final volume of 2 ml, a typical reaction mixture contained 1 mg protein, 1 mM DTZ as substrate, and a regenerating system composed of NAD^+ (26 mM), NADP^+ (2 mM), glucose 6-phosphate (53 mM), and glucose 6-phosphate dehydrogenase (4 units). Because of the different protein content of the homogenates (lower at the earliest ages), the required volume of 1.15% (w/v) KCl-0.01 M phosphate buffer (pH 7.4) was added to the mixture to yield a final volume of 2 ml. Incubations were conducted in glass vials in an oscillating water bath at 37°C for 10 min in air. At the end of this period, an aliquot of $50 \mu\text{l}$ was withdrawn from the incubation mixture and put on a tube containing 20 ml cold (-20°C) extraction medium (see HPLC assay) and assayed for MA and M1 concentrations. Vials containing the same composition, but without the NADPH regenerating system, were used to assess DTZ deacetylase activity.

For measuring enzyme activities in blood, incubation mixtures consisted of 2 ml whole blood containing DTZ (1 mM). At the end of the incubation (3 min) an aliquot of $100 \mu\text{l}$ was withdrawn from the incubation mixture and immediately haemolyzed by addition of $100 \mu\text{l}$ cold water and added to 20 ml cold (-20°C) extraction medium. As above, samples were assayed for M1 and MA concentrations.

Chemical breakdown of DTZ, MA and M1 was assessed in vials containing the same composition but inactivated by heat (5 min at 75°C). DTZ and its metabolites (M1 and MA) were spiked to the incubation mixture to yield concentrations of 1, 0.1 and 0.01 mM respectively. Serial samples were drawn at 0, 10, 30 and 60 min and assayed by HPLC. The amount of M1 or MA formed enzymatically was then calculated by subtracting the amount formed by chemical breakdown from that formed during incubations of DTZ with the enzyme source. DTZ deacetylase or demethylase activity in homogenates or whole blood was expressed as $\text{nmol min}^{-1} \text{mg}^{-1}$ protein or as $\text{nmol min}^{-1} \text{ml}^{-1}$ blood respectively.

In addition, two separate studies on the stability of M1 and MA in whole blood and homogenates were carried out. MA was spiked to the incubation mixtures to yield a final concentration close to that observed in the main experiments in the presence of an NADPH-regenerating system or in whole blood. Aliquots of 50 μl were sampled from the incubation medium at 0, 3, 5 and 10 min and assayed for MA concentrations. Similarly, the stability of M1 was checked but without the presence of NADPH. Finally, the formation of M1 and MA over time was studied. Briefly, under the conditions described above, incubations were stopped at 0, 3, 5, 10, 15, 20 and 30 min in each organ and age studied.

HPLC assay of DTZ and its metabolites

DTZ and its metabolites (M1 and MA) were assayed in blood and tissue homogenates, after hexane:isobutanol (98:2 v/v) extraction, according to the HPLC method of Yeung *et al.* (1989). The HPLC system consisted of an isocratic pump (Model 145, Water Associates, Mildford, MA, USA), a 5- μm reversed-phase column (Novapack C18, 150 $\times$ 3.9 mm i.d.), and a UV-VIS detector set at 237 nm (Model 484, both Water Associates). Quantitation was performed using a calibration curve of spiked blank blood or tissue homogenates over the range of concentrations assayed. The method was shown to be linear for concentrations ranging from 10 and 5000, 10 to 1000 and 10 to 500 ng ml^{-1} DTZ, M1 and MA respectively. The detection limit was 10, 5 and 10 ng ml^{-1} for DTZ, M1 and MA respectively. Recovery was determined as > 90% for each analyte. Inter- and intra-assay precision, determined by injection of control standards, was 7 and 6% respectively.

Statistics

All data are presented as mean $\pm$ SD. Differences between ages were assessed using analysis of variance. If a difference was detected, Duncan's multiple range test was used for multiple comparison. All other comparisons were performed using Student's paired *t*-test with equal variance. Significance was accepted at $p < 0.05$.

Results

Controls for body weight of the does and for growth rate of their offspring indicated no differences compared with routine controls measured in our laboratory. The does gained no weight throughout the study, but their offspring grew according to a logarithmic pattern, indicating that suckling was adequate (data not shown).

The time-course of DTZ blood concentration during chronic administration to the pregnant rabbit is showed in figure 1. DTZ was consistently detected throughout the chronic infusion period. Up to day 8 of the beginning of infusion (gestation day 23), DTZ blood concentrations were maintained between the proposed range (~ 10 –20 ng ml^{-1}). However, a sharp increase in DTZ concentration could be observed at the end of the infusion period (gestation day 30), with levels ranging from 35 to 60 ng ml^{-1} ($p < 0.05$).

The greatest protein concentration in homogenates of the unexposed rabbit was measured in the liver, followed by the intestine, lung and brain, whatever the age examined (table 1). The protein content tended to increase with postnatal age in all tissues examined. However, only significant differences in the protein content of 1-day-old rabbit homogenates as compared with adults were detected. The protein concentration in homogenates of the exposed rabbit was so close to the described for the unexposed animals that we were unable to observe significant differences between them (data not shown).

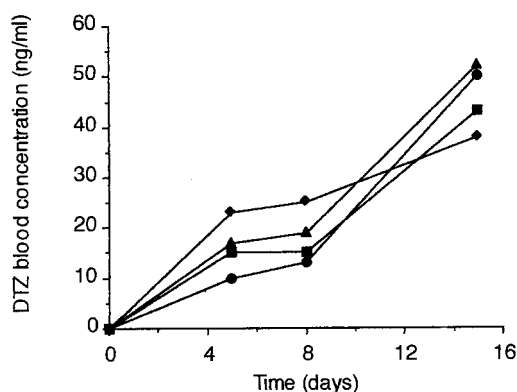


Figure 1. DTZ blood concentrations following DTZ administration to four pregnant rabbits (from gestation day 15 to delivery). A mini-osmotic pump was used (see the Materials and methods) providing an infusion rate of $600 \text{ ng min}^{-1} \text{ kg}^{-1}$ of this drug.

Table 1. Total protein concentration (mg g^{-1} tissue) in the 9000g supernatant of several tissue homogenates from rabbits of untreated does at different post-natal ages.

	Post-natal stage (days)				
	1	8	16	30	Adult
Liver	64.4 ± 9.1^a	71.7 ± 17.0	81.2 ± 6.1	87.3 ± 6.0	94.0 ± 15.0
Lung	43.8 ± 5.7^a	45.1 ± 4.2	47.3 ± 8.0	47.3 ± 7.0	56.5 ± 8.2
Intestine	49.0 ± 7.7^a	53.2 ± 8.5	59.5 ± 8.6	61.1 ± 7.0	71.1 ± 11.7
Brain	17.5 ± 2.1^a	17.5 ± 2.4	24.0 ± 1.3	23.2 ± 6.0	22.7 ± 4.6

^a Significantly different from the adult ($p < 0.05$).

Data are mean $\pm$ SD of four experiments.

DTZ chemical breakdown was $< 2\%$ of the initial amount after 60-min incubation in all the tissues and ages examined, indicating that most of the M1 and MA formed under the present experimental conditions was due to enzymatic formation. In addition, it could not be observed that any appreciable chemical degradation of either M1 or MA in the experimental conditions (data not shown).

A substantial DTZ deacetylase activity in liver, lung, gut and brain homogenates and in whole blood from the untreated newborn was observed whatever the age examined (figure 2). This activity was highest in the brain homogenates, followed by the lung, liver and gut. Blood was not included in this comparison because of the unequal composition of the incubation mixture. The effect of intra-uterine exposure to DTZ on DTZ deacetylase activity observed in newborns depends on the tissue studied (figure 2). Thus, the activity observed in whole blood was not altered by intra-uterine chronic exposure to DTZ. However, the DTZ deacetylase activity observed in liver, lung and brain homogenates from 1-, 8- and 16-day-old rabbits was significant lower in exposed animals. This effect could not be detected in 30-day-old rabbits. In gut homogenates, the effect was not so clear. In fact, differences between treatments were not detected in 1- and 30-day-old rabbits. However, 8- and 16-day-old exposed rabbits showed a lower DTZ deacetylase activity when compared with unexposed animals.

DTZ demethylase activity was only detected in liver and gut homogenates and in blood (figure 3). The activity observed in the other tissues (brain and lung) was

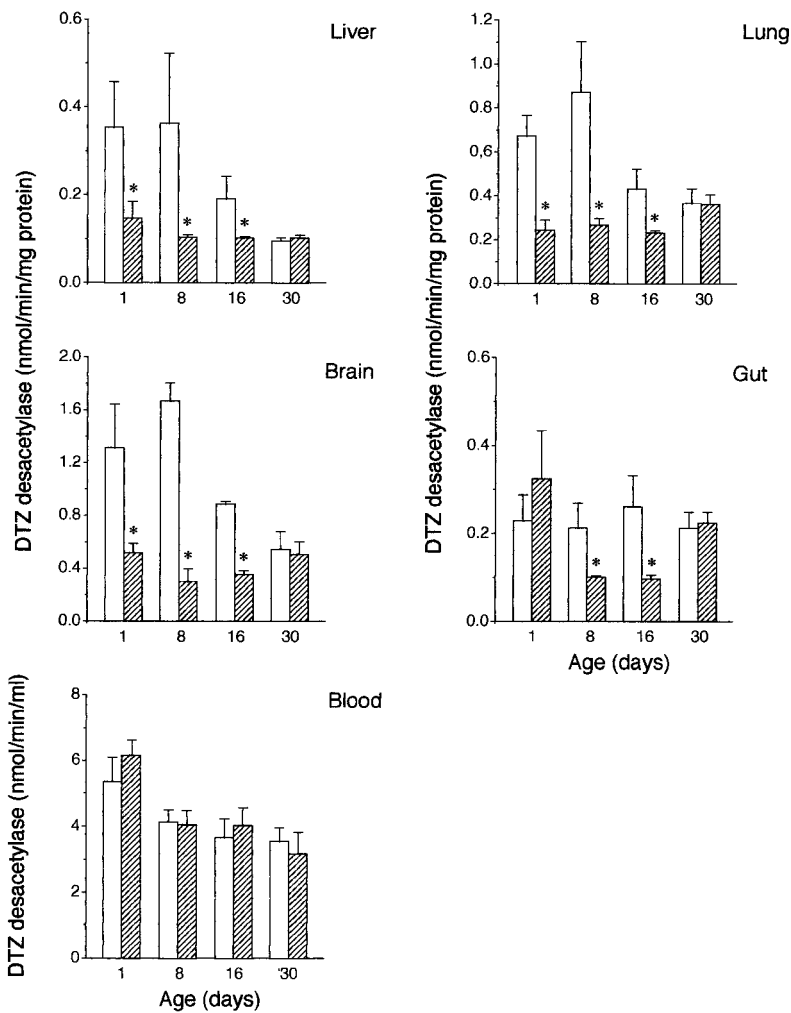


Figure 2. DTZ deacetylase activity measured in the 9000g supernatant of several tissues homogenates and in whole blood from rabbits at different post-natal ages whose mothers received (▨) or did not receive (□) chronic treatment with DTZ from mid-gestation to term. The incubation conditions are described in the Materials and methods and data are given as mean $\pm$ SD ($n = 4$). * Significantly different from control ($p < 0.05$).

negligible. Liver and gut homogenates from 1-, 8- and 16-day-old exposed rabbits displayed $\sim 25\%$ of the activity observed in adults ($p < 0.05$). However, the DTZ demethylase activity observed in blood was unchanged throughout the ages studied. Comparison between the DTZ demethylase activity observed in tissue homogenates and in whole blood from treated and untreated newborn rabbits is also shown in figure 3. As can be observed, no differences were apparent between exposed and unexposed rabbits.

Discussion

Newborn and young rabbits showed a high DTZ deacetylase activity in hepatic and extrahepatic tissues, confirming that different organs can metabolize DTZ and

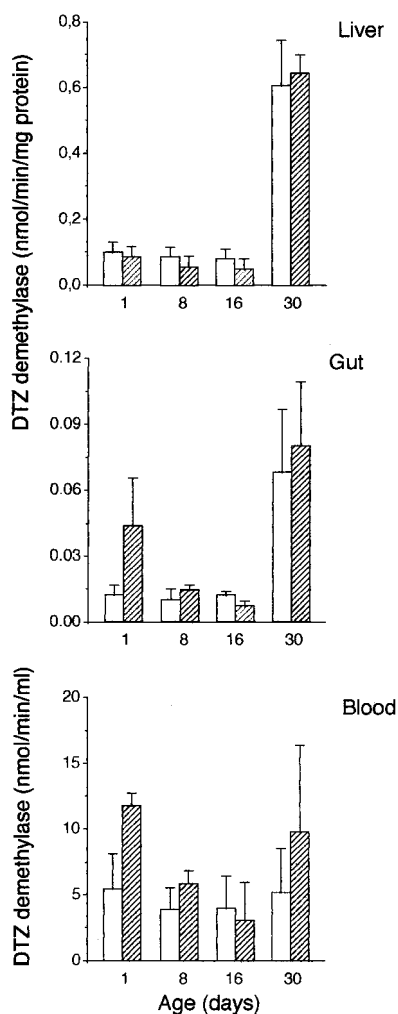


Figure 3. DTZ demethylase activity measured in the 9000g supernatant of several tissues homogenates and in whole blood from rabbits at different post-natal ages whose mothers received (▨) or did not receive (□) chronic treatment with DTZ from mid-gestation to term. The incubation conditions are described in the Materials and methods and data are given as mean $\pm$ SD ($n = 4$). * Significantly different from unexposed rabbits ($p < 0.05$).

thus contribute to the overall elimination of the drug, early in development. As described, degradation of DTZ through the deacetylation pathway is either enhanced or shows no changes in newborn and young rabbits if compared with values observed in adults (Fraile *et al.* 1997).

For the first time, it has been described that intra-uterine chronic exposure to DTZ affects its own metabolism in offspring. It has been demonstrated that an impaired formation of M1 in neonatal and young rabbits whose mothers received DTZ chronic administration from mid-gestation to term, compared with unexposed newborns. Lefebvre *et al.* (1996) reported that the production of M1 was reduced in lung and intestinal homogenates of animals receiving a chronic administration of DTZ. The present results support these findings and demonstrate that the formation of M1 was also reduced in the liver under the experi-

mental conditions. This effect seems to disappear after 30 days of life. A possible explanation for this latter experimental finding may be the physiological turnover of the esterase involved in DTZ metabolism. So, the new esterase formed will be able to metabolize efficiently this drug.

Intra-uterine exposure to DTZ does not seem to alter the neonatal DTZ demethylase activity as compared with unexposed rabbits. The findings disagree with previously published reports on DTZ metabolism inhibition after chronic treatment. In fact, adult patients chronically administered with this drug undergo a rise in DTZ blood levels after some weeks of treatment (Kates 1983, Hermann and Morselli 1985). Furthermore, chronic administration of DTZ has been related to altered clearance of drugs which are metabolized throughout common metabolic pathways. In fact, it is now well established that in adults DTZ inhibits its own metabolism through interaction with enzymes from the P450 superfamily (CYP3A), but the ultimate mechanism is not yet understood although this aspect has been discussed (Sutton *et al.* 1997, Jones *et al.* 1999).

It is now conventional knowledge that, at birth, many functions are developed at a rate lower than that predicted on size basis (Nouws 1992). In general, this rule holds in processes involving drug elimination. In fact, it is widely accepted that during development, microsomal mixed-function oxidase activity increases over several weeks, depending on the particular enzyme and species, after which it plateaus or decreases slightly to reach the adult level (Schwark 1992). In this sense, the lack of effect of chronic exposure to DTZ on DTZ N-demethylase activity may be the consequence of the lack of previous activity of the enzymes. However, other factors may contribute to explain this result. For example, DTZ concentrations in foetuses may not be high enough to yield an appreciable reduction on DTZ demethylation in young rabbits. Alternatively, a more prolonged time of exposure could be required to yield a postnatal alteration in this reaction.

The findings reported here suggest that intra-uterine chronic exposure to DTZ has a large and prolonged effect on newborn metabolism deacetylase activity compared with unexposed rabbits.

Acknowledgements

The work was supported by the Spanish DGICYT (PB92-0350), L. J. F. is supported by the Diputacion General de Aragón (BCB-17/93). The authors thank Dr R. Latini and the Istituto di Ricerche Farmacologiche 'Mario Negri' for providing the diltiazem metabolites.

References

- BREGANTE, M. A., ARAMAYONA, J. J., FRAILE, L. J., GARCIA, M. A. and SOLANS, C., 2000, Diltiazem blood pharmacokinetics in pregnant and non-pregnant rabbits. Maternal and fetal tissue levels. *Xenobiotica*, **30**, 831-841.
- FRAILE, L. J., ARAMAYONA, J. J., BREGANTE, M. A., GARCIA, M. A. and ABADIA, A. R., 1996, Deacetylation of diltiazem by several rabbit tissues. *Pharmaceutical Research*, **13**, 1874-1879.
- FRAILE, L. J., ARAMAYONA, J. J., GARCIA, M. A. and BREGANTE, M. A., 1997, Enhanced diltiazem deacetylase activity in pre-term and full-term rabbits compared with adult rabbits. *Biology of the Neonate*, **72**, 51-61.
- HERMANN, P. and MORSELLI, P. L., 1985, Pharmacokinetics of diltiazem and other calcium channel blockers. *Acta Pharmacologica and Toxicologica*, **57**, 10-20.

- HOLBROOK, R. H., GIBSON, R. N. and VOSS, E. M., 1988, Tocolytic and cardiovascular effects of the calcium antagonist diltiazem in the near-term pregnant rabbit. *American Journal of Obstetrics and Gynecology*, **159**, 591-595.
- HOMSY, W., LEFEBVRE, M., CAILLE, G. and DU SOUICH, P., 1995, Metabolism of diltiazem in hepatic and extrahepatic tissues of rabbits: *in vitro* studies. *Pharmaceutical Research*, **12**, 609-614.
- HUSSAIN, M. D., TAM, Y. K., GRAY, M. R. and COUTTS, R. T., 1994, Mechanisms of time-dependent kinetics of diltiazem in the isolated perfused rat liver. *Drug Metabolism and Disposition*, **22**, 36-42.
- JONES, D. R., GORSKI, J. C., HAMAN, M. A., MAYHEW, B. S., RIDER, S. and HALL, S. D., 1999, Diltiazem inhibition of cytochrome P-450 3A activity is due to metabolite intermediate complex formation. *Journal of Pharmacology and Experimental Therapeutics*, **290**, 1116-1125.
- KATES, R. E., 1983, Calcium antagonists. Pharmacokinetic properties. *Drugs*, **25**, 113-124.
- LEBOEUF, E. and GRECH-BELANGER, O., 1987, Deacetylation of diltiazem by rat liver. *Drug Metabolism and Disposition*, **15**, 122-126.
- LEFEBVRE, M., CAILLE, G. and DU SOUICH, P., 1996, Organ-specific pattern of inhibition of diltiazem metabolism at steady state in rabbits. *Journal of Pharmacology and Experimental Therapeutics*, **279**, 902-907.
- LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. and RANDALL, R. J., 1951, Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry*, **193**, 265-275.
- MURRAY, M. and BUTLER, A. M., 1996, Enhanced inhibition of microsomal cytochrome P450 3A2 in rat liver during diltiazem biotransformation. *Journal of Pharmacology and Experimental Therapeutics*, **279**, 1447-1452.
- NOUWS, J. F. M., 1992, Pharmacokinetics in immature animals: a review. *Journal of Animal Science*, **70**, 3627-3634.
- PICHARD, L., GILLET, G., FABRE, I., DALET-BELUCHE, I., BONLS, C., THENOT, J. P. and MAUREL, P., 1990, Identification of the rabbit and human cytochromes P-450III A as the major enzymes involved in the N-demethylation of diltiazem. *Drug Metabolism and Disposition*, **18**, 711-719.
- RENTON, K. W., 1985, Inhibition of hepatic microsomal drug metabolism by the calcium channel blockers diltiazem and verapamil. *Biochemical Pharmacology*, **34**, 2549-2553.
- SCHWARK, W. S., 1992, Factors that affect drug disposition in food-producing animals during maturation. *Journal of Animal Science*, **70**, 3635-3645.
- SUGAWARA, Y., NAKAMURA, S., USUKI, S., ITO, Y., SUZUKI, T., OHASHI, M. and HARIGAYA, S., 1988, Metabolism of diltiazem. II. Metabolic profile in rat, dog and man. *Journal of Pharmacobiodynamics*, **11**, 224-233.
- SUTTON, D., BUTLER, A. M., NADIN, L. and MURRAY, M., 1997, Role of CYP3A4 in human hepatic diltiazem N-demethylation: Inhibition of CYP3A4 activity by oxidized diltiazem metabolites. *Journal of Pharmacology and Experimental Therapeutics*, **282**, 294-300.
- WEIR, M. R., 1995, Diltiazem: ten years of clinical experience in the treatment of hypertension. *Journal of Clinical Pharmacology*, **35**, 220-232.
- YABANA, H., NAGAO, T. and SATO, M., 1985, Cardiovascular effects of the metabolites of diltiazem in dogs. *Journal of Cardiovascular Pharmacology*, **7**, 152-157.
- YEUNG, P. K. F., FENG, J. Z. and BUCKLEY, S. J., 1998, Pharmacokinetics and hypotensive effect of diltiazem in rabbits: comparison of diltiazem with its major metabolites. *Journal of Pharmacy and Pharmacology*, **50**, 1247-1253.
- YEUNG, P. K. F., MONTAGUE, T. J., TSUI, B. and MCGREGOR, C., 1989, High-performance liquid chromatographic assay of diltiazem and six of its metabolites in plasma: application to a pharmacokinetic study in healthy volunteers. *Journal of Pharmaceutical Science*, **78**, 592-597.
- YEUNG, P. K. F., MOSHER, S. J., QUILLIAM, M. A. and MONTAGUE, T. J., 1990, Species comparison of pharmacokinetics and metabolism of diltiazem in humans, dogs, rabbits and rats. *Drug Metabolism and Disposition*, **18**, 1055-1059.



Metabolism of 2,5-bis(trifluoromethyl)-7-benzyloxy-4-trifluoromethylcoumarin by human hepatic CYP isoforms: evidence for selectivity towards CYP3A4

A. B. RENWICK†, D. F. V. LEWIS‡, S. FULFORD§,
D. SURRY§, B. WILLIAMS§, P. D. WORBOYS§,
X. CAI¶, R. W. WANG¶, R. J. PRICE†,
B. G. LAKE†* and D. C. EVANS¶

† TNO BIBRA International Ltd, Woodmansterne Road, Carshalton, Surrey
SM5 4DS, UK

‡ School of Biological Sciences, University of Surrey, Guildford, Surrey GU2 5XH, UK
§ Merck Sharp and Dohme Ltd, Neuroscience Research Centre, Terlings Park, Eastwick
Road, Harlow, Essex CM20 2QR, UK

¶ Drug Metabolism, Merck & Co., 126 East Lincoln Avenue, Rahway, NJ 07065, USA

Received 7 November 2000

1. The metabolism of 2,5-bis(trifluoromethyl)-7-benzyloxy-4-trifluoromethylcoumarin (BFBFC) to 7-hydroxy-4-trifluoromethylcoumarin (HFC) was studied in human liver microsomes and in cDNA-expressed human liver CYP isoforms. For purposes of comparison, some limited studies were also performed with 7-benzyloxyquinoline (7BQ).

2. Initial interactive docking studies with a homology model of human CYP3A4 indicated that BFBFC was likely to be a selective substrate for CYP3A4 with a relatively high binding affinity, due to the presence of several key hydrogen bonds with active site amino acid residues.

3. Kinetic analysis of NADPH-dependent BFBFC metabolism to HFC in three preparations of pooled human liver microsomes revealed mean ($\pm$ SEM) K_m and $V_{max} = 4.6 \pm 0.3 \mu M$ and 20.0 ± 3.8 pmol/min/mg protein, respectively.

4. The metabolism of BFBFC to HFC was determined in a characterized bank of 24 individual human liver microsomal preparations employing a BFBFC substrate concentration of $10 \mu M$ (i.e. around twice K_m). Good correlations ($r^2 = 0.736-0.904$) were observed between BFBFC metabolism and markers of CYP3A isoforms.

5. While $10 \mu M$ BFBFC was metabolized to HFC by cDNA-expressed CYP3A4, little or no metabolism was observed with cDNA-expressed CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6 and CYP2E1.

6. The metabolism of $10 \mu M$ BFBFC in human liver microsomes was markedly inhibited by 5-50 μM troleandomycin and 0.2-5 μM ketoconazole, but stimulated by 0.2-10 μM α -naphthoflavone. The metabolism of $10 \mu M$ BFBFC in human liver microsomes was also markedly inhibited by an antibody to CYP3A4.

7. Kinetic analysis of NADPH-dependent 7BQ metabolism to 7-hydroxyquinoline (7HQ) in human liver microsomes revealed K_m and $V_{max} = 70 \mu M$ and 3.39 nmol/min/mg protein, respectively.

8. While $80 \mu M$ 7BQ was metabolized to 7HQ by cDNA-expressed CYP3A4, only low rates of metabolism were observed with cDNA-expressed CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6 and CYP2E1.

9. In summary, by correlation analysis, the use of cDNA-expressed CYP isoforms, chemical inhibition and inhibitory antibodies, BFBFC metabolism in human liver

* Author for correspondence; e-mail: blake@tnobibra.co.uk

microsomes appears to be primarily catalysed by CYP3A4. BFBFC may be a useful fluorescent probe substrate for human hepatic CYP3A4, but compared with 7BQ has only a low rate of metabolism in human liver microsomes.

Introduction

Isoforms of the cytochrome P450 (CYP) superfamily are known to play a major role in the oxidative metabolism of both xenobiotics and certain endogenous compounds (Parkinson 1996). Many studies have demonstrated that members of the human hepatic CYP1A, CYP2A, CYP2B, CYP2C, CYP2D, CYP2E and CYP3A subfamilies can metabolize a wide variety of therapeutic agents (Wrighton *et al.* 1993, Nelson *et al.* 1996, Parkinson 1996, Pelkonen *et al.* 1998). In recent years extensive use has been made of *in vitro* techniques employing human liver microsomes and cDNA-expressed human liver CYP isoforms in order to identify which CYP isoforms will be involved in the metabolism of a new chemical entity and to gain information on the potential for *in vivo* drug interactions (Wrighton *et al.* 1993, Crespi *et al.* 1997, 1998, Clarke 1998, Pelkonen *et al.* 1998, Rodrigues 1999).

One requirement for conducting *in vitro* metabolism studies is the availability of suitable probe substrates for the CYP isoforms of interest. In this respect substrates that are metabolized to highly fluorescent products are more amenable for use in rapid screens than substrates which require chromatographic separation of the metabolites prior to quantitation by spectroscopic, radiometric or mass spectrometry techniques.

Various fluorescent substrates are available as metabolic probes for human hepatic CYP isoforms. For example, 7-ethoxyresorufin is often employed as a probe substrate for CYP1A2 in liver microsomes (Clarke 1998, Pelkonen *et al.* 1998). Other studies have demonstrated that derivatives of 7-hydroxy-4-trifluoromethylcoumarin (HFC) may also be used as substrates for human hepatic CYP isoforms. For example, 7-ethoxy-4-trifluoromethylcoumarin has been reported to be metabolized by a number of CYP isoforms including CYP1A2, CYP2B6, CYP2C9 and CYP2E1 (Buters *et al.* 1993, Code *et al.* 1997), while 7-methoxy-4-trifluoromethylcoumarin and 7-benzyloxy-4-trifluoromethylcoumarin (BFC; figure 1) have been reported to be good substrates, respectively, for cDNA-expressed CYP2C9 and CYP3A4 (Crespi *et al.* 1999, Stresser *et al.* 2000). With respect to CYP3A isoforms, 3-(3,4-difluorobenzyloxy)-5,5-dimethyl-4-(4-methylsulphonylphenyl)-5H-furan-2-one (DFB) has been reported to be a specific fluorescent probe for CYP3A4/5 in human liver microsomes (Chauret *et al.* 1999), while BFC, 7-benzyloxyresorufin (7BR) and 7-benzyloxyquinoline (7BQ) have all been used as fluorescent substrates for cDNA-expressed CYP3A4 (Crespi *et al.* 1997, 1998, 1999, Stresser *et al.* 2000). However, recent studies on the metabolism of BFC by human hepatic CYP isoforms demonstrated that BFC was not a specific probe for CYP3A4, but rather was metabolized by CYP1A2, CYP3A4 and to a lesser extent by other CYP isoforms (Renwick *et al.* 2000). While a correlation was reported between 7BR metabolism and CYP3A4 apoprotein levels in human liver microsomes (Forrester *et al.* 1992), studies with inhibitory antibodies suggested that 7BR was not a specific substrate for CYP3A isoforms (Burke *et al.* 1994). Additional molecular modelling studies and investigations with cDNA-expressed CYP isoforms have demonstrated that 7BR is

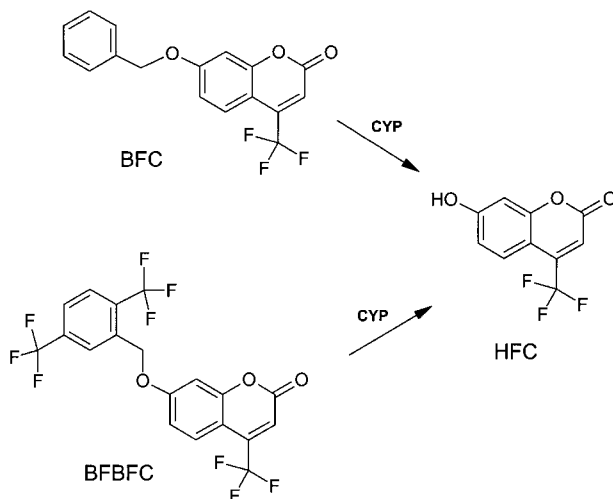


Figure 1. Metabolism of BFC and BFBFC to HFC.

metabolized by a number of CYP isoforms including CYP2B6 and CYP3A4 (Waxman *et al.* 1991, Ono *et al.* 1996, Lewis *et al.* 1999a).

The aim of the present study was to develop a more specific HFC derivative as a fluorescent probe substrate for CYP3A isoforms in human liver microsomes. Initial *in vitro* metabolism and molecular modelling studies with a number of BFC analogues (Lake, Lewis, Williams and Evans, unpublished observations) suggested that 2,5-bis(trifluoromethyl)-7-benzyloxy-4-trifluoromethylcoumarin (BFBFC; figure 1) may be metabolized by human hepatic CYP3A4. The human hepatic CYP isoform specificity of BFBFC was investigated by 'reaction phenotyping' studies, employing correlation analysis, the use of cDNA-expressed human CYP isoforms, chemical inhibition and inhibitory antibodies. In addition, interactive docking studies were performed with a homology model of human CYP3A4. Finally, some limited studies were also conducted to examine the human CYP isoform specificity of 7BQ.

Materials and methods

Materials

NADPH, NADP⁺, glucose 6-phosphate, glucose 6-phosphate dehydrogenase, Tris, α -naphthoflavone, diethyldithiocarbamate, quinidine, sulphaphenazole and troleandomycin were from Sigma-Aldrich Co. (Poole, UK), BFC, HFC, ketoconazole and *S*(+)-mephenytoin were from Salford Ultrafine Chemicals and Research Ltd (Manchester, UK) and 7BQ and 7HQ were from GENTEST Corporation (Woburn, MA, USA). Samples of BFBFC (L-871,408; purity > 98%) and its 3,5-isomer, namely 3,5-bis(trifluoromethyl)-7-benzyloxy-4-trifluoromethylcoumarin (BTBFC; L-871,420; purity > 98%), together with inhibitory antibodies against CYP2C8/9, CYP2D6 and CYP3A4, were provided by MSD Research Laboratories (Harlow, UK). The chemicals and the inhibitory antibodies were stored at -20 and -80°C , respectively. The specificity and use of the CYP3A4 antibody has been described by Mei *et al.* (1999). Microsomes from human B-lymphoblastoid cells containing cDNA-expressed human CYP isoforms (GENTEST) were from Cambridge Bioscience (UK) and stored at -80°C . The samples of human B-lymphoblastoid cell microsomes comprised control cell microsomes (i.e. no transfected human CYP isoform cDNA but contains native CYP1A1 activity) and cell microsomes containing CYP1A2, CYP2A6+ OR (i.e. CYP2A6 cDNA plus human NADPH-cytochrome P450 reductase (OR) cDNA), CYP2B6, CYP2C8+ OR, CYP2C9+ OR, CYP2C19, CYP2D6+ OR, CYP2E1+ OR and CYP3A4+ OR. Microsomes from baculovirus-infected insect cells containing cDNA-expressed

human CYP isoforms (all + OR) were provided by Merck Research Laboratories (West Point, PA, USA) and were used for the studies with 7BQ.

Molecular modelling

The three-dimensional structure of CYP3A4 was derived by homology modelling using crystallographic coordinates of the substrate-bound CYP102 haemoprotein domain (Li and Poulos 1997) via methods described by Lewis *et al.* (1996, 1999a, b). The putative substrate, BFBFC, was constructed from a molecular model of BFC, a known non-specific substrate of CYP3A4, which had previously been built from pre-minimized substructural fragments using the Sybyl Fragment Library (Tripos Associates, St Louis, MO, USA). Interactive docking of these substrate molecules was undertaken via the routines (e.g. DOCK) implemented in the Sybyl molecular modelling package (Tripos Associates) prior to energy minimization of the enzyme-substrate complex. All molecular modelling procedures were carried out on a Silicon Graphics Indigo² IMPACT 10000 graphics workstation operating under UNIX.

Human liver microsomes

Samples of human liver (surplus to transplant requirements) were transported on ice to TNO BIBRA from other institutions and were stored at -80°C . Washed microsomal fractions were prepared in 0.154 M KCl containing 50 mM Tris-HCl, pH 7.4, as described (Lake 1987). Liver microsomal fractions were assayed for total CYP content (Lake 1987) and for protein (Lowry *et al.* 1951) employing bovine serum albumin as standard. The washed microsomal fractions were diluted to 10 mg protein ml^{-1} and aliquots stored at -80°C . Three separate batches, designated pools A, B and C, of pooled human liver microsomes were prepared. Each batch was prepared by pooling liver samples from five subjects. Pool A comprised liver samples from males aged 15, 17 and 29 years and females aged 31 and 42 years; pool B comprised liver samples from males aged 2.5 and 58 years and females aged 14, 59 and 74 years and pool C comprised liver samples from male subjects aged 44 and 50 years and female subjects aged 11, 31 and 55 years. Studies were also conducted with a bank of 24 individual human liver microsomal preparations (Renwick *et al.* 1998), which had been previously characterized for total CYP content and CYP isoform enzyme activities. For the studies with 7BQ only, a pool of human liver microsomes (pool D), comprising liver samples from males aged 48, 66 and 68 years and females aged 42 and 60 years, was provided by Merck Research Laboratories (Rahway, NJ, USA).

Metabolism of BFBFC by human liver microsomes

Incubations were performed in Nunc MicroWellTM (Life Technologies Ltd, Paisley, UK) polystyrene 96-well plates with conical wells. The NADPH-dependent metabolism of BFBFC was studied in incubation mixtures containing 2–30 μM BFBFC, 2–50 μg , microsomal protein and 70 mM phosphate buffer, pH 7.4, in a final volume of 100 μl . Stock solutions of BFBFC were prepared in acetonitrile and diluted 1:300 with 100 mM phosphate buffer, pH 7.4, so that the final acetonitrile concentration in the incubations was 0.2% (v/v). Acetonitrile was selected as the solvent for BFBFC and methanol for the inhibitors studied (see below) in order to minimize solvent effects on CYP isoform activities in human liver microsomes and cDNA-expressed human CYP isoform preparations (Chauret *et al.* 1998, Busby *et al.* 1999). For each BFBFC substrate concentration, microsomal protein concentration and incubation time, six wells were incubated with NADPH (tests) and two wells without NADPH (blanks). After mixing, the plates were pre-incubated for 10 min in a 37°C incubator and the reaction then initiated by the addition of 1 mM (final concentration) NADPH. Incubations were performed for 5–90 min at 37°C and were terminated by the addition of 100 μl Tris base in 60% (v/v) acetonitrile. The plates were centrifuged at 260g for 10 min at 4°C and a 150 μl aliquot removed from each well into a white Porvair 96-well plate (Perkin Elmer Ltd, Beaconsfield, UK). All fluorescence measurements were performed with a Perkin Elmer model LS-50B luminescence spectrophotometer equipped with a Perkin-Elmer model LS50 plate reader. Fluorescence measurements were made at excitation and emission wavelengths of 410 and 510 nm, respectively, employing 3 nm slits. Standard curves were constructed with 0–50 pmol HFC/well. The above incubation system was also employed to study the metabolism of 10 μM BTBFC and 20 μM BFC and the effect of BFBFC and BTBFC on the metabolism of 50 μM BFC.

BFBFC metabolism correlation analysis

Incubations were conducted as described above with 10 μM BFBFC (added in 0.2% (v/v) acetonitrile) and 15 μg , microsomal protein for 25 min. For each of the 24 preparations of human liver microsomes six wells were incubated with NADPH and two wells (blanks) without NADPH.

BFBFC metabolism by cDNA-expressed CYP isoforms

For each of the B-lymphoblastoid cell cDNA-expressed CYP isoform preparations and each incubation time, four wells were incubated with NADPH and one well (blank) without microsomal protein. Incubation mixtures contained 10 μM BFBFC (added in 0.2% (v/v) acetonitrile), 1 mM NADPH

and 100 mM phosphate buffer, pH 7.4, in a final volume of 100 μ l. After a 10-min pre-incubation at 37°C, the reaction was initiated with 100 μ g, B-lymphoblastoid cell microsomal protein and incubations terminated after 40 min.

BFBFC metabolism inhibition studies

Incubations were conducted as described above with 10 μ M BFBFC, 15 μ g, human liver microsomal protein and 70 mM phosphate buffer, pH 7.4, for 25 min. For each inhibitor concentration six wells were incubated with BFBFC and two wells (blanks) without substrate. To study the effect of the mechanism-based inhibitors, diethyldithiocarbamate (CYP2E1) and troleandomycin (CYP3A4), the compounds were pre-incubated for 30 min at 37°C with 1 mM NADPH and liver microsomes prior to the addition of BFBFC and a further aliquot of 1 mM NADPH. In the studies with the inhibitors α -naphthoflavone (CYP1A2), sulphaphenazole (CYP2C9), quinidine (CYP2D6) and ketoconazole (CYP3A4) and the substrate *S*-mephenytoin (CYP2C19), the compounds were pre-incubated for 10 min at 37°C with 1 mM NADPH and liver microsomes prior to the addition of BFBFC. Stock solutions of the inhibitors and *S*-mephenytoin were prepared in methanol and added to give a final solvent concentration (i.e. acetonitrile and methanol) in the incubation mixtures of either 0.4 or 0.45% (v/v). The effects of antibodies to CYP2C8/9, CYP2D6 and CYP3A4 were studied in incubation mixtures containing 10 μ M BFBFC, 1 mM NADPH, 15 μ g, human liver microsomal protein, 70 mM phosphate buffer, pH 7.4, and dilutions of each antibody (0.001, 0.01, 0.05 or 0.5 μ l/well) for 25 min. For each concentration of each antibody six wells were incubated with BFBFC and two wells (blanks) without substrate. The antibodies were pre-incubated with the liver microsomes and buffer for 10 min at 37°C prior to the addition of BFBFC.

Metabolism of 7BQ by human liver microsomes and cDNA-expressed CYP isoforms

Incubations were conducted with either 50 μ g human liver microsomal protein or 10 pmol cDNA-expressed human CYP isoforms with 5–800 μ M 7BQ, 1.3 mM NADP⁺, 3.5 mM glucose 6-phosphate, 0.28 units glucose 6-phosphate dehydrogenase, 3.3 mM MgCl₂ and 200 mM phosphate buffer, pH 7.4, in a final volume of 200 μ l. The incubations were performed for either 10 min (cDNA-expressed CYP isoforms) or 20 min (human liver microsomes) in a shaking incubator at 37°C. The formation of 7HQ was monitored at excitation and emission wavelengths of 420 and 530 nm, respectively.

Results

Molecular modelling of BFBFC with CYP3A4

Molecular modelling studies with BFBFC were performed with a model of human hepatic CYP3A4 described previously (Lewis *et al.* 1996, 1999b). Figure 2 shows the preferred orientation of BFBFC within the putative active site of CYP3A4, where a combination of π -stacking and hydrogen-bonded interactions with complementary amino acid residues position the molecule for oxidation at the experimentally observed site, namely, at the benzylic position. In particular, a tyrosine residue (TYR 266) in the putative I helix distal to the haem moiety forms a key hydrogen bond with one of the trifluoromethyl groups. In addition, there is hydrogen bonding between an asparagine sidechain (ASN 74), located within substrate recognition site 1 (Gotoh 1992) and lying within the haem environment, and both the second trifluoromethyl group on the substrate and the oxygen of the coumarin nucleus. Adjacent to this asparagine, a phenylalanine sidechain (PHE 72) forms a π - π interaction with the coumarin nucleus, and a further hydrogen bond between a tyrosine residue (TYR 69) with the trifluoromethyl moiety of the coumarin nucleus serves to 'anchor' this substructure within the CYP3A4 reactive centre, as indicated in figure 2. In contrast, molecular modelling studies with the 3,5-isomer of BFBFC, namely BTBFC, revealed a greatly diminished ability to form hydrogen bonds with the trifluoromethyl groups in the putative active site of CYP3A4 (data not shown).

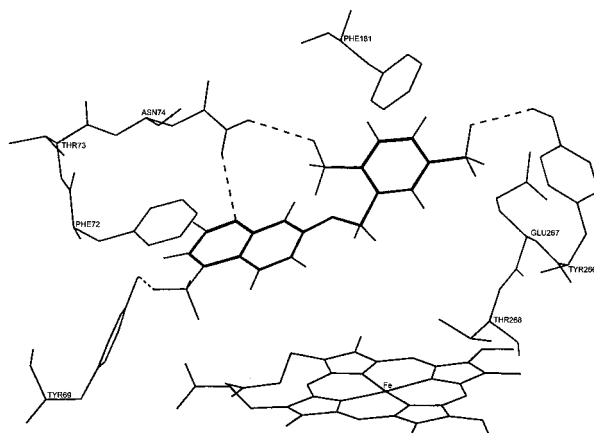


Figure 2. Interaction of BFBFC with the putative active site of human CYP3A4. The putative active site of CYP3A4 is shown with BFBFC docked in the preferred orientation for oxidation at the benzylic position. Hydrogen bonds between BFBFC and key CYP3A4 active site amino acid residues are shown by the dotted lines. The central iron atom of the haem moiety of CYP3A4 is also indicated. For further details see the Results.

Kinetics of BFBFC metabolism in human liver microsomes

Initial BFBFC metabolism studies were conducted with a substrate concentration of $25\text{ }\mu\text{M}$. BFBFC was metabolized by human liver microsomes in the presence of NADPH to HFC (figure 1). The formation of HFC was linear with respect to incubation time for at least 25 min and microsomal protein concentration up to at least $20\text{ }\mu\text{g}$, protein/well (data not shown).

Using three pooled human liver microsomal preparations, the metabolism of BFBFC was investigated over a substrate concentration range of $2\text{--}30\text{ }\mu\text{M}$ (figure 3). Kinetic analysis of Eadie-Hofstee plots revealed $K_m = 4.1$, 4.5 and $5.1\text{ }\mu\text{M}$ for the pool A, B and C human liver microsomal preparations, respectively. Corresponding $V_{\max} = 13.3$, 26.4 and $20.2\text{ pmol/min/mg protein}$, respectively. The observed $V_{\max}$ appeared to correlate with the total CYP content of the pooled liver microsomal preparations, which were 0.39 , 0.72 and $0.51\text{ nmol/mg protein}$ for the A, B and C pools, respectively. For the three pooled human liver microsomal preparations mean ($\pm\text{SEM}$) K_m and $V_{\max} = 4.6 \pm 0.3\text{ }\mu\text{M}$ and $20.0 \pm 3.8\text{ pmol/min/mg protein}$, respectively. A BFBFC substrate concentration of $10\text{ }\mu\text{M}$ (i.e. approximately twice K_m) was selected for the subsequent studies described below.

Comparison of BFBFC metabolism with BFC and BTBFC

The rate of metabolism of BFBFC in human liver microsomes was compared with both BFC and the 3,5-isomer of BFBFC, namely BTBFC. The studies were performed with substrate concentrations of $10\text{ }\mu\text{M}$ for BFBFC (i.e. around twice K_m) and BTBFC and $20\text{ }\mu\text{M}$ for BFC (i.e. around twice the K_m for BFC in human liver microsomes; Renwick *et al.* 2000). Both BFC (figure 4A) and BFBFC (figure 4B) were metabolized to HFC in a time-dependent manner, whereas only trace amounts of BTBFC metabolism was observed (figure 4B). Under the incubation conditions employed, the rate of BFBFC metabolism was only $\sim 6\%$ of that of BFC

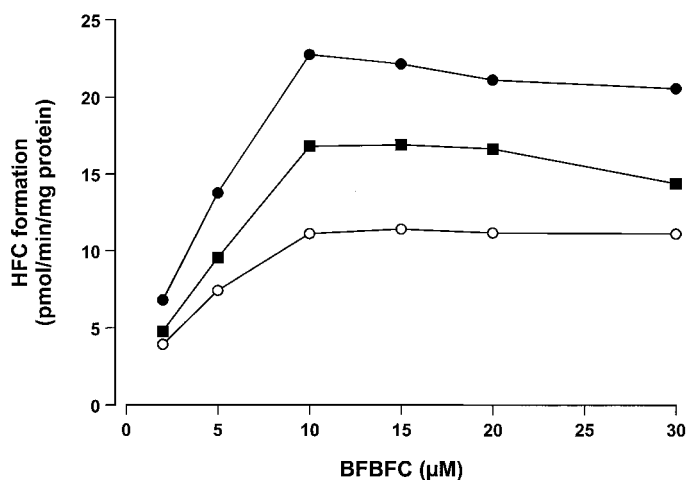


Figure 3. Kinetics of BFBFC metabolism to HFC in three pooled human liver microsomal preparations. Incubations ($n = 6$ wells for each BFBFC substrate concentration) were performed with 15 μg , protein/well of pools A (○), B (●) and C (■) microsomal preparations with 2–30 μM BFBFC for 25 min.

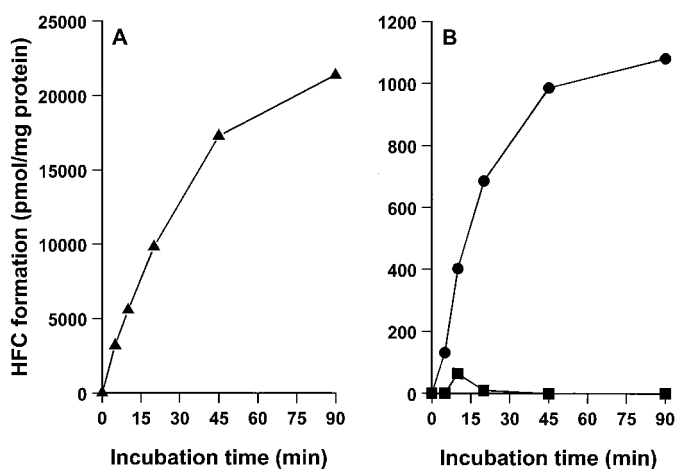


Figure 4. Comparison of the metabolism of BFC, BFBFC and BTBFC to HFC in human liver microsomes. Incubations ($n = 6$ wells for each substrate and time point) were performed with 20 μM BFC (A, ▲), 10 μM BFBFC (B, ●) and 10 μM BTBFC (B, ■) with 15 μg /well microsomal protein for 5–90 min. Results are mean of data obtained with the pool B and C human liver microsomal preparations.

(figure 4A and B). In some additional studies the effect of BFBFC and BTBFC on the metabolism of 50 μM BFC (i.e. a substrate concentration where metabolism is predominantly catalysed by CYP3A4; Renwick *et al.* 2000) in human liver microsomes was studied. BFC metabolism was only inhibited to ~64% of control (mean of studies with pool B and C liver microsome preparations) by either 100 μM BFBFC or 100 μM BTBFC (data not shown).

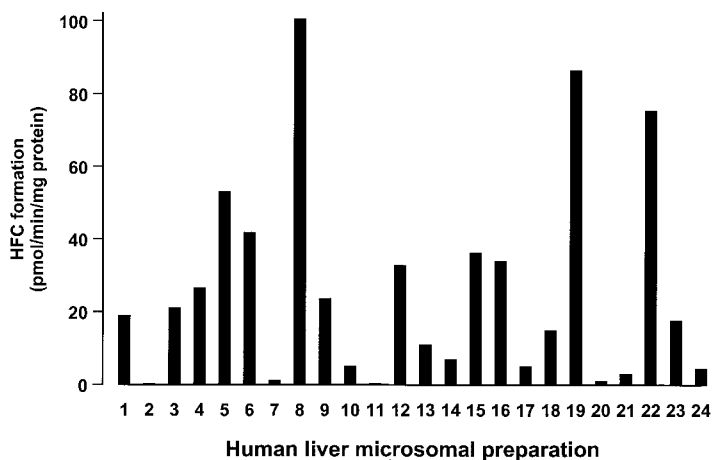


Figure 5. Variability of BFBFC metabolism to HFC in a panel of 24 characterized human liver microsomal preparations. Incubations ($n = 6$ wells for each microsomal preparation) were performed with $10\ \mu\text{M}$ BFBFC and $15\ \mu\text{g/well}$ microsomal protein for 25 min.

BFBFC metabolism correlation analysis

The metabolism of $10\ \mu\text{M}$ BFBFC was examined with a characterized bank of 24 human liver microsomal preparations (Renwick *et al.* 1998). BFBFC was metabolized to HFC by 22 of the 24 liver microsomal preparations examined (figure 5). Microsomal BFBFC metabolism varied from 1 to 101 pmol/min/mg protein.

The rates of BFBFC metabolism were correlated with values for total CYP content and a range of CYP isoform enzyme activities (table 1). Good correlations ($r^2 = 0.736\text{--}0.904$) were observed between BFBFC metabolism to HFC and a number of enzyme activities catalysed by CYP3A isoforms including testosterone 6β -hydroxylase ($r^2 = 0.904$; figure 6H). A good correlation ($r^2 = 0.834$) was also observed between BFBFC metabolism and total CYP content (table 1) whereas a lower correlation ($r^2 = 0.532$) was observed between BFBFC metabolism and CYP2B6 catalysed *S*-mephenytoin N-demethylase activity (figure 6C). Only low correlations ($r^2 = 0.076\text{--}0.328$) were observed between BFBFC metabolism and markers of enzyme activities catalysed by CYP1A2, CYP2A6, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP4A9/11 isoforms (figure 6 and table 1).

Metabolism of BFBFC by cDNA-expressed CYP isoforms

The metabolism of $10\ \mu\text{M}$ BFBFC was examined in GENTEST B-lymphoblastoid cell microsomes containing cDNA-expressed CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4, together with microsomes from control (wild-type) cells. BFBFC was metabolized to HFC by the cDNA-expressed CYP3A4 preparation (figure 7), whereas only very low rates of BFBFC metabolism were observed with the cDNA-expressed CYP2B6 preparation and with the control cell microsomes (which contain native CYP1A1). In contrast, BFBFC was not metabolized to HFC by the cDNA-expressed CYP1A2, CYP2A6, CYP2C8, CYP2C9, CYP2C19, CYP2D6 and CYP2E1 preparations (figure 7).

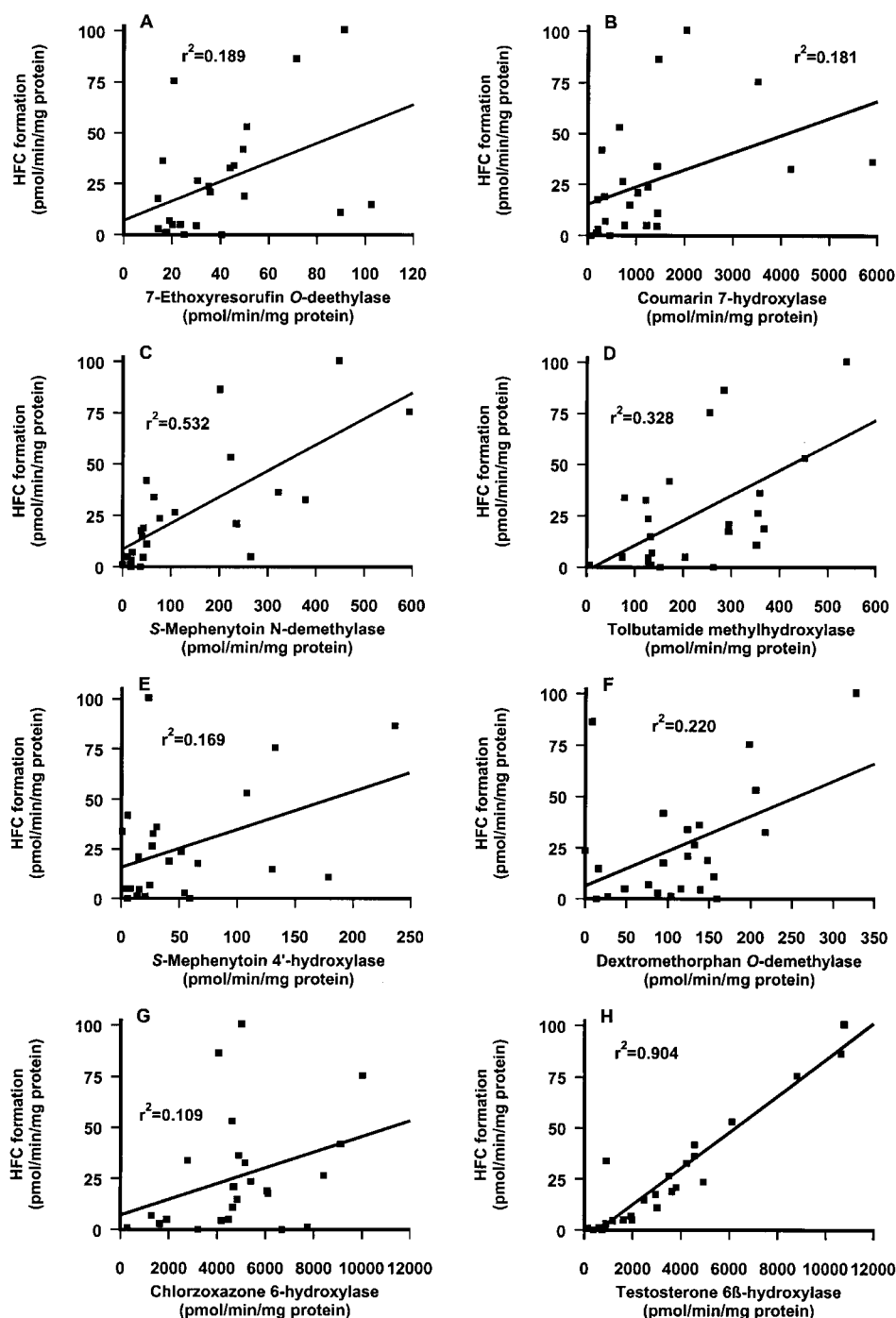


Figure 6. Correlation between BFBFC metabolism to HFC and some CYP isoform enzyme activities in a panel of 24 characterized human liver microsomal preparations. Incubations ($n = 6$ wells for each microsomal preparation) were performed with $10 \mu\text{M}$ BFBFC and $15 \mu\text{g/well}$ microsomal protein for 25 min. The metabolism of BFBFC was compared with enzymatic markers of CYP1A2 (A), CYP2A6 (B), CYP2B6 (C), CYP2C9 (D), CYP2C19 (E), CYP2D6 (F), CYP2E1 (G) and CYP3A4 (H).

Table 1. Correlation analysis for BFBFC metabolism to HFC with various CYP isoform-dependent enzyme activities in human liver microsomes.

CYP isoform	Enzyme activity ^a	r^2
-	total CYP	0.834
1A2	7-ethoxyresorufin <i>O</i> -deethylase	0.189
	caffeine <i>N</i> 3-demethylase	0.232
2A6	coumarin 7-hydroxylase	0.181
2B6	<i>S</i> -methenytol <i>N</i> -demethylase	0.532
2C9	tolbutamide methylhydroxylase	0.328
2C19	<i>S</i> -mephenytoin 4'-hydroxylase	0.169
2D6	dextromethorphan <i>O</i> -demethylase	0.220
2E1	chlorzoxazone 6-hydroxylase	0.109
3A4/5	testosterone 6 β -hydroxylase	0.904
	testosterone 2 β -hydroxylase	0.897
	testosterone 15 β -hydroxylase	0.897
	diazepam 3-hydroxylase	0.893
	caffeine 8-hydroxylase	0.844
	dextromethorphan <i>N</i> -demethylase	0.736
4A9/11	lauric acid 12-hydroxylase	0.076

^a Levels of total CYP content and CYP isoform enzyme activities were determined in a panel of 24 individual human liver microsomal preparations (Renwick *et al.* 1998). These values (plotted on the *x* axis) were then plotted against the data obtained for the metabolism of 10 μ M BFBFC (figure 5) by the same panel of human liver microsomes (plotted on the *y*-axis) and the various correlations determined.

Inhibition of BFBFC metabolism

The effect of some known CYP inhibitors and one substrate (*S*-mephenytoin) on the metabolism of 10 μ M BFBFC in human liver microsomes (pool B) was studied. For the mechanism-based inhibitors (Newton *et al.* 1995) diethyldithiocarbamate and troleandomycin, the compounds were pre-incubated with NADPH and the liver microsomes for 30 min prior to the addition of BFBFC and another aliquot of NADPH. While treatment with 10–50 μ M diethyldithiocarbamate had little effect on BFBFC metabolism, the addition of 5 μ M troleandomycin inhibited BFBFC metabolism to < 2% of control, with a complete inhibition of enzyme activity being observed with 10–50 μ M troleandomycin (figure 8A). The addition of either 50–500 μ M *S*-mephenytoin or 2–20 μ M quinidine had little effect on BFBFC metabolism, whereas some inhibition of enzyme activity was observed by treatment with 2–20 μ M sulphaphenazole (figure 8B). In contrast, BFBFC metabolism was markedly stimulated to up to 325% of control by treatment with 0.2–10 μ M α -naphthoflavone (figure 8B). Ketoconazole was a potent inhibitor of BFBFC metabolism, inhibiting enzyme activity to 46% of control at a concentration of 0.2 μ M, with a complete inhibition of enzyme activity being observed by the addition of 0.5–5 μ M ketoconazole (figure 8B).

The effect of three available inhibitory antibodies to CYP2C8/9, CYP2D6 and CYP3A4 on BFBFC metabolism by human liver microsomes was also investigated. Using two pooled human liver microsomal preparations (pools B and C), treatment with 0.001–0.5 μ l/well (i.e. 0.001–0.5 μ l/100 μ l incubation mixture) of either the CYP2C8/9 or the CYP2D6 antibody had little effect on BFBFC metabolism (figure 9A and B). In contrast, the addition of 0.01 μ l/well of the CYP3A4 antibody had a marked effect on BFBFC metabolism, with HFC

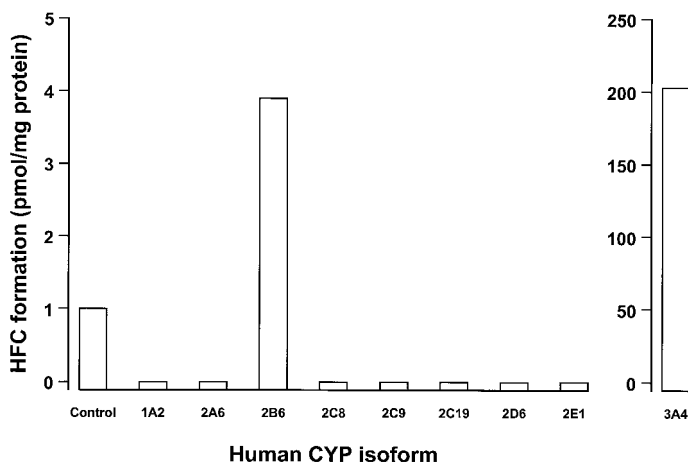


Figure 7. Metabolism of BFBFC by B-lymphoblastoid cell microsomes containing cDNA-expressed human CYP isoforms. Incubations ($n = 4$ wells for each microsomal preparation) were performed with $10 \mu\text{M}$ BFBFC and $100 \mu\text{g/well}$ B-lymphoblastoid cell microsomal protein for 40 min.

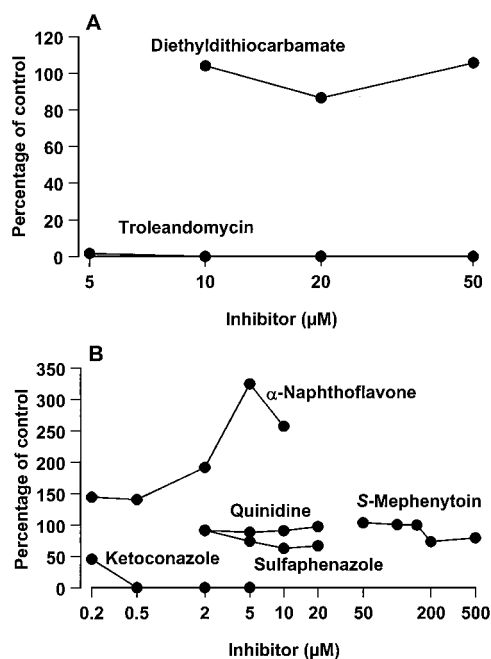


Figure 8. Inhibition of BFBFC metabolism in human liver microsomes. To study the effects of 10 – $50 \mu\text{M}$ diethyldithiocarbamate and 5 – $50 \mu\text{M}$ troleandomycin (A), but not 0.2 – $10 \mu\text{M}$ α -naphthoflavone, 2 – $20 \mu\text{M}$ sulphaphenazole, 50 – $500 \mu\text{M}$ *S*-mephenytoin, 2 – $20 \mu\text{M}$ quinidine and 0.2 – $5 \mu\text{M}$ ketoconazole (B), the compounds were pre-incubated with liver microsomes and NADPH for 30 min at 37°C prior to the addition of $10 \mu\text{M}$ BFBFC. Incubations ($n = 6$ wells for each inhibitor concentration) were performed with $15 \mu\text{g}$ protein/well of the pool B liver microsomal preparation for 25 min and the results are expressed as percentage of control (no inhibitor added) incubations. Control rates of metabolism of BFBFC to HFC were 9.1 and $10.1 \text{ pmol/min/mg protein}$ for the mechanism-based inhibitors (A) and the other compounds (B) respectively.

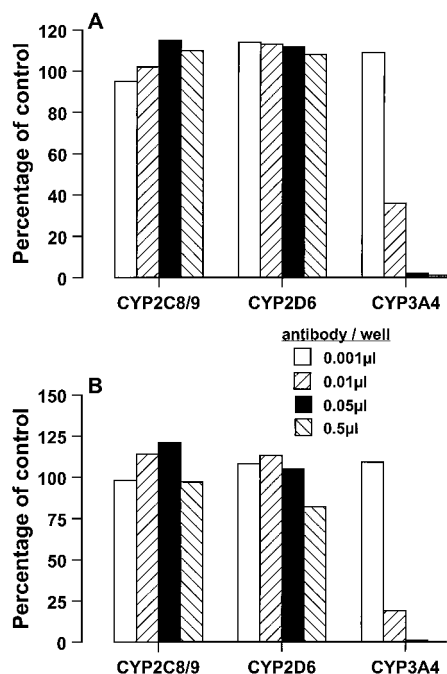


Figure 9. Effect of inhibitory antibodies on BFBFC metabolism in human liver microsomes. Inhibitory antibodies (0.001, 0.01, 0.05 and 0.5 μ l/well) to CYP2C8/9, CYP2D6 and CYP3A4 were pre-incubated with the pool B (A) and C (B) liver microsomal preparations for 10 min at 37°C prior to addition of 10 μ M BFBFC. Incubations ($n = 6$ wells for each inhibitory antibody concentration) were performed with 15 μ g/well microsomal protein for 25 min and the results are expressed as percentage of control (no antibody added) incubations. Control rates of BFBFC metabolism to HFC by the pool B (A) and C (B) human liver microsomal preparations were 18.3 and 13.5 pmol min/mg protein, respectively.

formation being inhibited by $\geq 98\%$ by the addition of 0.05 and 0.5 μ l/well of the CYP3A4 inhibitory antibody (figure 9A and B). Additional studies demonstrated that CYP2C9 catalysed tolbutamide methylhydroxylase was inhibited to 25% of control by the addition of 0.5 μ l/100 μ l incubation mixture of the CYP2C8/9 antibody, whereas no inhibition was observed by the addition of (1.0 μ l/100 μ l incubation mixture of the CYP3A4 antibody (data not shown).

Kinetics of 7BQ metabolism in human liver microsomes

The NADPH-dependent metabolism of 7BQ to 7HQ was studied with the pool D liver microsomal preparation over a substrate concentration range of 5–800 μ M (figure 10). Observed K_m and $V_{max} = 70 \mu$ M and 3.39 nmol/min/mg protein, respectively.

Metabolism of 7BQ by cDNA-expressed CYP isoforms

The metabolism of 80 μ M 7BQ (i.e. a substrate concentration similar to the K_m for 7BQ metabolism in human liver microsomes) was studied in baculovirus-infected insect cell microsomes containing cDNA-expressed CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4 (figure

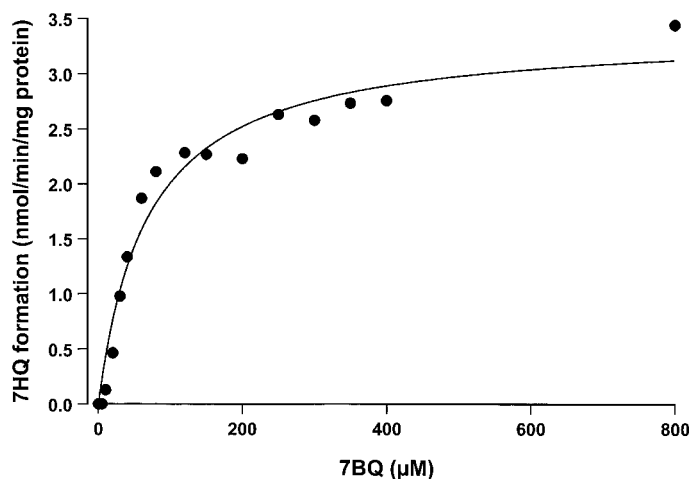


Figure 10. Kinetics of 7BQ metabolism to 7HQ in the pool D human liver microsomal preparation. Duplicate incubations were performed with 5-800 μM 7BQ substrate and 50 μg , human liver microsomal protein for 20 min.

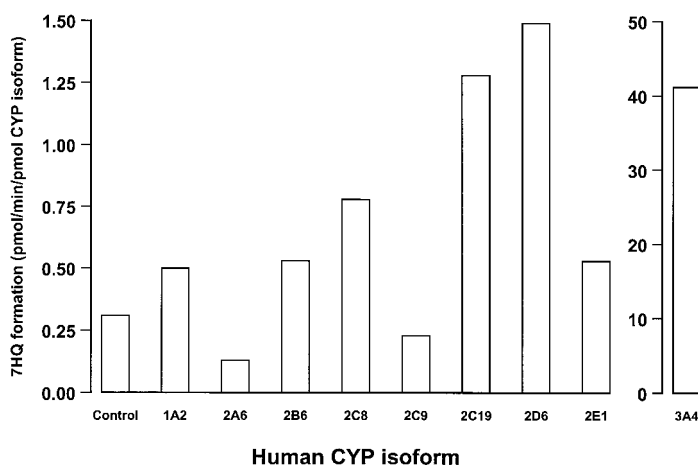


Figure 11. Metabolism of 7BQ by baculovirus-infected insect cell microsomes containing cDNA-expressed human CYP isoforms. Duplicate incubations were performed with 80 μM 7BQ substrate and 10 pmol of the cDNA-expressed CYP isoforms for 10 min.

11). 7BQ was metabolized to 7HQ by the cDNA-expressed CYP3A4 preparation, whereas only very low rates of 7BQ metabolism were observed with the control cell microsomes and the other cDNA-expressed CYP isoforms.

Discussion

In this study the metabolism of BFBFC to HFC (figure 1) by human liver microsomal preparations and cDNA-expressed CYP isoforms has been investigated. For purposes of comparison some more limited studies were performed with 7BQ.

In a previous study conducted in our laboratory BFC was shown not to be a specific substrate for CYP3A4 in human liver microsomes (Renwick *et al.* 2000). However, in this investigation initial molecular modelling studies demonstrated that BFBFC could be docked in the putative active site of our CYP3A4 model (Lewis *et al.* 1996, 1999b) in an orientation for oxidation at the experimentally observed site, namely at the benzylic site (figure 2). In this position, the substrate is held in place by a number of key hydrogen bonds, and a π - π stacking interaction, with nearby amino acid residues. No molecular modelling studies were performed with 7BQ.

BFBFC metabolism to HFC was observed in three preparations of pooled liver microsomes. Additional studies would be required to determine if BFBFC could be metabolized to products other than HFC in human liver microsomes. Investigations into the kinetics of BFBFC metabolism to HFC in human liver microsomes revealed a mean ($\pm$ SEM, $n = 3$) $K_m = 4.6 \pm 0.3 \mu\text{M}$. The K_m for BFBFC metabolism is thus similar to that of BFC, where values of $8.3 \pm 1.3 \mu\text{M}$ (mean $\pm$ SEM, $n = 4$; Renwick *et al.* 2000) and $14.2 \pm 3.4 \mu\text{M}$ (mean $\pm$ SEM, $n = 3$; Surry and Evans, unpublished data) were previously observed. Despite the similarity in K_m , the rate of BFBFC metabolism to HFC in human liver microsomes is much lower than that of BFC (figure 4). The 3,5-isomer of BFBFC, namely BTBFC, was also synthesized. However, this compound is clearly not suitable as a CYP isoform fluorescent substrate, as it was not metabolized to HFC in human liver microsomes to any significant extent (figure 4). Moreover, neither BTBFC nor BFBFC were potent inhibitors of BFC metabolism in human liver microsomes.

In this study 'reaction phenotyping' (Parkinson 1996, Rodrigues 1999) for BFBFC metabolism was performed by correlation analysis, the use of cDNA-expressed CYP isoforms, chemical inhibition and the use of inhibitory antibodies. The studies were conducted with $10 \mu\text{M}$ BFBFC (i.e. around twice K_m). The best correlations ($r^2 = 0.736$ - 0.904) with BFBFC metabolism to HFC were obtained with testosterone 2β -, 6β - and 15β -hydroxylases, diazepam 3-hydroxylase, caffeine 8-hydroxylase and dextromethorphan *N*-demethylase (table 1). These enzyme activities are considered to be catalysed by CYP3A isoforms in human liver (Wrighton *et al.* 1993, Gorski *et al.* 1994, Tassaneeyakul *et al.* 1994, Parkinson 1996, Yang *et al.* 1999). A good correlation ($r^2 = 0.834$) was also observed between BFBFC metabolism and total CYP content. This may be attributable to the fact that CYP3A isoforms are relatively abundant in human liver microsomes (Wrighton *et al.* 1993, Shimada *et al.* 1994, Clarke 1998, Rodrigues 1999). In contrast to the CYP3A isoform markers, poorer correlations ($r^2 = 0.076$ - 0.532) were observed between BFBFC metabolism and the enzymatic markers of CYP1A2, CYP2A6, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP4A9/11 studied (table 1).

The metabolism of $10 \mu\text{M}$ BFBFC by cDNA-expressed CYP isoforms was also examined. While BFBFC was extensively metabolized to HFC by the cDNA-expressed CYP3A4 preparation, no detectable BFBFC metabolism was observed with the CYP1A2, CYP2A6, CYP2C8, CYP2C9, CYP2C19, CYP2D6 and CYP2E1 preparations (figure 7). Although detectable BFBFC metabolism was observed with the control B-lymphoblastoid cell microsomes (which contain native CYP1A1) and cDNA-expressed CYP2B6, the rates of BFBFC metabolism were only 0.5 and 2.0%, respectively, of that of cDNA-expressed CYP3A4 when expressed per unit of protein. The activity of the CYP2B6 preparation was only

around 1.0% of that of CYP3A4 when calculated per unit of cDNA-expressed CYP.

The effect of chemical inhibitors and inhibitory antibodies on BFBFC metabolism in human liver microsomes was also investigated. Previous work has demonstrated α -naphthoflavone, sulphaphenazole, *S*-mephenytoin, quinidine, diethyldithiocarbamate and troleandomycin may be considered either inhibitors or substrates of human hepatic CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4, respectively (Wrighton *et al.* 1993, Chang *et al.* 1994, Newton *et al.* 1995, Parkinson 1996, Clarke 1998, Pelkonen *et al.* 1998, Rodrigues 1999). In addition, at low concentrations, ketoconazole is an inhibitor of human hepatic CYP3A4 (Newton *et al.* 1995). BFBFC metabolism to HFC was inhibited by $\geq 98\%$ by 5–50 μM of the CYP3A4 mechanism-based inhibitor troleandomycin and by 0.5–5 μM ketoconazole (figure 8). In contrast, 0.2–10 μM α -naphthoflavone stimulated BFBFC metabolism in human liver microsomes. Other studies have demonstrated that α -naphthoflavone and some other flavonoids can stimulate the metabolism of certain CYP3A substrates in human liver microsomes (Ueng *et al.* 1997, Pelkonen *et al.* 1998). BFBFC metabolism was essentially unaffected by 10–50 μM diethyldithiocarbamate, 50–500 μM *S*-mephenytoin and 5–20 μM quinidine, whereas some inhibition was observed with 5–20 μM sulphaphenazole. However, the relatively small effect of sulphaphenazole was not supported by the other studies (i.e. correlation analysis, use of cDNA-expressed CYP isoforms and inhibitory antibodies). The involvement of CYP3A4 in BFBFC metabolism in human liver microsomes was also demonstrated by the studies with the inhibitory antibodies. At concentrations of 0.01–0.5 $\mu\text{l/well}$ the CYP3A4 antibody inhibited BFBFC metabolism by 64–100%, whereas little effect was observed with the CYP2C8/9 and CYP2D6 antibodies (figure. 9).

Overall, the results of the reaction phenotyping studies demonstrate that BFBFC is primarily metabolized by CYP3A4 in human liver microsomes. Good correlations were obtained between BFBFC metabolism and known CYP3A4-dependent enzyme activities. BFBFC was metabolized by cDNA-expressed CYP3A4 and BFBFC metabolism was inhibited by ketoconazole, troleandomycin and a CYP3A4 inhibitory antibody. These studies were performed with a BFBFC substrate concentration of 10 μM . Additional investigations would be required to ascertain whether BFBFC would be a low affinity substrate for other CYP isoforms at higher substrate concentrations. Like BFBFC, in some limited studies with human cDNA-expressed CYP isoforms, 7BQ was found to be a relatively specific substrate for CYP3A isoforms. Additional reaction phenotyping studies would be required to confirm the CYP isoform specificity of this substrate.

The aim of the current study was to develop a fluorescent substrate with a high specificity for CYP3A4 in human liver microsomes. Although there are many CYP3A4 probe substrates available (Clarke 1998, Pelkonen *et al.* 1998, Kenworthy *et al.* 1999), most are unsuitable for rapid screening purposes as they require chromatographic separation of the metabolites prior to quantitation by spectroscopic, radiometric or mass spectrometry techniques. While cDNA-expressed CYP3A4 can be assayed in rapid 96-well plate format inhibition screens with fluorescent substrates such as BFC and 7-benzoyloxyresorufin (Crespi *et al.* 1997, 1998, 1999), these substrates lack the required specificity for studies with human liver microsomes (Waxman *et al.* 1991, Burke *et al.* 1994, Ono *et al.* 1996, Lewis *et al.* 1999a, Renwick *et al.* 2000). One exception is DFB, which has been recently

reported to be a specific fluorescent probe for CYP3A4 inhibition studies utilizing human liver microsomes in a 96-well plate format (Chauret *et al.* 1999).

The K_m for BFBFC metabolism in human liver microsomes of $4.6\mu\text{M}$ is somewhat lower than the $26\mu\text{M}$ reported for DFB (Chauret *et al.* 1999) and lower than the $70\mu\text{M}$ observed in the present study for 7BQ. However, the reported V_{max} for DFB (Chauret *et al.* 1999) and 7BQ (this study) are 1.0 and $3.39\text{ nmol/min/mg protein}$, respectively, which are much greater than the $V_{\text{max}} = 20.0\text{ pmol/min/mg protein}$ observed for BFBFC. Thus although BFBFC is a specific substrate for CYP3A4, it has only a low turnover which may limit the usefulness of this fluorescent probe. Although the rate of BFBFC metabolism appears low compared with DFB and 7BQ, it may be worthwhile examining whether BFBFC is also a suitable fluorescent probe substrate for rapid CYP3A4 inhibition screening studies using human liver microsomes. One potential advantage of BFBFC over DFB (Chauret *et al.* 1999), is that NADPH does not interfere with the measurement of the HFC product. The use of BFBFC as a fluorescent substrate for CYP isoform induction studies is currently being investigated in our laboratories.

In summary, the present results demonstrate that BFBFC is metabolized to HFC in human liver microsomes. From the results of reaction phenotyping studies BFBFC appears to be a specific fluorescent probe substrate for human hepatic CYP3A4. This study also demonstrates the usefulness of human CYP isoform homology models in the design of new specific CYP substrates. Finally, based on limited studies with cDNA-expressed CYP isoforms 7BQ also appears to be a relatively specific substrate for CYP3A4.

Acknowledgements

The authors are grateful to MSD Research Laboratories, Harlow, Essex, for the provision of a research grant. They thank Dr T. A. Baillie, Ms Q. Mei, Dr T. H. Rushmore and Dr M. Shou, Merck Research Laboratories, West Point, PA, for the generous gifts of the CYP2C8/9, CYP2D6 and CYP3A4 inhibitory antibodies and the cDNA-expressed human CYP isoforms.

References

- BURKE, M. D., THOMPSON, S., WEAVER, R. J., WOLF, C. R. and MAYER, R. T., 1994, Cytochrome P450 specificities of alkoxyresorufin O-dealkylation in human liver microsomes. *Biochemical Pharmacology*, **48**, 923-936.
- BUSBY, W. F., JR, ACKERMANN, J. M. and CRESPI, C. L., 1999, Effect of methanol, ethanol, dimethyl sulphoxide, and acetonitrile on *in vitro* activities of cDNA-expressed human cytochromes P-450. *Drug Metabolism and Disposition*, **27**, 246-249.
- BUTERS, J. T. M., SCHILLER, C. D. and CHOU, R. C., 1993, A highly sensitive tool for the assay of cytochrome P450 enzyme activity in rat, dog and man. Direct fluorescence monitoring of the deethylation of 7-ethoxy-4-trifluoromethylcoumarin. *Biochemical Pharmacology*, **46**, 1577-1584.
- CHANG, T. K. H., GONZALEZ, F. J. and WAXMAN, D. J., 1994, Evaluation of triacetyloandomycin, α -naphthoflavone and diethyldithiocarbamate as selective chemical probes for inhibition of human cytochromes P450. *Archives of Biochemistry and Biophysics*, **311**, 437-442.
- CHAURET, N., GAUTHIER, A. and NICOLL-GRIFFITH, D. A., 1998, Effect of common organic solvents on *in vitro* cytochrome P450-mediated metabolic activities in human liver microsomes. *Drug Metabolism and Disposition*, **26**, 1-4.

- CHAURET, N., TREMBLAY, N., LACKMAN, R. L., GAUTHIER, J.-Y., SILVA, J. M., MAROIS, J., YERGEY, J. Y. and NICOLL-GRIFFITH, D. A., 1999, Description of a 96-well plate assay to measure cytochrome P4503A inhibition in human liver microsomes using a selective fluorescent probe. *Analytical Biochemistry*, **276**, 215-226.
- CLARKE, S. E., 1998, *In vitro* assessment of human cytochrome P450. *Xenobiotica*, **28**, 1167-1202.
- CODE, E. L., CRESPI, C. L., PENMAN, B. W., GONZALEZ, F. J., CHANG, T. K. H. and WAXMAN, D. J., 1997, Human cytochrome P4502B6. Interindividual hepatic expression, substrate specificity, and role in precarcinogen activation. *Drug Metabolism and Disposition*, **25**, 985-993.
- CRESPI, C. L., MILLER, V. P., ACKERMANN, J. M., STRESSER, D. M. and BUSBY, W. F., JR, 1999, Novel high throughput fluorescent cytochrome P450 assays. *Toxicological Sciences*, **48**(1-S), 69.
- CRESPI, C. L., MILLER, V. P. and PENMAN, B. W., 1997, Microtiter plate assays for inhibition of human, drug-metabolizing cytochromes P450. *Analytical Biochemistry*, **248**, 188-190.
- CRESPI, C. L., MILLER, V. P. and PENMAN, B. W., 1998, High throughput screening for inhibition of cytochrome P450 metabolism. *Medical Chemistry Research*, **8**, 457-471.
- FORRESTER, L. M., HENDERSON, C. J., GLANCEY, M. J., BACK, D. J., PARK, B. K., BALL, S. E., KITTERINGHAM, M. R., MCCLAREN, A. W., MILES, J. S., SKETT, P. and WOLF, C. R., 1992, Relative expression of cytochrome P450 isoenzymes in human liver and association with the metabolism of drugs and xenobiotics. *Biochemical Journal*, **281**, 359-368.
- GORSKI, J. C., JONES, D. R., WRIGHTON, S. A. and HALL, S. D., 1994, Characterization of dextromethorphan N-demethylation by human liver microsomes. Contribution of the cytochrome P450 3A (CYP3A) subfamily. *Biochemical Pharmacology*, **48**, 173-182.
- GOTOH, O., 1992, Substrate recognition sites in cytochrome P450 family 2 (CYP2) proteins inferred from comparative analyses of amino acid and coding nucleotide sequences. *Journal of Biological Chemistry*, **267**, 83-90.
- KENWORTHY, K. E., BLOOMER, J. C., CLARKE, S. E. and HOUSTON, J. B., 1999, CYP3A4 drug interactions: correlation of 10 *in vitro* probe substrates. *British Journal of Clinical Pharmacology*, **48**, 716-727.
- LAKE, B. G., 1987, Preparation and characterisation of microsomal fractions for studies of xenobiotic metabolism. In K. Snell and B. Mullock (eds), *Biochemical Toxicology: A Practical Approach* (Oxford: IRL Press), pp. 183-215.
- LEWIS, D. F. V., DICKINS, M., EDDERSHAW, P. J., TARBIT, M. H. and GOLDFARB, P. S., 1999b, Cytochrome P450 substrate specificities, substrate structural templates and enzyme active site geometries. *Drug Metabolism and Drug Interactions*, **15**, 1-49.
- LEWIS, D. F. V., EDDERSHAW, P. J., GOLDFARB, P. S. and TARBIT, M. H., 1996, Molecular modelling of CYP3A4 from an alignment with CYP102: identification of key interactions between putative active site residues and CYP3A-specific chemicals. *Xenobiotica*, **26**, 1067-1086.
- LEWIS, D. F. V., LAKE, B. G., DICKINS, M., EDDERSHAW, P. J., TARBIT, M. H. and GOLDFARB, P. S., 1999a, Molecular modelling of CYP2B6, the human CYP2B isoform, by homology with the substrate-bound CYP102 crystal structure: evaluation of CYP2B6 substrate characteristics, the cytochrome b₅ binding site and comparisons with CYP2B1 and CYP2B4. *Xenobiotica*, **29**, 361-393.
- LI, H. and POULOS, T. L., 1997, The structure of the cytochrome P450_{BM3} haem domain complexed with the fatty acid substrate, palmitoleic acid. *Nature Structural Biology*, **4**, 140-146.
- LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. and RANDALL, R. J. 1951, Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry*, **193**, 265-275.
- MEI, Q., TANG, C., ASSANG, C., LIN, Y., SLAUGHTER, D., RODRIGUES, A. D., BAILLIE, T. A., RUSHMORE, T. H. and SHOU, M., 1999, Role of a potent inhibitory monoclonal antibody to cytochrome P-450 3A4 in assessment of human drug metabolism. *Journal of Pharmacology and Experimental Therapeutics*, **291**, 749-759.
- NELSON, D. R., KOYMANS, L., KAMATAKI, T., STEGEMAN, J. J., FEYEREISEN, R., WAXMAN, D. J., WATERMAN, M. R., GOTOH, O., COON, M. J., ESTABROOK, R. W., GUNSALUS, I. C. and NEBERT, D. W., 1996, P450 superfamily: update on new sequences, gene mapping, accession numbers and nomenclature. *Pharmacogenetics*, **6**, 1-42.
- NEWTON, D. J., WANG, R. W. and LU, A. Y. H., 1995, Cytochrome P450 inhibitors. Evaluation of specificities in the *in vitro* metabolism of therapeutic agents by human liver microsomes. *Drug Metabolism and Disposition*, **23**, 154-158.
- ONO, S., HATANAKA, T., HOTTA, H., SATOH, T., GONZALEZ, F. J. and TSUTSUI, M., 1996, Specificity of substrate and inhibitor probes for cytochrome P450s: evaluation of *in vitro* metabolism using cDNA-expressed human P450s and human liver microsomes. *Xenobiotica*, **26**, 681-693.
- PARKINSON, A., 1996, Biotransformation of xenobiotics. In C. D. Klaassen (ed.), *Casarett and Doull's Toxicology: The Basic Science of Poisons*, 5th edn (New York: McGraw-Hill), pp. 113-186.
- PELKONEN, O., MÄENPÄÄ, J., TAAVITSAINEN, P., RAUTIO, A. and RAUNIO, H., 1998, Inhibition and induction of human cytochrome P450 (CYP) enzymes. *Xenobiotica*, **28**, 1203-1253.
- RENWICK, A. B., MISTRY, H., BALL, S. E., WALTERS, D. G., KAO, J. and LAKE, B. G., 1998, Metabolism of zaleplon by human hepatic cytochrome P450 isoforms. *Xenobiotica*, **28**, 337-348.

- RENWICK, A. B., SURRY, D., PRICE, R. J., LAKE, B. G. and EVANS, D. C., 2000, Metabolism of 7-benzyloxy-4-trifluoromethylcoumarin by human hepatic cytochrome P450 isoforms. *Xenobiotica*, **30**, 955-969.
- RODRIGUES, A. D., 1999, Integrated cytochrome P450 reaction phenotyping. Attempting to bridge the gap between cDNA-expressed cytochromes P450 and native human liver microsomes. *Biochemical Pharmacology*, **57**, 465-480.
- SHIMADA, T., YAMAZAKI, H., MIMURA, M., INUI, Y. and GUENGERICH, F. P., 1994, Interindividual variations in human liver cytochrome P-450 enzymes involved in the oxidation of drugs, carcinogens and toxic chemicals: studies with liver microsomes of 30 Japanese and 30 Caucasians. *Journal of Pharmacology and Experimental Therapeutics*, **270**, 414-423.
- STRESSER, D. M., MILLER, V. P., BLANCHARD, A., ACKERMANN, J. M., DANDENEAU, A. and CRESPI, C. L., 2000, Cytochrome P450 inhibition assays - the challenges in interpreting data for CYP3A4. *Toxicological Sciences*, **54(1-S)**, 101.
- TASSANEYAKUL, W., BIRKETT, D. J., MCMANUS, M. E., TASSANEYAKUL, W., VERONESE, M. E., ANDERSSON, T., TUKEY, R. H. and MINERS, J. O., 1994, Caffeine metabolism by human hepatic cytochromes P450: contributions of 1A2, 2E1 and 3A isoforms. *Biochemical Pharmacology*, **47**, 1767-1776.
- UENG, Y.-F., KUWABARA, T., CHUN, Y.-J. and GUENGERICH, F. P., 1997, Cooperativity in oxidations catalysed by cytochrome P450 3A4. *Biochemistry*, **36**, 370-381.
- WAXMAN, D. J., LAPENSON, D. P., AOYAMA, T., GELBOIN, H. V., GONZALEZ, F. J. and KORZEKWA, K., 1991, Steroid hormone hydroxylase specificities of eleven cDNA-expressed human cytochrome P450s. *Archives of Biochemistry and Biophysics*, **290**, 160-166.
- WRIGHTON, S. A., VANDENBRANDEN, M., STEVENS, J. C., SHIPLEY, LA., RING, B. J., RETTIE, A. E. and CASHMAN, J. R., 1993, *In vitro* methods for assessing human hepatic drug metabolism: their use in drug development. *Drug Metabolism Reviews*, **25**, 453-484.
- YANG, T. J., KRAUSZ, K. W., SAI, Y., GONZALEZ, F. J. and GELBOIN, H. V., 1999, Eight inhibitory monoclonal antibodies define the role of individual P-450s in human liver microsomal diazepam, 7-ethoxycoumarin and imipramine metabolism. *Drug Metabolism and Disposition*, **27**, 102-109.

Different expression of hepatic and renal cytochrome P450s between the streptozotocin-induced diabetic mouse and rat

T. SAKUMA, R. HONMA, S. MAGUCHI, H. TAMAKI and N. NEMOTO*

Department of Toxicology, Faculty of Pharmaceutical Sciences, Toyama Medical and Pharmaceutical University, Sugitani 2630, Toyama 930-0194, Japan.

Received 11 December 2000

1. Since limited information is available about alterations of cytochrome P450 levels in diabetic animals other than rat, expression of P450s in the liver and kidney of the streptozotocin (STZ)-induced diabetic mouse was investigated.

2. The mRNA levels of CYP2B10, 3A11, 4A10 and 4A14 in the liver were increased in the STZ-induced diabetic mouse of both sexes. The CYP2B9 mRNA level was increased in the liver of the male diabetic mouse. These alterations were observed even at 2 weeks after administration. Insulin treatment restored these changes. The findings were consistent with changes reported in rat.

3. The levels of hepatic CYP1A2 and 2E1 and renal 2E1 and 4A did not change in the diabetic mouse at any time-point examined. No changes were seen in CYP2A- or 2C-related proteins in the diabetic mouse. These findings were in contrast to those in rat.

4. The results indicate that mouse P450s respond to insulin-dependent diabetes mellitus differently from those of the rat, and suggest that the expression of P450s in diabetes is not generally the same across animal species.

Introduction

Microsomal P450 catalyses the oxidative metabolism of a wide variety of lipophilic compounds such as drugs and carcinogens as well as endogenous steroids and fatty acids (Guengerich 1991, Porter and Coon 1991). Therefore, levels of expression of each isoform are important factors determining the biological consequences of xenobiotics. Expression of P450 is altered not only by exposure to various chemicals, but also by diverse hormonal conditions caused by diseases such as diabetes and hypertension (Schenkman *et al.* 1989, Ioannides *et al.* 1996). Among these diseases, extensive studies have concentrated on the effects of type I insulin-dependent diabetes mellitus (IDDM), and the mechanisms involved are reasonably well understood (Shimojo 1994, Ioannides *et al.* 1996). IDDM induces CYP1A2, 2A1, 2B, 2C6, 2C7, 3A1, 3A2, 4A1, 4A2, 4A3 and particularly 2E1 in the rat liver. In contrast, IDDM suppress the expression of CYP2C11, 2C13 and 2A2. These alterations have been explained, at least in part, by the altered pathophysiological conditions in diabetes. Increased levels of ketone bodies appear to induce CYP2E1, 1A2 and 2B (Barnett *et al.* 1988, 1990a, 1992a, Bellward *et al.* 1988, Favreau and Schenkman 1988a,b, Dong *et al.* 1988). Increased levels of free fatty acid seem to induce CYP4A (Kimura *et al.* 1989).

* Author for correspondence; e-mail: tsakuma@ms.toyama-mpu.ac.jp

Decreased levels of pituitary growth hormone appear to account for induction of 2E1, 2A1, 2B and 3A1, and suppression of 2C11 (Favreau and Schenkman 1988a,b, Yamazoe *et al.* 1989a,b, Thummel and Schenkman 1990, Barnett *et al.* 1990b, 1993). Decreased levels of testosterone seem to induce 2A1 and 2C12 (Thummel and Schenkman 1990). Decreased levels of thyroid hormone appear to induce 2A1 and 2B1 (Yamazoe *et al.* 1990).

However, these isoforms have been extensively studied only in the rat, and little information is available about alterations in P450 expression in other species, including humans. Therefore, it has been assumed that the observations in rat can be extrapolated to humans. However, taking into account the large species differences in P450 expression, it is of substantial interest to clarify whether similar alterations are observed in expression of orthologous genes or members in the same gene subfamily in other animal species. Therefore, the authors set out to examine this problem in a genetically related species—the mouse. With regard to the effects of diabetes on the expression of murine P450, although there have been a few reports describing changes in mouse drug-metabolizing enzyme activities in both IDDM and NIDDM models using diagnostic substrates (Knodell *et al.* 1984, Barnett *et al.* 1992b), there have been no studies of alterations in expression levels of extensive P450 isoforms in the IDDM model mouse.

In the current study, we have examined changes in mRNA and protein levels of P450s and of catalytic activities in the liver and kidney of the streptozotocin-induced diabetic ddY mouse.

Materials and methods

Materials

Streptozotocin, methoxyresorufin, pentoxyresorufin and erythromycin were obtained from Sigma Chemical Co. (St Louis, MO, USA). Aniline hydrochloride was from Wako Pure Chemical Industries (Osaka, Japan), a *TaKaRa* RNA PCR kit (AMV) v.2.1 from TaKaRa Shuzo (Shiga, Japan), γ - ^{32} P ATP was from Amersham (Aylesbury, UK), and aqueous suspension of recombinant human crystalline insulin zinc (Novoline U, 40 IU ml $^{-1}$) was from Novo Nordisk A/S (Bagsvaerd, Denmark). Antibodies against rat CYP1A1, rat 2A1, rat 2B1 and rat 2D1 were generous gifts from Dr Y. Funae, Osaka City University. Antibodies against rat CYP2C11, rat 2E1, rat 3A2 and rat 4A1 were purchased from Daiichi Pure Chemicals (Tokyo, Japan). Other chemicals were of the highest grade commercially available.

Animals and treatment

Seven-week old male and female ddY mice and male SD rats were purchased from Sankyo Labo Service (Tokyo, Japan). Mice were given a single i.v. injection of streptozotocin (150 mg kg $^{-1}$ body weight) dissolved in saline and killed 2, 3 or 4 weeks later. Rats were given a single i.v. injection of streptozotocin (65 mg kg $^{-1}$ body weight) and killed 4 weeks later. The animals showing urinary glucose levels of > 100 mg dl $^{-1}$ with Pretest 7a (Wako Pure Chemical Industries, Osaka, Japan) and were judged to be diabetic at 1 week after injection. In some diabetic mice, insulin treatment (100 IU kg day $^{-1}$, s.c.) was started 1 week after the streptozotocin injection. Livers and kidneys were excised immediately after killing the animals and were used for preparation of total RNA and microsomes. Serum glucose was measured by the glucose oxidase method (Schmidt 1961) using a glucose test kit (New Blood Sugar Test, Boehringer Mannheim, Mannheim, Germany).

Analyses of enzyme activities

Liver microsomes were prepared as described by Kamataki and Kitagawa (1974). The protein concentration was determined by the method of Bradford (1976) using Protein Assay (BioRad Laboratories, Inc., Hercules, CA, USA) with bovine serum albumin as a standard. MROD and PROD activities were determined as described by Sakuma *et al.* (1999). The incubation mixture for the assay of aniline *p*-hydroxylase and erythromycin *N*-demethylase activities consisted of 100 mM potassium phosphate (pH 7.4), 0.1 mM EDTA, 1 mg microsomal protein and substrate (5 mM aniline-

HCl or erythromycin). The reaction was started by addition of an NADPH-generating system (0.5 mM NADP⁺, 5 mM glucose 6-phosphate, 1 unit glucose 6-phosphate dehydrogenase, and 5 mM MgCl₂) in a final volume of 1 ml. The activities of aniline *p*-hydroxylase and erythromycin *N*-demethylase were estimated by determination of *p*-aminophenol (Schenkman *et al.* 1967) and formaldehyde (Nash 1953), respectively.

Immunoblotting analysis

Microsomal protein (3 µg) was resolved by SDS-polyacrylamide gel electrophoresis and then transferred onto a nitrocellulose membrane with a blotting system (Transblot-SD, BioRad Laboratories). The localized P450 species were reacted with a specific antibody from a rabbit or goat and stained with a Vectastain ABC kit with horseradish peroxidase (Vector Laboratories, Burlingame, CA, USA). The bases of identification of immunoreactive bands in mouse liver microsomes were as follows. In the case of CYP1A2, it was previously observed that the indicated band appeared at the area estimated from the deduced amino acid sequence, and that changes in the levels of the band corresponded to the changes in the levels of CYP1A2 mRNA (Sakuma *et al.* 1999), while CYP1A1, the other isoform of CYP1A subfamily, appeared at a higher molecular weight. Similarly, in the case of CYP2B9 and 2B10, it was previously observed that the indicated bands appeared at the area estimated from the deduced amino acid sequences, and that changes in the levels of the bands corresponded to the changes in the levels of respective mRNA species (Jarukamjorn *et al.* 1999, Sakuma *et al.* 1999). It is well known that most animal species including the mouse have only one member of the CYP2E subfamily, namely CYP2E1. The sole band, thus, appeared at the area estimated from the deduced amino acid sequence was inferred to be CYP2E1. We have previously demonstrated that both CYP3A11 and 3A41 are major isoforms of the CYP3A subfamily in the adult mouse liver and others are minor ones. CYP3A41 is expressed in only the female, whereas 3A11 is expressed in both sexes. Changes of the levels of the smaller female specific and the higher molecular weight bands observed in both sexes corresponded to changes in CYP3A41 and 3A11 mRNA species, respectively. Thus, we have considered that the higher and lower molecular weight bands are referred to as CYP3A11 and 3A41, respectively (Sakuma *et al.* 2000). In the case of CYP4A, three murine isoforms, CYP4A10, 4A12, and 4A14, have been isolated to date. In the current study, it is demonstrated that CYP4A10 mRNA is expressed in the liver of both sexes, whereas 4A14 mRNA is expressed in the liver of female. Additionally, Bell *et al.* (1993) reported the male-specific expression of CYP4A12 mRNA in the liver, and the same result was also observed in the present preliminary experiments (data not shown). Since two immunoreactive bands detected in the current study have been observed in either sex of the untreated mouse, it is likely that the antibodies can not detect CYP4A10 protein or that the expression level of the isoform is under the limit of detection. Thus, we have considered that the higher male-specific and the lower female-specific bands in hepatic microsomes corresponded to CYP4A14 and 4A12, respectively. On the other hand, we could not identify two immunoreactive bands observed in renal microsomes since sex-specificity of expression of each CYP4A isoform in the kidney has not been confirmed. Therefore, we did not indicate proposed isoforms in immunoblotting of renal microsomes.

Thus, we have indicated the proposed individual isoforms of CYP1A, 2B, 2E, 3A and 4A subfamilies. On the other hand, the existence of at least four, one and seven murine isoforms have been reported to date in the CYP2A, 2C and 2D gene subfamilies, respectively (appeared in the homepage of Dr Nelson: <http://drnelson.utmem.edu/CytochromeP450.html>). However, since informative data about the relative expression level and mobility of each isoform on immunoblotting has not been obtained, we could not identify either band. Thus, in the case of the CYP2A, 2C and 2D gene subfamilies, the position of immunoreactive bands is indicated by arrowheads.

Semiquantitative RT-PCR analysis of mouse P450 mRNA

Semiquantitative RT-PCR was performed using a *TaKaRa* RNA PCR kit (AMV) v.2.1 under the conditions recommended by the supplier. Total RNA was prepared from the livers of mice by the method of Chomczynski and Sacchi (1987) using TRIzol reagent (Life Technologies, Gaithersburg, MD, USA). Amplification and verification of cDNA of members of mouse CYP2B and 3A gene subfamilies, i.e. CYP2B9, 2B10, 3A11, 3A13, 3A16 and 3A41, has been described elsewhere (Nemoto *et al.* 1995, Sakuma *et al.* 2000). Mouse CYP4A-specific primers were selected according to Bell *et al.* (1993) and Henderson *et al.* (1994) using Genetyx-Mac software. The nucleotide sequences of the primers were: CYP4A10/12 sense, 5'-ATGTTTCGAGGGCCAGACAC-3'; 4A10 antisense, 5'-CTTCGGGTTGTGGTGGAGA-3'; 4A12 antisense, 5'-AAAAGGACAGCATGACATGGAT-3'; 4A14 sense, 5'-ATGTTTTCGAGGGTCATGACACTA-3'; 4A14 antisense, 5'-GCATGGC-TATGGTGAGAAGAA-3'. PCR was performed for 18 cycles, consisting of 1 min at 94°C, 1.5 min at 60°C and 2 min at 72°C. PCR of hepatic RNA samples proceeded exponentially under these conditions. The products amplified from CYP4A10, 4A12, 4A14 mRNA were 315, 292 and 383bp in length, respectively. To normalize the applied RNA quantity, aliquots of the cDNA samples were simultaneously amplified by PCR using primers for GAPDH cDNA. The nucleotide sequences of

primers for amplification of GAPDH cDNA and PCR conditions have been described Nemoto *et al.* (1995).

Other Methods

The contents of P450 and cytochrome *b*₅ in liver microsomes were determined according to the method of Omura and Sato (1964). The activity of NADPH-P450 reductase was measured using cytochrome *c* as an electron acceptor (Phillips and Langdon 1962). One unit of reductase was defined as the amount of enzyme that catalysed reduction of 1 μmol cytochrome *c* min⁻¹.

Results

The concentrations of serum glucose in the streptozotocin-induced diabetic ddY mouse are shown in figure 1. The administration of streptozotocin raised the glucose concentration, which remained high for the 4 weeks of the study period. Treatment with insulin (100 IU kg⁻¹ day⁻¹ s.c.) starting 1 week after streptozotocin injection suppressed the increase in glucose concentration in the males in the 2 weeks group. However, other insulin-treated groups showed no significant reductions of the increased glucose concentrations at the time points examined.

Figure 2 shows the contents of P450 and cytochrome *b*₅ and activity of NADPH-P450 reductase in liver microsomes of the streptozotocin-induced diabetic mouse. Although small, but statistically significant, changes of total P450 contents were observed in some groups and no clear alterations were observed. The contents of cytochrome *b*₅ in liver microsomes were not changed by streptozotocin-induced diabetes. This change was in contrast to that in the streptozotocin-induced diabetic rat in which the contents were significantly increased (figure 2, extreme right column in the bottom chart) (Barnett *et al.* 1993). On the other hand, activities of NADPH-P450 reductase in liver microsomes of the streptozotocin-induced diabetic mouse were higher than those in the control mouse. The increased level was restored by treatment with insulin in all males and females at 4 weeks. This change was in contrast to that in the streptozotocin-induced diabetic rat in which the activity was significantly decreased (figure 2, extreme right column in the middle chart).

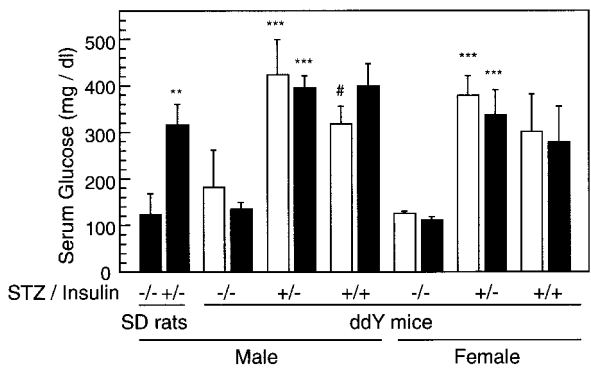


Figure 1. Concentrations of serum glucose in the streptozotocin-induced diabetic mouse. Values are means ± SD of five mice or rats. ** *p* < 0.01, *** *p* < 0.001 relative to control; # *p* < 0.05 relative to diabetes. Duration of diabetes: □, 2 weeks; ■, 4 weeks. Treatment of mice or rats: -/-, no treatment with either streptozotocin or insulin; +/-, treatment with streptozotocin only; +/+, treatment with both streptozotocin and insulin.

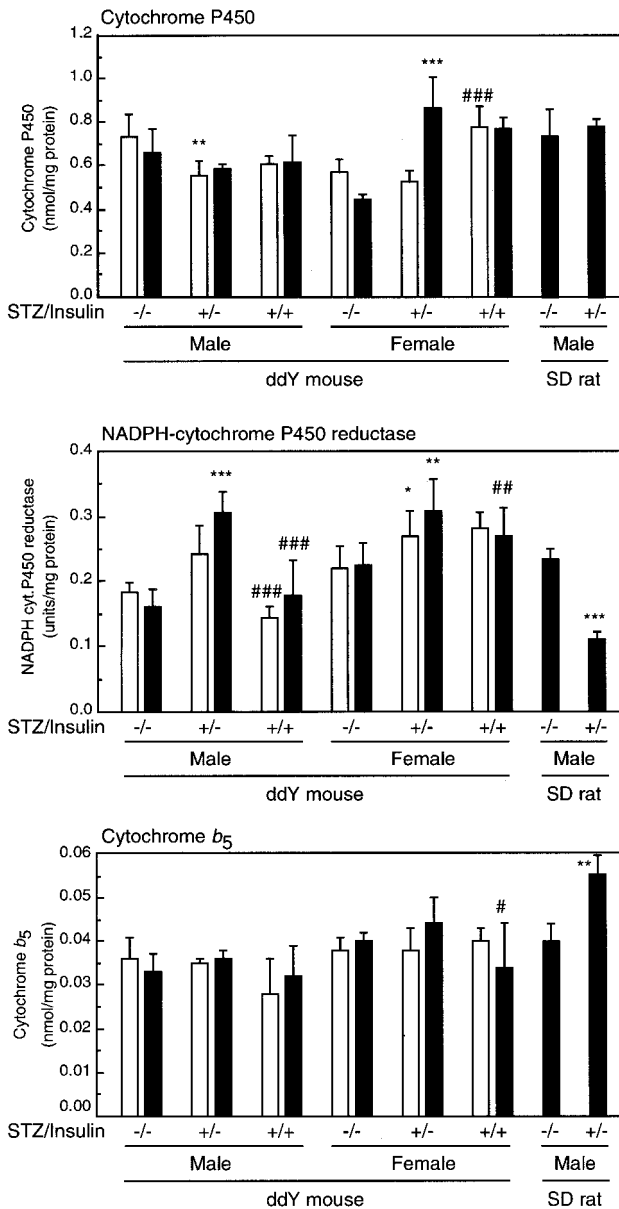


Figure 2. Levels of spectrally determined P450, cytochrome b_5 and activity of NADPH-P450 reductase in liver microsomes of the streptozotocin-induced diabetic mouse. Values are means $\pm$ SD of five mice or rats. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ relative to control; # $p < 0.05$, ### $p < 0.01$, #### $p < 0.001$ relative to diabetes. Duration of diabetes; □, 2 weeks; ■, 4 weeks. Treatment of mice or rats: -/-, no treatment with either streptozotocin or insulin; +/-, treatment with streptozotocin only; +/+ treatment with both streptozotocin and insulin.

The catalytic activities of hepatic microsomes of the streptozotocin-induced diabetic mouse are shown in figure 3. The activities of PROD, an indicator of CYP2B, and erythromycin *N*-demethylase, an indicator of CYP3A, were significantly higher in the streptozotocin-induced diabetic mouse of both sexes after 2 and 4 weeks than in the respective control group. Diabetic mice treated with

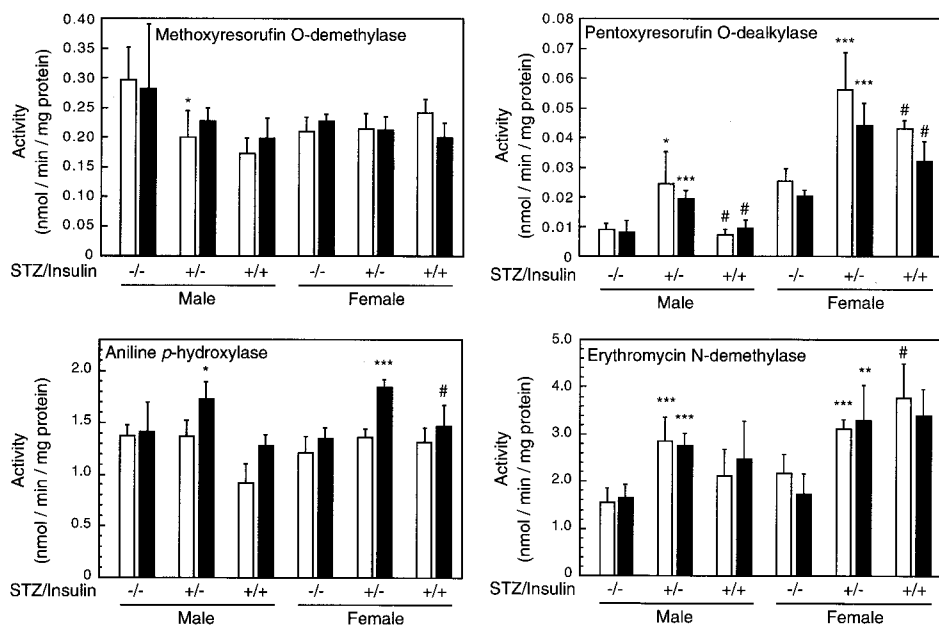


Figure 3. Microsomal activities of methoxyresorufin *O*-demethylase, pentoxyresorufin *O*-dealkylase, aniline *p*-hydroxylase and erythromycin *N*-demethylase in the liver of the streptozotocin-induced diabetic mouse. Values are means $\pm$ SD of five mice or rats. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ relative to control; # $p < 0.05$ relative to diabetes. Duration of diabetes: □, 2 weeks; ■, 4 weeks. Treatment of mice or rats: -/-, no treatment with either streptozotocin or insulin; +/-, treatment with streptozotocin only; +/+ treatment with both streptozotocin and insulin.

insulin had lower PROD activity than that without treatment. However, erythromycin *N*-demethylase activity in the female mouse treated with insulin did not change, whereas a tendency toward restoration of the activity was observed in the male. A small but statistically significant increase in the activity of aniline hydroxylase, an indicator of CYP2E1, was observed 4 weeks after injection in both sexes, and this increase was restored by treatment with insulin. No clear changes in the effect of streptozotocin-induced diabetes on the activity of MROD, an indicator of CYP1A2, were observed in either sex, whereas a statistically significant decrease in the activity was observed in the male streptozotocin-induced diabetic mouse at 2 weeks.

The effects of streptozotocin-induced diabetes on the level of P450 protein in liver microsomes were examined by immunoblotting analysis (figure 4). In accordance with the microsomal enzyme activity, an immunoreactive band predicted to be CYP2B10 was induced by streptozotocin-induced diabetes. Two CYP3A-related bands were detected. The larger band indicated to be CYP3A11 was present in both sexes, whereas the smaller band indicated to be CYP3A41 was detected only in females. In contrast to the microsomal enzyme activity, neither band showed a clear increase in the streptozotocin-induced diabetic mouse. Similarly, two CYP4A-related bands were detected, and the larger indicated to be CYP4A12 and smaller bands indicated to be CYP4A14 were expressed specifically in males and females, respectively. Expression of the smaller band was induced and enhanced by streptozotocin-induced diabetes in the male and female

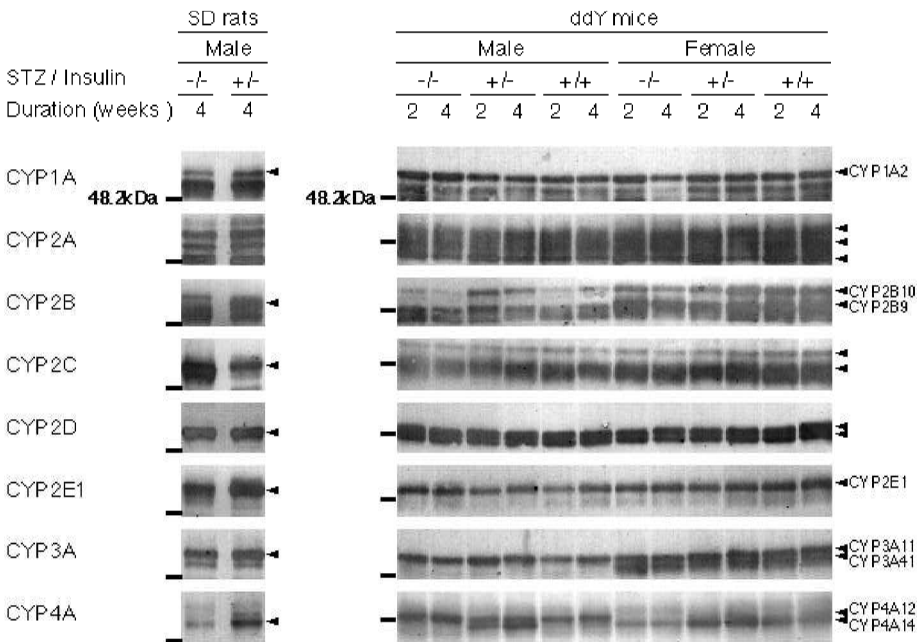


Figure 4. Immunoblotting analysis of liver microsomes from the streptozotocin-induced diabetic mouse. Aliquots of 3 μ g of liver microsomes were used for immunoblotting analysis with antibodies against rat isoforms. The blots are representative of the expression of each subfamily in five animals per treatment group. Arrowheads indicate immunoreactive proteins including both identified and as yet unidentified species. The thick line on the left of each image indicates the position of a molecular weight marker of 48.2 kDa. Treatment of mice or rats: -/-, no treatment with either streptozotocin or insulin; +/-, treatment with streptozotocin only; +/+ treatment with both streptozotocin and insulin.

liver, respectively. These changes were restored by insulin treatment. Two CYP2D-related bands were detected using antibodies against rat 2D1. Streptozotocin-induced diabetes did not alter the intensity of either band. These findings were consistent with the observations about members of the same gene subfamilies in rats (figure 4, left panel) (Ioannides *et al.* 1996). In contrast, the results of the other forms examined, such as CYPs 1A2, 2E1, 2A and 2C, were not consistent with those observed in rat (figure 4, left panel). In agreement with the microsomal activity, CYP1A2 protein was not induced by diabetes. The level of CYP2E1 protein in liver microsomes was not increased in either sex by streptozotocin-induced diabetes even at 4 weeks after administration, while it was slightly induced in the insulin-treated female diabetic mouse. Three and two immunoreactive bands were detected in liver microsomes of mouse using antibodies against rat CYP2A1 and 2C11, respectively. The amounts of those immunoreactive proteins were not changed by streptozotocin-induced diabetes. These results were not consistent with rat CYP2A and 2C subfamilies (figure 4, left panel), in which 2A1 was induced and 2C11 and 2C13 were suppressed by streptozotocin (Favreau and Schenkman, 1988a,b; Thummel and Schenkman 1990, Shimojo *et al.* 1993).

To verify which isoforms changed on treatment, semiquantitative RT-PCR analyses of the members of CYP2B, 3A and 4A gene subfamilies were carried out. Figure 5 shows the changes in CYP2B9 and 2B10 mRNA levels. Induction of CYP2B9 mRNA by streptozotocin-induced diabetes was observed in males,

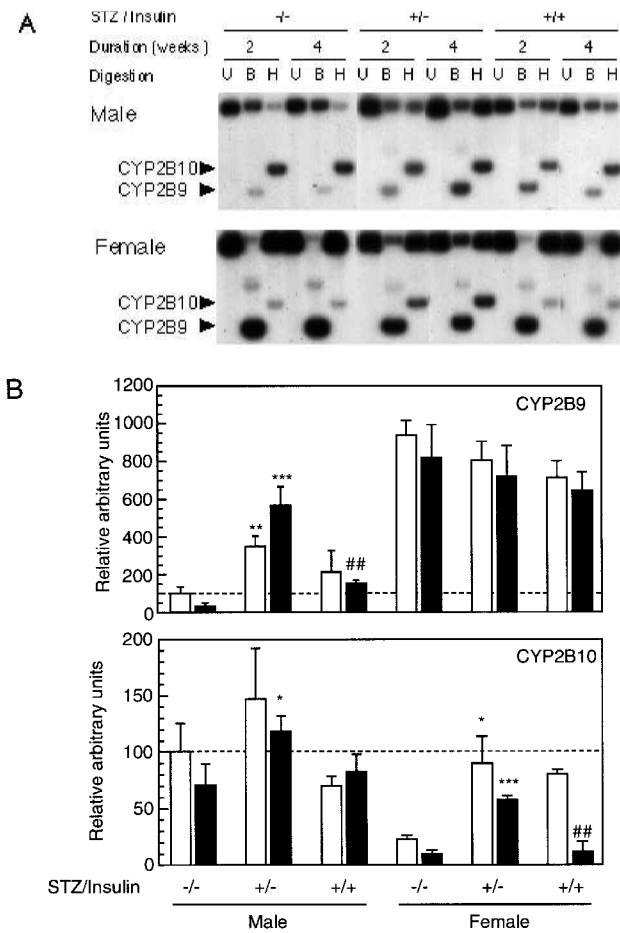


Figure 5. Verification of CYP2B isoforms induced by diabetes in the liver of the streptozotocin-induced diabetic mouse. RT-PCR was performed using hepatic total RNA. The 5'-end labelled antisense primer was used. RT-PCR products were digested with *Bgl* II and *Hha* I, subjected to 12% polyacrylamide gel electrophoresis. Signal intensity was analysed with a Fujix BAS2000 image analyser, and then exposed to X-ray film. The cDNA of CYP2B9 could not be digested by *Hha* I, but could be cut by *Bgl* II, whereas that of 2B10 had the opposite characteristics. (A) Separation of RT-PCR products by polyacrylamide gel electrophoresis. Representative autoradiograms of gel-exposed X-ray films are shown. (B) Relative levels of CYP2B9 and 2B10 mRNAs in the liver. Each CYP2B mRNA level was normalized to the level of GAPDH mRNA and is shown as the relative level in the non-treated male mouse (100). Each column represents the mean $\pm$ SD ($n = 5$). Significance was examined using Student's *t*-test between the control and diabetic mouse and between the diabetic mouse without treatment and that treated with insulin separated according to sex; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with the control mouse, ## $p < 0.01$ compared with the diabetic mouse. U, untreated; B, *Bgl* II digested; H, *Hha* I digested. Treatment of mice or rats: -/-, no treatment with either streptozotocin or insulin; +/-, treatment with streptozotocin only; +/+ treatment with both streptozotocin and insulin.

whereas no changes were observed in females. In accordance with the results of immunoblotting analysis, induction of CYP2B10 mRNA by streptozotocin-induced diabetes was also observed in both sexes. Induction of both isoforms was restored by insulin treatment. We then examined four CYP3A isoforms, i. e. 3A11, 3A13, 3A16 and 3A41, and three CYP4A isoforms, i. e. 4A10, 4A12 and 4A14. Among the members of these subfamilies, three isoforms were affected

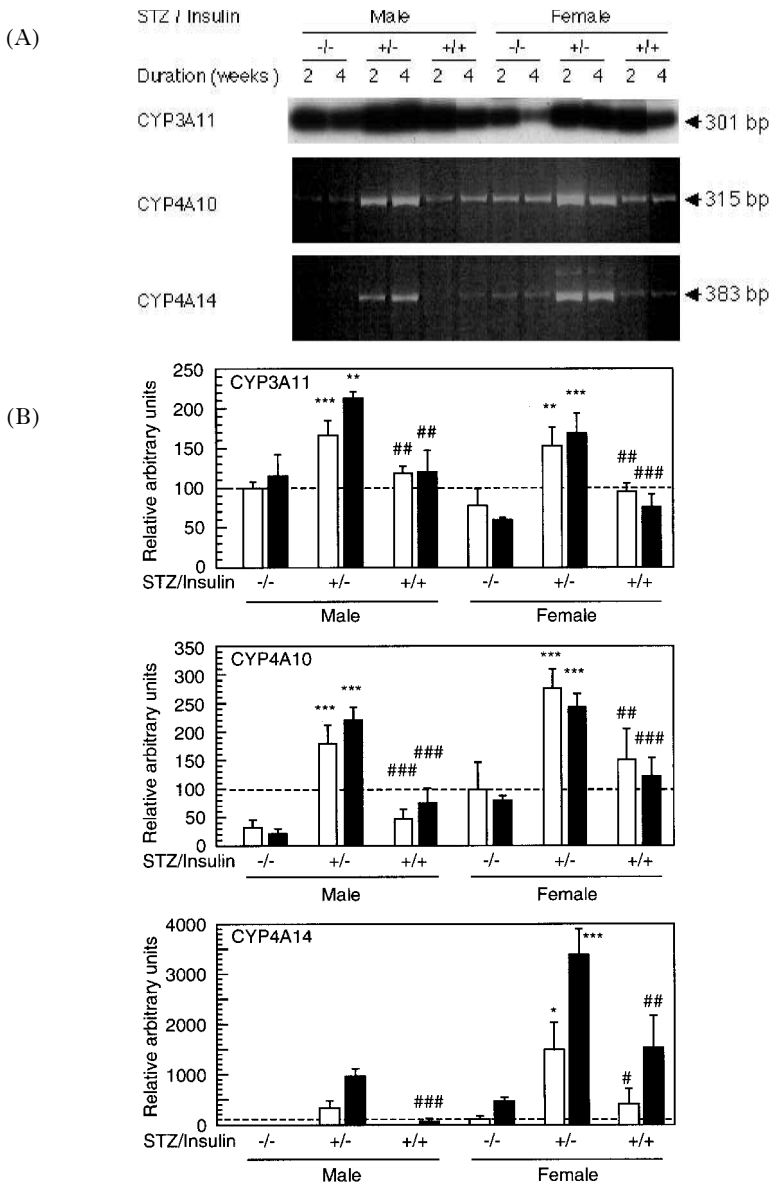


Figure 6. Verification of CYP3A and CYP4A isoforms induced by diabetes in the liver of the streptozotocin-induced diabetic mouse. RT-PCR was performed using hepatic total RNA. In the case of CYP3A subfamily, the 5'-end-labelled antisense primer was used. Distinction of each 3A isoform was carried out by digestion with *Hind* III or *Hinc* II. *Hind* III produces signals of 301, 185 and 96 bases from CYP3A11, 3A13 and 3A16 mRNA, respectively. *Hinc* II produces a signal of 287 bases from CYP3A41 mRNA. Digests were resolved by 5.5% polyacrylamide gel electrophoresis, and then signal intensity was analysed with a Fujix BAS2000 image analyser after

by streptozotocin-induced diabetes (figure 6). CYP3A11 was induced in the streptozotocin-induced diabetic mouse of both sexes, and was restored by insulin treatment. Similar changes, i.e. induction by streptozotocin-induced diabetes and restoration by insulin treatment, were also observed for CYP4A10 and 4A14.

the gel-exposed imaging plate was scanned with a BAS2000 Scanner. In the case of CYP4A, isoform-specific primer sets were used. Thus, the amount of RT-PCR product reflected the amount of mRNA of each isoform in respective samples. RT-PCR products were resolved by 5.5% polyacrylamide gel electrophoresis, and the signal intensity was determined using NIH image software after the ethidium bromide-staining gel image was obtained by ImageDoc™ Documentation System (Sawady Technology, Tokyo, Japan). (A) Separation of RT-PCR products on polyacrylamide gel electrophoresis. Representative autoradiogram (CYP3A11) or images of ethidium bromide-staining gel (CYP4A10 and 4A14) are shown. (B) Relative levels of CYP3A11, 4A10 and 4A14 mRNAs in the liver. CYP mRNA level was normalized to the level of GAPDH mRNA and is shown as the relative level in the non-treated male mouse (100). Each column represents the mean ± SD (n = 5). The Student's *t*-test was used to examine the significance of differences between the control and diabetic mouse and between the diabetic mouse without treatment and that treated with insulin separated according to sex; **p* < 0.05, ***p* < 0.01, ****p* < 0.001 compared with the control mouse; #*p* < 0.05, ##*p* < 0.01, ###*p* < 0.001 compared with the diabetic mouse. Treatment of mice or rats: -/-, no treatment with either streptozotocin or insulin; +/-, treatment with streptozotocin only; ++ treatment with both streptozotocin and insulin.

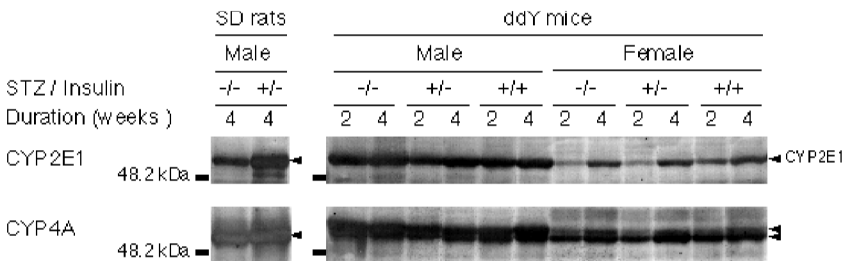


Figure 7. Immunoblotting analysis of renal microsomes from the streptozotocin-induced diabetic mouse. Aliquots of 30 µg renal microsomes were used for immunoblotting analysis with antibodies against rat P450E1 and 4A1. The blots are representative of the expression of each subfamily in five animals per treatment group. Arrowheads indicate immunoreactive bands corresponding to P450 isoforms. The thick line on the left of each image indicates the position of a molecular weight marker of 48.2 kDa. Treatment of mice or rats: -/-, no treatment with either streptozotocin or insulin; +/-, treatment with streptozotocin only; ++ treatment with both streptozotocin and insulin.

The expression of renal CYP2E1 and 4A is also affected by diabetes (Shimojo *et al.* 1993). Therefore, we examined expression of these isoforms by immunoblotting analysis (figure 7). Expression of CYP2E1 in renal microsomes was sexually dimorphic, with higher expression in the male than in the female mouse. However, the level of CYP2E1 was not affected by streptozotocin-induced diabetes. In contrast to hepatic microsomes, two constitutively expressed CYP4A-related bands were detected in renal microsomes from the control mouse of both sexes. However, no diabetes-related changes were observed.

Discussion

This study gave findings indicating marked species differences in P450 expression between rat and mouse in a streptozotocin-induced model of diabetes. Based on the results of studies in rat, it is generally accepted that the changes in P450 in diabetic animals are attributable to metabolic and hormonal alterations (Schenkman *et al.* 1989, Shimojo 1994, Ioannides *et al.* 1996). However, since the underlying mechanisms are complicated and many hormonal interactions and

feedback controls are associated with the pathophysiological condition, it is difficult to identify which factor(s) are responsible for the species differences.

In the current study, treatment with insulin did not show significant reductions of the increased glucose concentrations at the time points examined except in the 2 weeks male. The lack of a clear effect of insulin treatment on serum glucose level in the other groups was probably due to the interval of approximately 1 day between the last injection and the measurement of serum glucose level, although the insulin preparation used was a slow release type.

It has been demonstrated that CYP1A2 level is increased by 2- or 3-fold in rat made diabetic by alloxan or streptozotocin treatment (Yamazoe *et al.* 1988, 1989b, Barnett *et al.* 1990a). In contrast, our immunochemical and catalytic analyses demonstrated that the level of CYP1A2 was not altered in the streptozotocin-induced diabetic ddY mouse, indicating a species-related difference in CYP1A2 expression in diabetic animals. In rat, since diet-induced hyperketonemia enhanced the activity and increased the apoprotein level of CYP1A2 (Barnett *et al.* 1988, 1990a), and acetone treatment induced this P450 (Barnett *et al.*, 1992a), elevated levels of ketone bodies are thought to be a major factor responsible for the induction. However, CYP2E1, another isoform known to be induced by hyperketonemia in rat, also was not induced in the streptozotocin-induced ddY mouse. This species-related difference may be explained by the following two possibilities: lack of machinery to respond to elevated ketone levels of mouse CYP1A2 itself or the streptozotocin-induced diabetic ddY mouse may not show hyperketonemia, at least within the time frame examined in the present study.

It has been demonstrated that microsomal aniline *p*-hydroxylase activity in the rat liver is increased by approximately 1.7-fold even at 2 weeks after administration (Shimojo *et al.* 1993). During the same period, 2.5-8.5-fold induction of CYP2E1 protein was observed (Favreau and Schenkman 1988a, Thummel and Schenkman 1990, Shimojo *et al.* 1993). In contrast, the levels of CYP2E1 protein and its activity in the streptozotocin-induced diabetic ddY mouse were not induced at 2 weeks after administration. CYP2E1 protein was not induced at 4 weeks, whereas slight enhancement of the activity was observed. These results suggested that mouse CYP2E1 is not inducible by streptozotocin-induced diabetes or that factor(s) responsible for induction of P450 were not sufficient to induce this P450 at least within the present experimental period. Similarly to the rat CYP1A2, a number of studies have indicated the major factor responsible for the induction of rat CYP2E1 is elevated levels of ketone bodies (Barnett *et al.* 1988, 1990a, 1992a, Bellward *et al.* 1988, Dong *et al.* 1988, Favreau and Schenkman 1988a, b). Thus, ketone bodies seem to be one of factors which is not elevated to a sufficient level to trigger the induction. In the streptozotocin-treated rat, induction of CYP2E1 reached a maximal level within 4 weeks after the onset of diabetes and was maintained for at least 15 weeks (Favreau and Schenkman, 1988a). Therefore, to clarify whether the kinetics of CYP2E1 changes in diabetic animals are different in mouse and rat, further investigation of the level of CYP2E1 over 4 weeks after the onset of diabetes is necessary. The enhancement of the enzyme at 4 weeks after administration may reflect the contributions of other enzymes that are capable of catalysing the reaction, but this remains to be resolved in future studies.

In rat, in addition to elevated ketone body level, decreased growth hormone secretion has been suggested to be an important factor responsible for the induction of CYP2E1 (Barnett *et al.* 1993). On the other hand, Hong *et al.*

(1990) reported that hypophysectomy has no major effect on the amount of CYP2E1. Therefore, in contrast to rat, mouse CYP2E1 does not seem to be altered by decreased growth hormone secretion even though the streptozotocin-induced diabetic ddY mouse showed low growth hormone levels (Murao *et al.* 1995).

In rat, induction of CYP2B by diabetes is more prominent in males than in females. This sex-related difference has been attributed to the more prominent decrease of growth hormone secretion in the male rat, since the degree of hyperketonemia, another important factor responsible for CYP2B induction in rat, is similar in both sexes (Barnett *et al.* 1993). In the present study, induction of mouse CYP2B9 was observed only in the male mouse, suggesting that factor(s) showing sexual dimorphism may be responsible for the prominent induction in males. On the other hand, induction of CYP2B10 expression by streptozotocin-induced diabetes was observed in both sexes, and this was additive to basal male-dominant expression. Therefore, the induction of the gene may be attributed to factor(s) showing no sex-related differences. One possible factor showing sexual dimorphism is decreased growth hormone secretion as in rat. The possibility that the female-specific expression of CYP2B9 is attributed, at least in part, to the suppressive effect of pulsatile growth hormone, which is characteristic of males, has been demonstrated by the studies of *stat5* genes (Waxman *et al.* 1995, Park *et al.* 1999). Therefore, it is highly possible that the induction of CYP2B9 in the liver of the male diabetic mouse is due to changes of growth hormone secretion as described for CYP2C11 in rat (Favreau and Schenkman 1988a,b, Yamazoe *et al.* 1989b).

In the mouse liver, five isoforms of the CYP3A subfamily are expressed at the mRNA level: 3A11, 13, 16, 25 and 41, and mRNA expression was analysed in four of these isoforms (all except 3A25). Of the isoforms examined, only CYP3A11 showed induction in the livers of the streptozotocin-induced diabetic ddY mouse and restoration by insulin treatment, whereas the other three isoforms were not altered. This finding indicated that each CYP3A isoform has a different sensitivity to diabetes and these CYP3A isoforms are not controlled by uniform regulatory mechanisms. On immunoblotting analysis, the immunoreactive band predicted to be CYP3A11 did not show a clear increase in liver microsomes of the streptozotocin-induced diabetic mouse. The apparent discrepancy between apoprotein and mRNA levels may be because the band included other unaltered CYP3A-related proteins.

In rat, induction of CYP3A isoforms can be attributed to a decrease in pulsatile growth hormone secretion in the male liver. Thus, expression of CYP3A was only stimulated by streptozotocin in the male rat (Barnett *et al.* 1993). On the other hand, induction of mouse CYP3A11 was observed in both sexes, suggesting that the most important factor responsible for the induction is not a decrease in growth hormone secretion.

In rat, induction of CYP4A1, 4A2 and 4A3 is attributable to alterations in fatty acid metabolism (Kimura *et al.* 1989). In the current study, levels of mRNA expression of two isoforms, CYP4A10 and 4A14, were increased by the streptozotocin-induced diabetes in ddY mouse. It has been demonstrated that both isoforms are transcriptionally activated by peroxisome proliferators such as the hypolipidemic drug clofibrate through activation of PPAR α (Heng *et al.* 1997, Barclay *et al.* 1999). In addition to peroxisome proliferators, endogenous com-

pounds such as fatty acids bind and activate PPAR α (Göttlicher *et al.* 1992, Forman *et al.* 1997). Kroetz *et al.* (1998) demonstrated that CYP4A mRNA did not increase in the streptozotocin-induced diabetic PPAR α -deficient mouse. Taking these observations into account, it is likely that the induction of CYP4A10 and 4A14 in the streptozotocin-induced diabetic mouse is the result of activation of PPAR α by increases in free fatty acids levels that occur during the progression of diabetes.

With regard to the species-related differences of drug metabolizing enzymes in other animals, it has been demonstrated that acute and chronic streptozotocin-induced diabetes can induce CYP2E1 and 1A and suppress 2B in the hamster kidney and liver (Chen *et al.* 1996). This latter paper and the present observations indicate the presence of large species-related differences even among rodents. Since many P450 isoforms are involved in the biotransformation of many drugs and carcinogens, it will be important to investigate the ability of diabetes to regulate those xenobiotic metabolizing enzymes in humans.

Acknowledgements

The work was supported in part by Grants-in-Aid for Cancer Research from the Ministry of Education, Culture, Sports and Science of Japan, and by the Smoking Research Foundation. The authors express special thanks to Dr Y. Funae, Osaka City University for generously supplying antibodies against rat CYP1A1, 2A1, 2B1 and 2D1.

References

- BARCLAY, T. B., PETERS, J. M., SEWER, M. B., FERRARI, L., GONZALEZ, F. J. and MORGAN, E. T., 1999, Modulation of cytochrome P-450 gene expression in endotoxemic mice is tissue specific and peroxisome proliferator-activated receptor- α dependent. *Journal of Pharmacology and Experimental Therapeutics*, **290**, 1250-1257.
- BARNETT, C. R., ABBOTT, R. A., BAILEY, C. J., FLATT, P. R. and IOANNIDES, C., 1992b, Cytochrome P450-dependent mixed-function oxidase and glutathione *S*-transferase activities in spontaneous obesity-diabetes. *Biochemical Pharmacology*, **43**, 1868-1871.
- BARNETT, C. R., FLATT, P. R. and IOANNIDES, C., 1988, Role of ketone bodies in the diabetes-induced changes in hepatic mixed-function oxidase activities. *Biochimica Biophysica Acta*, **967**, 250-254.
- BARNETT, C. R., FLATT, P. R. and IOANNIDES, C., 1990a, Induction of hepatic microsomal P450 I and IIB proteins by hyperketonaemia. *Biochemical Pharmacology*, **40**, 393-397.
- BARNETT, C. R., GIBSON, G. G., WOLF, C. R., FLATT, P. R. and IOANNIDES, C., 1990b, Induction of cytochrome P450III and P450IV family proteins in streptozotocin-induced diabetes. *Biochemical Journal*, **268**, 765-769.
- BARNETT, C. R., PETRIDES, L., WILSON, J., FLATT, P. R. and IOANNIDES, C., 1992a, Induction of rat hepatic mixed-function oxidases by acetone and other physiological ketones: their role in diabetes-induced changes in cytochrome P450 proteins. *Xenobiotica*, **22**, 1441-1450.
- BARNETT, C. R., RUDD, S., FLATT, P. R. and IOANNIDES, C., 1993, Sex differences in the diabetes-induced modulation of rat hepatic cytochrome P450 proteins. *Biochemical Pharmacology*, **45**, 313-319.
- BELL, D. R., PLANT, N. J., RIDER, C. G., NA, L., BROWN, S., ATEITALLA, I., ACHARYA, S. K., DAVIES, M. H., ELIAS, E., JENKINS, N. A., GILBERT, D. J., COPELAND, N. G. and ELCOMBE, C. R., 1993, Species-specific induction of cytochrome P-450 4A RNAs: PCR cloning of partial guinea-pig, human and mouse CYP4A cDNAs. *Biochemical Journal*, **294**, 173-180.
- BELLWARD, G. D., CHANG, T., RODRIGUES, B., MCNEILL, J. H., MAINES, S., RYAN, D. E., LEVIN, W. and THOMAS, P. E., 1988, Hepatic cytochrome P450j induction in the spontaneously diabetic BB rat. *Molecular Pharmacology*, **33**, 140-143.
- BRADFORD, M. M., 1976, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, **72**, 248-254.

- CHEN, T.-L., CHEN, S.-H., TAI, T.-Y., CHAO, C.-C., PARK, S. S., GUENGERICH, P. F. and UENG, T.-H., 1996, Induction and suppression of renal and hepatic cytochrome P450-dependent monooxygenases by acute and chronic streptozotocin diabetes in hamsters. *Archives in Toxicology*, **70**, 202-208.
- CHOMCZYNSKI, P. and SACCHI, N., 1987, Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Analytical Biochemistry*, **162**, 156-159.
- DONG, Z., HONG, J., MA, Q., LI, D., BULLOCK, J., GONZALEZ, F. J., PARH, S. S., GELBOIN, H. V. and YANG, C. S., 1988, Mechanism of induction of cytochrome P450ac (P450j) in chemically induced and spontaneously diabetic rats. *Archives in Biochemistry and Biophysics*, **263**, 29-35.
- FAVREAU, L. V. and SCHENKMAN, J. B., 1988a, Composition changes in hepatic microsomal cytochrome P-450 during onset of streptozocin-induced diabetes and during insulin treatment. *Diabetes*, **37**, 577-584.
- FAVREAU, L. V. and SCHENKMAN, J. B., 1988b, Cytochrome P-450 alterations in the BB/Wor spontaneously diabetic rat. *Biochemical Pharmacology*, **37**, 3505-3509.
- FORMAN, B. M., CHEN, J. M. and EVANS, R. M., 1997, Hypolipidemic drugs polyunsaturated fatty acids, and eicosanoids are ligands for peroxisome proliferator-activated receptors α and γ . *Proceedings of the National Academy of Sciences, USA*, **94**, 4312-4317.
- GÖTTLICHER, M., WIDMARK, E., LI, Q. and GUSTAFSSON, J.-Å., 1992, Fatty acid activate a chimera of the clofibrate acid-activated receptor and the glucocorticoid receptor. *Proceedings of the National Academy of Sciences, USA*, **89**, 4653-4657.
- GUENGERICH, P. F., 1991, Reactions and significance of cytochrome P-450 enzymes. *Journal of Biological Chemistry*, **266**, 10019-10022.
- HENDERSON, C. J., BAMMLER, T. and WOLF, C. R., 1994, Deduced amino acid sequence of a murine cytochrome P-450 Cyp4a protein: developmental and hormonal regulation in liver and kidney. *Biochimica et Biophysica Acta*, **1200**, 182-190.
- HENG, Y. M., SHARON KUO, C.-W., JONES, P. S., SAVORY, R., SCHULZ, R. M., TOMLINSON, S. R., GRAY, T. J. B. and BELL, D. R., 1997, A novel murine P-450 gene, Cyp4a14, is part of a cluster of Cyp4a and Cyp4b, but not of CYP4F, genes in mouse and humans. *Biochemical Journal*, **325**, 741-749.
- HONG, J. -Y., NING, S. M., MA, B.-L., LEE, M.-J., PAN, J. and YANG, C. S., 1990, Role of pituitary hormones in the regulation of hepatic cytochrome P450IIE1 in rats and mice. *Archives of Biochemistry and Biophysics*, **281**, 132-138.
- IOANNIDES, C., BARNETT, C. R., IRIZAR, A. and FLATT, P. R., 1996, Expression of cytochrome P450 proteins in disease. In C. Ioannides (ed.), *Cytochrome P450: Metabolic and Toxicological Aspects* (Boca Raton: CRC Press), pp. 301-327.
- JARUKAMJORN, K., SAKUMA, T., MIYAURA, J. and NEMOTO, N., 1999, Different regulation of the expression of mouse hepatic cytochrome P450 2B enzymes by glucocorticoid and phenobarbital. *Archives of Biochemistry and Biophysics*, **369**, 89-99.
- KAMATAKI, T. and KITAGAWA, H., 1974, Effects of lyophilization and storage of rat liver microsomes on activity of aniline hydroxylase, contents of cytochrome b_5 and cytochrome P-450 and aniline-induced P-450 difference spectrum. *Japanese Journal of Pharmacology*, **24**, 195-203.
- KIMURA, S., HARDWICK, J. P., KOZAK, C. A. and GONZALEZ F. J., 1989, The rat clofibrate-inducible CYP4A subfamily. II. cDNA sequence of IVA3, mapping of the Cyp4a locus to mouse chromosome 4, and coordinate and tissue-specific regulation of the CYP4A genes. *DNA*, **8**, 517-525.
- KNODELL, R. G., HANDWERGER, B. S., MORLEY, J. E., LEVINE, A. S. and BROWN, D. M., 1984, Separate influences of insulin and hyperglycemia on hepatic drug metabolism in mice with genetic and chemically induced diabetes mellitus. *Journal of Pharmacology and Experimental Therapeutics*, **230**, 256-262.
- KROETZ, D. L., YOOK, P., COSTET, P., BIANCHI, P. and PINEAU, T., 1998, Peroxisome proliferator-activated receptor α controls the hepatic CYP4A induction adaptive response to starvation and diabetes. *Journal of Biological Chemistry*, **273**, 31581-31589.
- MURAO, S., SATO, M., TAMAKI, M., NIIMI, M., ISHIDA, T. and TAKAHARA, J., 1995, Suppression of episodic growth hormone secretion in the streptozotocin-induced diabetic mouse: time-course studies on the hypothalamic pituitary axis. *Endocrinology*, **136**, 4498-4504.
- NASH, T., 1953, The colorimetric estimation of formaldehyde by means of the Hantzsch reaction. *Biochemical Journal*, **55**, 416-421.
- NEMOTO, N. and SAKURAI, J., 1993, Activation of Cyp1a1 and Cyp1a2 genes in adult mouse hepatocytes in primary culture. *Japanese Journal of Cancer Research*, **84**, 272-278.
- NEMOTO, N., SAKURAI, J. and FUNAE, Y., 1995, Maintenance of phenobarbital-inducible Cyp2b gene expression in C57BL/6 mouse hepatocytes in primary culture as spheroids. *Archives of Biochemistry and Biophysics*, **316**, 362-369.
- NEMOTO, N., SAKURAI, J., TAZAWA, A. and ISHIKAWA, T., 1989, Proline-dependent expression of aryl hydrocarbon hydroxylase in C57BL/6 mouse hepatocytes in primary culture. *Cancer Research*, **49**, 5863-5869.

- OMURA, T. and SATO, R., 1964, The carbon monoxide-binding pigment of liver microsomes. I. Evidence for its haemoprotein nature. *Journal of Biological Chemistry*, **239**, 2370-2378.
- PARK, S.-H., LIU, X., HENNIGHAUSEN, L., DAVEY, H. W. and WAXMAN, D. J., 1999, Distinctive roles of STAT5a and STAT5b in sexual dimorphism of hepatic P450 gene expression: Impact of *Stat5a* gene disruption. *Journal of Biological Chemistry*, **274**, 7421-7430.
- PHILLIPS, A. H. and LANGDON, R. G., 1962, Hepatic triphosphopyridine nucleotide cytochrome *c* reductase: isolation, characterization, and kinetic studies. *Journal of Biological Chemistry*, **237**, 2652-2660.
- PORTER, T. D. and COON, M. J., 1991, Cytochrome P-450: multiplicity of isoforms, substrates, and catalytic and regulatory mechanisms. *Journal of Biological Chemistry*, **266**, 13469-13472.
- SAKUMA, T., OHTAKE, M., KATSURAYAMA, Y., JARUKAMJORN, K. and NEMOTO, N., 1999, Induction of CYP1A2 by phenobarbital in the livers of aryl hydrocarbon-responsive and -nonresponsive mice. *Drug Metabolism and Disposition*, **27**, 379-384.
- SAKUMA, T., TAKAI, M., ENDO, Y., KUROIWA, M., OHARA, A., JARUKAMJORN, K., HONMA, R. and NEMOTO, N., 2000, A novel female-specific member of the CYP3A gene subfamily in the mouse liver. *Archives of Biochemistry and Biophysics*, **377**, 153-162.
- SCHENKMAN, J. B., REMMER, H. and ESTABROOK, R. W., 1967, Spectral studies of drug interaction with hepatic microsomal cytochrome. *Molecular Pharmacology*, **3**, 113-123.
- SCHENKMAN, J. B., THUMMEL, K. E. and FAVREAU, L. V., 1989, Physiological and pathophysiological alterations in rat hepatic cytochrome P-450. *Drug Metabolism Reviews*, **20**, 557-584.
- SCHMIDT, F. H., 1961, Die enzymatische Bestimmung von Glucose und Fructose nebeneinander. *Klinische Wochenschrift*, **39**, 1244-1247.
- SHIMOJO, N., 1994, Cytochrome P450 changes in rats with streptozocin-induced diabetes. *International Journal of Biochemistry*, **26**, 1261-1268.
- SHIMOJO, N., ISHIZAKI, T., IMAOKA, S., FUNAE, Y., FUJII, S. and OKUDA, K., 1993, Changes in amounts of cytochrome P450 isozymes and levels of catalytic activities in hepatic and renal microsomes of rats with streptozotocin-induced diabetes. *Biochemical Pharmacology*, **46**, 621-627.
- THUMMEL, K. E. and SCHENKMAN, J. B., 1990, Effects of testosterone and growth hormone treatment on hepatic microsomal P450 expression in the diabetic rat. *Molecular Pharmacology*, **37**, 119-129.
- WAXMAN, D. J., RAM, P. A., PARK, S. H. and CHOI, H. K., 1995, Intermittent plasma growth hormone triggers tyrosine phosphorylation and nuclear translocation of a liver-expressed, Stat 5-related DNA binding protein. Proposed role as an intracellular regulator of male-specific liver gene transcription. *Journal of Biological Chemistry*, **270**, 13262-13270.
- YAMAZOE, Y., ABUZEID, M., YAMAUCHI, K., MURAYAMA, N., SHIMADA, M. and KATO, R., 1988, Enhancement by alloxan-diabetes of the rate of metabolic activation of three pyrolysate carcinogens via increase in the P-448-H content in rat liver. *Biochemical Pharmacology*, **37**, 2503-2506.
- YAMAZOE, Y., LING, X., MURAYAMA, N., GONG, D., NAGATA, K. and KATO, R., 1990, Modulation of hepatic level of microsomal testosterone 7 α -hydroxylase, P-450a (P450IIA), by thyroid hormone and growth hormone in rat liver. *Journal of Biochemistry*, **108**, 599-603.
- YAMAZOE, Y., MURAYAMA, N., SHIMADA, M., IMAOKA, S., FUNAE, Y. and KATO, R., 1989a, Suppression of hepatic levels of an ethanol-inducible P450DM/j by growth hormone: relationship between the increased level of P450DM/j and depletion of growth hormone in diabetes. *Molecular Pharmacology*, **36**, 716-722.
- YAMAZOE, Y., MURAYAMA, N., SHIMADA, M., YAMAUCHI, K. and KATO, R., 1989b, Cytochrome P-450 in livers of diabetic rats: regulation by growth hormone and insulin. *Archives of Biochemistry and Biophysics*, **268**, 567-575.



Metabolism of amiodarone (Part III): identification of rabbit cytochrome P450 isoforms involved in the hydroxylation of mono-*N*-desethylamiodarone

P. KOZLIK†, H. R. HA†*, B. STIEGER‡, L. BIGLER§
and F. FOLLATH‡

† Cardiovascular Therapy Research Unit and ‡ Clinical Pharmacology and Toxicology Division, Department of Internal Medicine, University Hospital of Zurich, Ramistrasse 100, CH-8091 Zurich, Switzerland

§ Institute of Organic Chemistry, University of Zurich, Winterthurerstrasse 190, CH-8057 Zurich, Switzerland

Received 7 November 2000

1. Amiodarone (AMI) is a potent anti-arrhythmic drug and mono-*N*-desethylamiodarone (MDEA) is its only known metabolite. It was found recently that in rabbit liver microsomes MDEA was biotransformed to *n*-3-hydroxybutyl-MDEA (3OH-MDEA).

2. In liver microsomes isolated from the untreated rabbit, the formation of 3OH-MDEA obeyed Michaelis-Menten enzyme kinetics with $K_m = 6.39 \pm 1.07 \mu\text{M}$ and $V_{\max} = 0.56 \pm 0.21 \text{ nmol min}^{-1} \text{ mg}^{-1} \text{ protein}$.

3. Furthermore, (1) among chemicals usually used as inhibitors of cytochrome P450, only midazolam (MDZ), cyclosporin A and ketoconazole inhibited the MDEA hydroxylase activity significantly (> 60% inhibition), (2) MDZ, a substrate of CYP3A, inhibited the 3OH-MDEA formation competitively ($K_i = 10 \pm 5 \mu\text{M}$), (3) the formation rates of 3OH-MDEA correlated positively with those of 1'-OH-MDZ ($r = 0.81$; $n = 6$), and (4) MDEA hydroxylase activity of microsomes isolated from rabbit rifampicin-induced cultured hepatocytes was 4-fold more active than the control.

4. Since CYP3A6 is mainly induced by rifampicin in rabbit-cultured hepatocytes, the data suggest that this isoform is involved in the biotransformation of MDEA to 3OH-MDEA.

5. Since α -naphthoflavone, cimetidine and quinidine also partially inhibited the MDEA hydroxylase activity, it is possible that other CYPs, such as 1A, 2C and 2D, may also be active in the metabolism of amiodarone.

Introduction

Amiodarone (AMI) has proved to be a potent anti-arrhythmic drug, but despite its frequent use, data on its metabolism are scarce. In humans and laboratory animals, mono-*N*-desethylamiodarone (MDEA) is known as the major metabolite of AMI. During long-term therapy, the serum concentration of this pharmacologically active metabolite is comparable with that of the parent drug, and cytochrome P4503A4 (CYP3A4) isoforms are involved in AMI dealkylation (Ha *et al.* 1992, 1996, Fabre *et al.* 1993). Previous data have shown that other metabolites of AMI may be produced. Storey *et al.* (1969) reported that, unlike in human and rat, the MDEA concentration in blood and tissue of rabbit receiving AMI (i.p. $40 \text{ mg kg}^{-1} \text{ day}^{-1}$ for 4 weeks) was very low. It was doubtful

* Author for correspondence; e-mail: huyriem.ha@dim.usz.ch

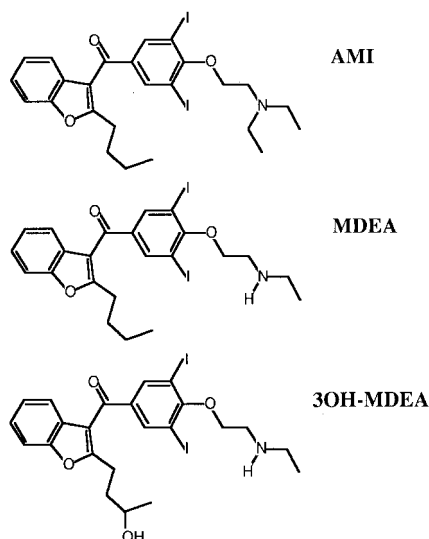


Figure 1. Chemical structure of amiodarone (AMI), mono-*N*-desethylamiodarone (MDEA) and *n*-3-hydroxybutyl-*N*-desethylamiodarone (3OH-MDEA).

that, in Storey's experiments, MDEA would have been further biotransformed to unknown product(s). Kannan *et al.* (1985) analysed the bile of rabbit receiving AMI (i.p. 20 mg kg⁻¹ for 6 weeks) and found two chromatographic peaks other than those corresponding to AMI and MDEA. The identity of these compounds remained unknown. From these data, it is possible that: (1) metabolic pathways other than dealkylation exist in AMI metabolism; (2) in rabbit the clearance of MDEA is particularly high; and (3) metabolism of AMI is species dependent. Recently, the present group has reported that in rabbit liver microsomes, MDEA is biotransformed to *n*-3-hydroxybutyl-MDEA (3OH-MDEA) (figure 1), and this compound may be detected in the liver, lung, kidney and heart of rat receiving AMI.HCl (Ha *et al.* 2001). In this paper, the identity of CYP isoenzyme(s) involved in the MDEA hydroxylation is investigated.

Materials and methods

Chemicals

Mono-*N*-desethylamiodarone hydrochloride (MDEA, purity > 98%) was a gift from Sanofi (Montpellier, France). Hydroxylated MDEA (3OH-MDEA, purity 97%) was isolated from the bioreaction using MDEA and rabbit liver microsomes as described by Ha *et al.* (2001). Midazolam (MDZ), 8-chloro-6-(2-fluorophenyl)-1-hydroxy-methyl-4H-imidazo(1,5-*a*)(1,4)-benzodiazepine (1'-OH-MDZ) and internal standard Ro 05-6669 (7-chloro-1,3-dihydro-5-(4-methoxyphenyl)-1-methyl-2H-1,4-benzodiazepin-2-one) were obtained from Hoffmann-La Roche (Basle, Switzerland). α -Naphthoflavone (ANP) was from Aldrich Chemical Co. (Buchs, Switzerland). Coumarin, cimetidine, cyclosporin A, diethyldithiocarbamic acid (DDC), methimazole, mephénytoin, sulphaphenazole, SKF525A (proadifen), quinidine, quinine, triacetyleandomycin (TAO), rifampicin (RIF), DL-isocitric acid, NADPH and isocitric dehydrogenase (EC 1.1.1.42) from porcine heart were purchased from Sigma Chemical Co. (Buchs, Switzerland). Other chemicals, such as diethyl ether, MgCl₂·6H₂O and Na₂HPO₄ were from Fluka Chemical Co. (Buchs, Switzerland).

3OH-MDEA standards

The off-line purified 3OH-MDEA (0.15 mg) from Ha *et al.* (2001) was dissolved in methanol and its UV spectrum was measured between 210 and 360 nm. Its concentration was calculated using the molar

extinction coefficient $\varepsilon = 440\,000\text{ M}^{-1}\text{ cm}^{-1}$ at 241 nm (Plomp 1991). The working standards were then prepared by diluting the stock solution with the incubation medium containing $200\text{ }\mu\text{g ml}^{-1}$ heat-deactivated rabbit liver microsomes to give final concentrations of 3OH-MDEA (base form) in the range $0.25\text{--}2.0\text{ }\mu\text{g ml}^{-1}$. The standards were stable for at least 1 month stored in aliquots of 2 ml at -20°C .

Quantitative HPLC assay for 3OH-MDEA

The performance of the quantitative HPLC assay being developed for 3OH-MDEA has been reported by Ha *et al.* (2000). Briefly, the separation of compounds was achieved using a LKB liquid chromatograph (Bromma, Sweden) connected to an integrator SP 4400 (Spectra-Physics, San Jose, CA, USA). The assay was operated under the following conditions: Nucleosil 100-5 Protect 1-250 $\times$ 4 mm (Macherey-Nagel, Oensingen, Switzerland) maintained at 45°C ; mobile phase, methanol:water:aqueous NH_3 25% (300:90:0.25 w/w); flow, 1.2 ml min^{-1} ; pressure, 190 bars; detection, 242 nm. The coefficients of variation of the between- and within-assay conditions were $< 7\%$.

Biological materials

Up to 5 g fresh liver of the untreated male New Zealand White rabbits (weighing 3-4 kg) was obtained from Dr H. Cristina, Institute for Laboratory Animal Science, University of Zurich. The blood in the sample was washed out with 0.25 M sucrose at 4°C . A rabbit liver microsome fraction was isolated by means of standard differential centrifugation as described for rat liver microsomes (Meier *et al.* 1981). Protein concentrations were measured using the method of Bensadoun *et al.* (1976).

3OH-MDEA hydroxylase activities

Rabbit liver microsomal protein ($200\text{ }\mu\text{g ml}^{-1}$) was incubated in 0.1 M sodium phosphate buffer, pH 7.4 (PB7.4) at 37°C in a final volume of 0.2 ml in the presence of a system that generated the reduced form of NADP (4.5 IU isocitrate dehydrogenase ml^{-1} , 5 mM isocitrate, 5 mM MgCl_2). Substrate MDEA was dissolved in ethanol, diluted with PB7.4 to desired concentrations and then added as a 2-fold concentrated solution in PB7.4. After a 5-min incubation at 37°C , the reaction was started by addition of $40\text{ }\mu\text{l}$ 5 mM NADPH. In the negative control incubation, rabbit liver microsomes were deactivated by heating at 90°C for 5 min.

After incubation (15 min at 37°C), the reaction was stopped by addition of 1 ml ice-cooled diethyl ether and vortexed. The tube was then capped and shaken horizontally for 10 min (400 rpm). The organic phase was separated by centrifugation and pipetted into another glass test tube. To ensure a quantitative result (recovery 95%), the extraction was repeated three times. The organic phases were pooled and evaporated to dryness at 21°C . The residue was then dissolved in $150\text{ }\mu\text{l}$ mobile phase, and $100\text{ }\mu\text{l}$ of the aliquot was injected onto the HPLC column.

Enzyme kinetics analysis

The linear relationships between 3OH-MDEA formation rates and MDEA concentrations ($2\text{--}50\text{ }\mu\text{M}$), microsomal protein ($25\text{--}3.500\text{ }\mu\text{g ml}^{-1}$) and time ($1\text{--}120\text{ min}$) were investigated. Sixteen different concentrations of MDEA, ranging from 2.5 to $40\text{ }\mu\text{M}$, were used in the kinetic experiments. The apparent enzyme kinetic parameters were first estimated using Lineweaver-Burk plots ($1/V$ versus $1/S$), and the values obtained were used as the first estimate for the non-linear least-squares regression analysis (ProFit software, QuantumSoft, Zurich, Switzerland). The Michaelis-Menten equation: $V = V_{\text{max}}^* S / (K_m + S)$, where V is the velocity of the metabolite formation, S is the MDEA concentration in the incubation, K_m is the affinity constant and V_{max}^* is the maximum enzyme velocity, was employed for the fitting. Eadie-Hofstee plots (V versus V/S) were then used to gain information regarding the involvement of the number of binding sites involved in the biotransformation. The inhibitory mechanisms of chemical inhibitors were investigated using Dixon plots ($1/V$ versus inhibitor concentrations).

Correlation study

Correlations between the MDEA hydroxylase activities and those of MDZ were investigated by comparing the formation rates of 3OH-MDEA and 1'OH-MDZ in six different rabbit liver microsome samples. The concentrations of 1'OH-MDZ were measured using the HPLC assay of Ha *et al.* (1993). The substrate concentrations of MDEA and MDZ were 25 and $20\text{ }\mu\text{M}$, respectively.

Inhibition study

The effects of various chemical inhibitor or substrate probes were also studied. Selective inhibitors used were: α -naphthoflavone (ANP) for CYP1A, coumarin (CYP2A6), sulphaphenazole (CYP2C9), cimetidine (CYP2C11), mephenytoin (2C19), quinidine (CYP2D6), DDC (CYP2E1) and TAO, ketoconazole (CYP3A). The substrate probes used were quinine and cyclosporin A for CYP3A. Except

for DDC, which was dissolved in water, all test substances were dissolved in an organic solvent, such as ethanol or DMSO, then diluted with PB7.4 to the desired concentration and added (20 μ l) to the standard incubation (10 μ M MDEA, 200 μ g ml⁻¹ microsomal protein, 1 mM NADPH, incubated at 37°C for 15 min). In the final mixture, the concentration of the organic solvent was < 1%. The effect of each compound on the formation of 3OH-MDEA at the respective inhibitor concentrations was compared with the control value determined from the standard incubation containing the same amount of the corresponding vehicle. The results were expressed as a percentage of the respective control. All measurements were performed in triplicate.

For assessing the mechanism of MDZ inhibition, four MDZ concentrations (50, 100, 150, 200 μ M) were added to three series of incubation samples containing 2.5, 5.0 and 10 μ M MDEA. The inhibition type was analysed using Dixon plots.

Effect of rifampicin induction

Rabbit liver microsomes, which were isolated from cultured hepatocytes [untreated (control; two samples) and treated with rifampicin (50 μ M for 4 days; two samples) as described by Daujat *et al.* (1987)], were generously provided by Dr. P. Gallier, Laboratoire de Pharmacologie-Toxicologie INRA, Toulouse. The samples were diluted with PB7.4 to a final concentration of 0.1 mg protein ml⁻¹ and incubated with 20 μ M MDEA for 5 min, as described above. The formation rates of 3OH-MDEA were compared with those of controls.

Results

In vitro, rabbit liver microsomes fortified by NADPH possibly biotransform MDEA to 3OH-MDEA and some other minor products. Using the described HPLC conditions, the retention times of 3OH-MDEA and MDEA were 4.26 and 7.23 min, respectively (figure 2). The formation rates of the metabolite were linear at 37°C for the incubation time up to 20 min with 5–10 μ M MDEA and up to 250 μ g ml⁻¹ microsomal protein (data not shown). Therefore, in the present studies, the conditions of 10 μ M MDEA, 200 μ g ml⁻¹ microsomal protein, 1 mM NADPH and 37°C for 15 min in a final volume of 0.2 ml were chosen for the standard incubation. Under these conditions, the 3OH-MDEA formation rates may be predicted using the classical Michaelis-Menten kinetic law (figure 3), and the apparent parameters were $K_m = 6.39 \pm 1.07 \mu$ M and $V_{max} = 0.56 \pm 0.21$ nmol min⁻¹ mg⁻¹ protein. Results shown are the mean $\pm$ SD obtained from three liver microsomes samples (duplicated determinations). The Eadie-Hofstee plot (figure 3, insert) suggests that the reaction is monophasic.

In order to determine whether CYP or flavin-containing monooxygenase (FMO) is involved in MDEA hydroxylation, SKF525A (non-specific CYP inhibitor) and methimazole (FMO inhibitor) were co-incubated with MDEA. At a concentration of 0.2 mM, SKF525A abolished the MDEA hydroxylase activity totally, whereas 1 mM methimazole suppressed only 20% of it (figure 4) suggesting that CYPs in rabbit liver microsomes are mainly involved in the reaction.

The inhibitory effect of various chemicals on MDEA hydroxylation was first investigated. In comparison with human and rat liver microsomes, those of rabbit are less frequently used for drug metabolism studies. Therefore, the effects of chemicals that may selectively inhibit the activity of CYPs in rabbit liver are not well documented. In the present study, these compounds were selected on the basis of published data showing their putatively selective inhibitors for human and/or rat CYPs. Among them coumarin, DDC and sulphaphenazole had essentially no effect (< 20% inhibition) on the MDEA hydroxylation. Thus, CYP isoforms 2A6, 2E1 and 2C9 may be considered as not being involved in the reaction. Mephenytoin, ANP and cimetidine were more active (30–50% inhibition) indicating that CYP1A, 2C11 and 2C19 may make a contribution for the MDEA

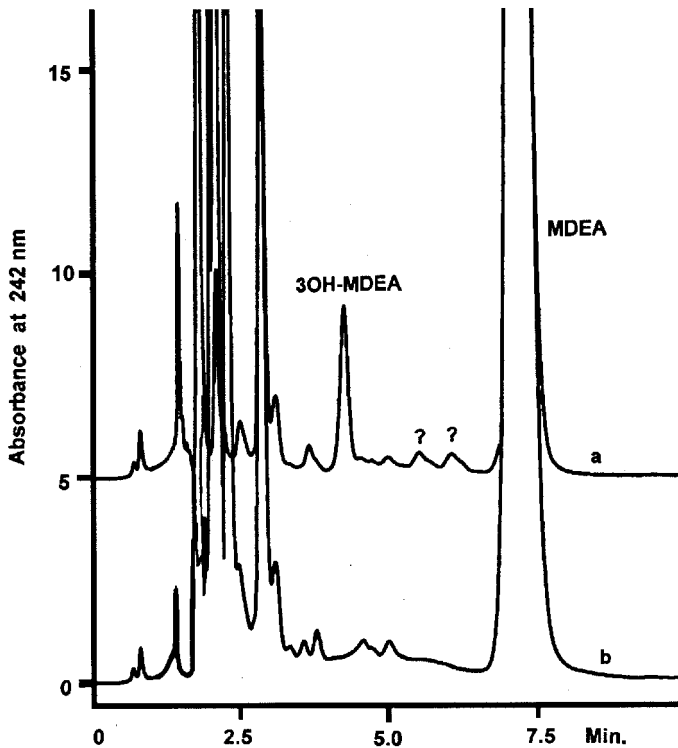


Figure 2. HPLC chromatogram (a) showing that in the standard incubation ($10\mu\text{M}$ MDEA, $200\mu\text{g ml}^{-1}$ microsomal protein, 1 mM NADPH, 0.1 M phosphate buffer, pH 7.4, incubated at 37°C for 15 min) MDEA was biotransformed to 3OH-MDEA and other minor products (?). Their retention times were 4.26, 5.67 and 6.07 min, respectively. The retention time of MDEA was 7.23 min. Negative control incubation using heat-deactivated microsomes (b).

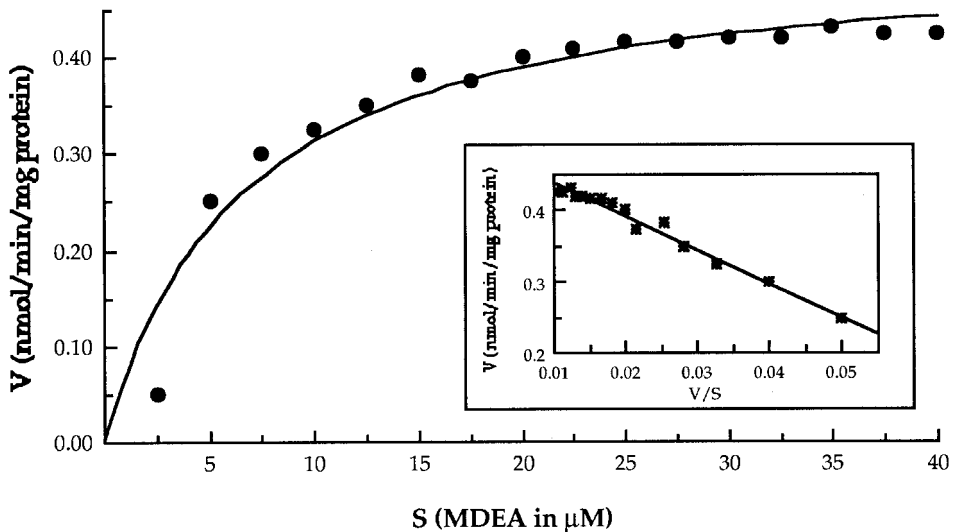


Figure 3. Representative enzyme kinetics of 3OH-hydroxylation of MDEA in rabbit liver microsomes. MDEA concentrations were in the range $2.5\text{--}40\mu\text{M}$. Other conditions (see figure 2) were unchanged. The estimates of Michaelis-Menten parameters were: $K_m = 6.45\mu\text{M}$; $V_{\max} = 0.51\text{ nmol min}^{-1}\text{ mg}^{-1}\text{ protein}$. Eadie-Hofstee plot (insert) suggests that the reaction is monophasic.

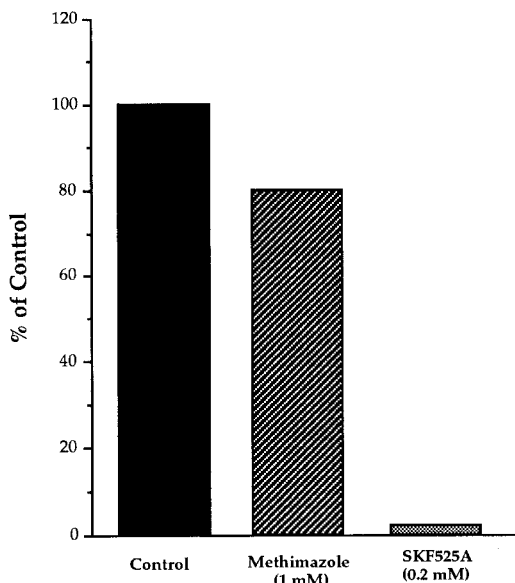


Figure 4. Effect of methimazole (FMO inhibitor) and SKF 525A (CYPs inhibitor) on MDEA-3-hydroxylase activity.

hydroxylation. Our data (figure 5) also showed that cyclosporin A, MDZ, quinidine, quinine and ketoconazole suppressed the initial formation rates of 3OH-MDEA by $> 60\%$, suggesting that MDEA hydroxylase belonged to CYP3A subfamily. Remarkably, the well-known inhibitor TAO for CYP3A isoforms in human and rat showed only a weakly inhibitory effect ($< 30\%$ inhibition) in the present study. This may be explained by the fact that, in contrast to rat, in rabbit TAO does not form significant amounts of the CYP3A-TAO nitroso-complex and, therefore, does not exhibit decreased monooxygenase activities (Wrighton *et al.* 1985).

Furthermore, quinidine is well known as an efficient inhibitor of the CYP2D6 isoform (Dayer *et al.* 1987). In the current studies, quinidine and its optical isomer, quinine, had comparable inhibitory effects on MDEA hydroxylation. Thus, the inhibitory effect of quinidine may not only have an effect on CYP2D6, but also have a result on non-specific inhibition of CYP3A, since quinidine (as well as quinine) is a known substrate for human CYP3A (Guengerich *et al.* 1986). The antimycotic agent ketoconazole is a well-known inhibitor of CYP3A isoforms (Balwin *et al.* 1995). Its estimated *in vitro* inhibition constant (K_i) with use of human liver microsomes for 1'OH-MDZ is $< 0.01 \mu\text{M}$ (von Moltke *et al.* 1996). Moreover, (1) the present liver microsome samples prepared from untreated rabbit also biotransformed MDZ to its 1'OH-MDZ ($K_m = 2.9 \mu\text{M}$) (data not shown); (2) the Dixon plot (figure 6a) for the inhibition of MDEA hydroxylase activity by MDZ had an intersection above the abscissa [suggesting that MDZ inhibits competitively ($K_i = 10 \pm 5 \mu\text{M}$) the 3OH-MDEA formation]; and (3) a positive correlation ($r = 0.81$) between the formation rates of 1'OH-MDZ and those of 3OH-MDEA measured in six different rabbit liver microsomes was observed (figure 6b).

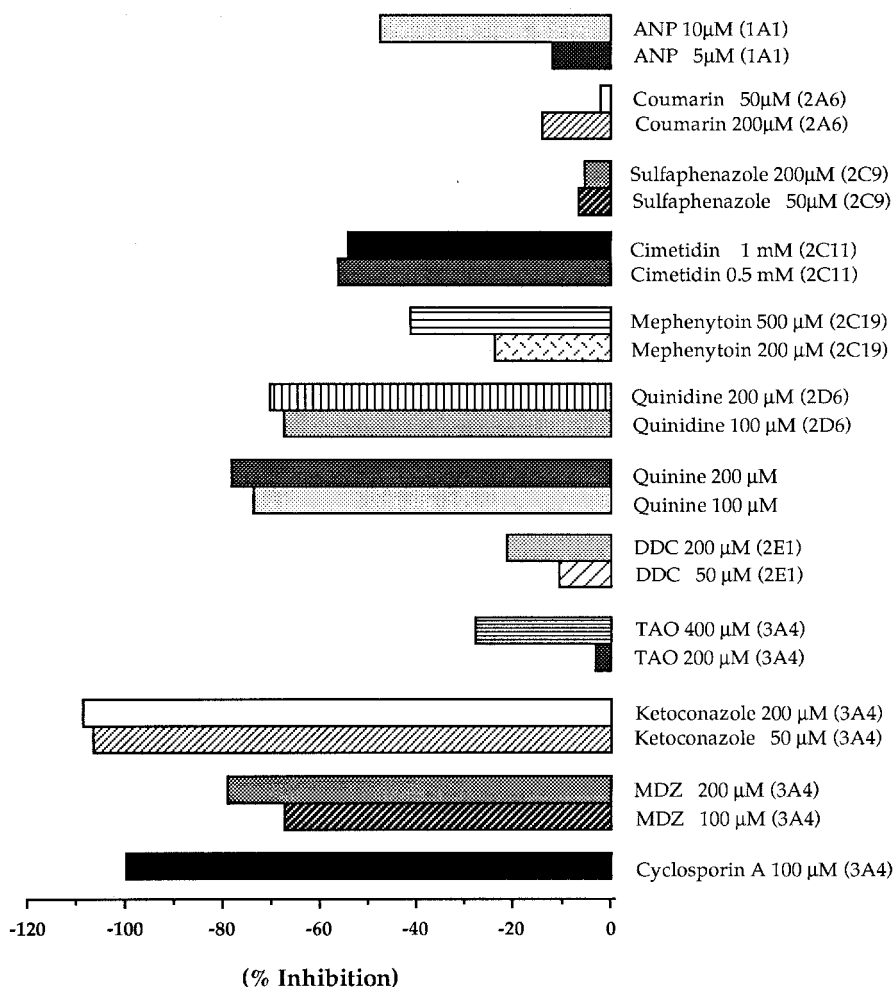


Figure 5. Effect of chemicals used as specific inhibitors and/or substrates on 3OH-MDEA hydroxylase. The tested compound was added to the standard incubation (see figure 2). The absolute value of the 100% activity (control) was $0.32 \text{ nmol min}^{-1} \text{ mg}^{-1}$ protein and results shown are the mean of three measurements.

In summary, the experiments performed using chemical inhibitors indicate that, in rabbit, isoforms belonging to the subfamily CYP3A are mainly responsible for the hydroxylation of MDEA. It is possible, however, that other CYPs such as 1A, 2C and 2D are also involved in this biotransformation.

In order to strengthen the probability that CYP3A isoforms are mainly involved in the studied reaction, a further experiment performed using biological reagents was carried out. The rabbit liver microsomes isolated from RIF-induced hepatocytes were used. The results in figure 7 show clearly that the RIF-induced microsomes are at least 4-fold more active than those of untreated samples. It is well documented that in cultured rabbit hepatocytes, the CYP3A6 isoform is RIF inducible (Daujatz *et al.* 1991); it is therefore possible that in our reaction this isoform is the one mainly involved. Moreover, (1) rat CYP3A2 polyclonal antisera (Gentest, Woburn, MA, USA) inhibited the formation of 3OH-MDEA in

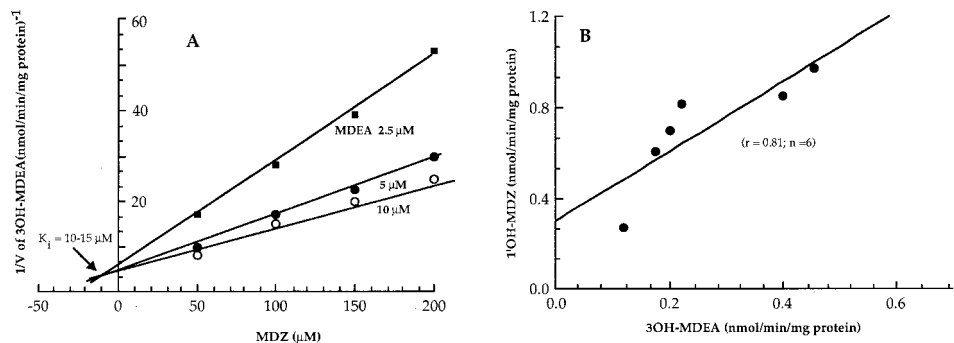


Figure 6. Dixon plot (A) for the inhibition of MDEA hydroxylase activity by midazolam (MDZ) has an intersection above the abscissa. This pattern is indicative of substrate competitive inhibition with $K_i = 10\text{--}15\text{ }\mu\text{M}$. Correlation ($r = 0.81$) between MDEA 3-hydroxylase activity and that of 1'OH-MDZ in rabbit liver microsomes (B).

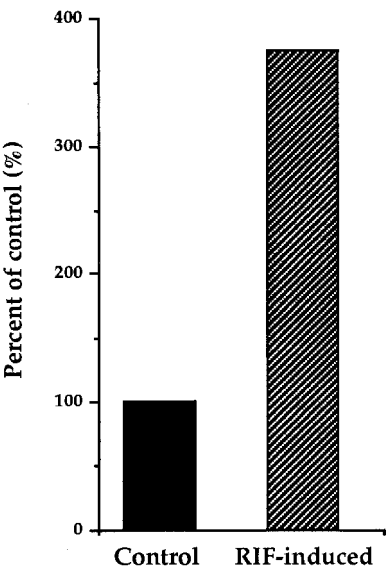


Figure 7. Representative influence of rifampicin treatment on MDEA 3-hydroxylase activity.

incubation using rat liver microsomes; and (2) human cDNA-expressed CYP3A4 isoforms purchased from the same source catalysed the hydroxylation of MDEA (submitted for publication). These observations support the notion that in mammals, CYP3A isoforms could mainly correspond to MDEA hydroxylase.

Discussion

Experiments performed with chemical inhibitors suggested that the CYP3A isoform in rabbit liver microsomes may be mainly involved in the MDEA hydroxylation. This assumption is supported by observations that: (1) substrates of CYP3A isoforms, such as MDZ, quinidine, quinine and cyclosporin A, inhibit the MDEA hydroxylase; (2) MDZ inhibits competitively the 3OH-MDEA hydroxylation; and (3) good correlation between the formation rates of 3OH-

MDEA and those of 1'-OH-MDZ is found. However, since other chemicals such as ANP, cimetidine and quinidine also have the ability to inhibit partially the MDEA hydroxylase activity, the contribution of other CYPs (1A, 2C, 2D) for the reaction cannot be completely ruled out. Furthermore, the present results also revealed that in accordance with previous observations (Wrighton *et al.* 1985) in rabbit, TAO is not a specific inhibitor of the CYP3A isoform.

Finally, the identity of CYP3A6 involved in the MDEA-3 hydroxylation can be determined by comparing the catalytic activities of microsomes isolated from RIF-induced rabbit hepatocytes with those from untreated cells. In rabbit, the gene coding for this isoform is on chromosome 6 in the q-ter region (Daujat *et al.* 1991). This enzyme, being investigated in the literature under several other names [P450IIIA4 (Daujat *et al.* 1987), P-450 LM_{3c} (Fabre *et al.* 1988), LM_{3c}, (Koop *et al.* 1981), P-450A7 (Pichard *et al.* 1990)], is known to be involved in the metabolism of many compounds such as cyclosporin A (Bertault-Peres *et al.* 1987), steroid (Waxman *et al.* 1988), midazolam (Fabre *et al.* 1988) and diltiazem (Pichard *et al.* 1990).

It is evident that the results of *in vitro* experiments must be used with great prudence to interpret the *in vivo* data. However, the findings in the present paper are in accordance with the increase of MDEA (and AMI) clearance, when AMI and RIF were co-administered to a patient (Zarembski *et al.* 1999). This drug interaction may be explained at least in part by the increase of the MDEA hydroxylase activity of RIF-induced enzyme(s). If so, in humans, the hydroxylation pathway would also be an important elimination route for AMI derivatives.

In conclusion, in rabbit the CYP3A6 isoform is mainly involved in the hydroxylation of MDEA. However, the contribution of other CYPs such as 1A, 2C and 2D to the reaction cannot be completely ruled out.

Acknowledgements

P. K. thanks the Cardiovascular Therapy Research Foundation, Department of Internal Medicine, University Hospital Zurich, for financial support. The authors thank Dr P. Gallier, Laboratoire de Pharmacologie-Toxicologie INRA, Toulouse, for supplying the rifampicin-treated liver microsomes and Dr H. Cristina, Institute for Laboratory Animal Science, University of Zurich, for supplying the rabbit liver. The authors also thank Dr Aileen McAinsh, Nashville, TN, for reviewing the manuscript. This publication represents, in part, the requirements of P. K.'s PhD thesis at the Swiss Federal Institute of Technology Zurich (ETHZ).

References

- BALDWIN, S., BLOOMER, J. C., SMITH, G. J., AYRTON, A. D., CLARKE, S. E. and CHENERY, R. J., 1995, Ketoconazole and sulphaphenazole as the respective selective inhibitors of P450 3A and 2C9. *Xenobiotica*, **25**, 261-270.
- BENSADOUN, A. and WEINSTEIN, D., 1976, Assay of protein in the presence of interfering materials. *Analytical Biochemistry*, **70**, 241-250.
- BERTAULT-PERES, I., BONLS, C., FABRE, G., JUST, S., CANO, J. P. and MAUREL, P., 1987, Metabolism of cyclosporin A. II. Implication of macrolide antibiotic inducible cytochrome P-450 3c from rabbit liver microsomes. *Drug Metabolism and Disposition*, **15**, 391-398.

- DAUJAT, M., CLAIR, P., ASTIER, C., FABRE, I., PINEAU, T., YERLE, M., GELLIN, J. and MAUREL, P., 1991, Induction, regulation and messenger half-life of cytochromes P450 1A1, 1A2 and 1A6 in primary cultures of rabbit hepatocytes. *European Journal Pharmacology*, **200**, 501-510.
- DAUJAT, M., PICHARD, L., DALET, C., LARROQUE, C., BONLS, C., POMPON, D., LI, D., GUZELIAN, P. S. and MAUREL, P., 1987, Expression of five forms of microsomal cytochrome P450 in primary cultures of rabbit hepatocytes treated with various classes of inducers. *Biochemical Pharmacology*, **36**, 3597-3606.
- DAYER, P., KRONBACH, T., EICHELBAUM, M., COVO, J. and MEYER, U. A., 1987, Enzymatic basis of the debrisoquine/sparteine-type genetic polymorphism of drug oxidation. Characterization of bufuralol 1'-hydroxylation in liver microsomes of *in vivo* phenotyped carriers of the genotoc deficiency. *Biochemical Pharmacology*, **36**, 4145-4152.
- FABRE, G., CREVATPISANO, P., DRAGNA, S., COVO, J., BARRA, Y. and CANO, J. P., 1988, Involvement of the macrolide antibiotic inducible cytochrome P-450 LM3c in the metabolism of midazolam by microsomal fractions prepared from rabbit liver. *Biochemical Pharmacology*, **37**, 1947-1953.
- FABRE, G., JULIAN, B., SANT-AUBERT, B., JOYEUX, H. and BERGER, Y., 1993, Evidence for CYP3A-mediated *N*-deethylation of amiodarone in human liver microsomal fractions. *Drug Metabolism and Disposition*, **21**, 978-985.
- GUENGERICH, F. P., MUELLER-ENOCH, D. and BLAIR, I. A., 1986, Oxidation of quinidine by human liver cytochrome P-450. *Molecular Pharmacology*, **30**, 287-295.
- HA, H. R., BIGLER, L., BINDER, M., KOZLIK, P., HESSE, M., ALTORFER, H. R. and FOLLATH, F., 2001, Metabolism of amiodarone (Part I): identification of a new hydroxylated metabolite of amiodarone. *Drug Metabolism and Disposition*, **29**, 152-158.
- HA, H. R., CANDINAS, R., STIEGER, B., MEYER, U. A. and FOLLATH, F., 1996, Interaction between amiodarone and lidocaine. *Journal of Cardiovascular Pharmacology*, **26**, 533-539.
- HA, H. R., KOZLIK, P., STIEGER, B., BIGLER, L. and FOLLATH, F., 2000, Metabolism of amiodarone (Part II): high-performance liquid chromatographic assay for mono-*N*-desethylamiodarone hydroxylation in liver microsomes. *Journal of Chromatography B, Biomedical Application* (in press, April 2001).
- HA, H. R., RENTSCH, K. M., KNEER, J. and VONDERSCHMITT, D. J., 1993, Determination of midazolam and its α -hydroxy metabolite in human plasma and urine by high-performance liquid chromatography. *Therapeutic Drug Monitoring*, **15**, 338-343.
- HA, H. R., WYSS, P., STIEGER, B., MEYER, U. A. and FOLLATH, F., 1992, Amiodarone metabolism in human liver microsomes. *FASEB Journal*, **6**, A1845 (abstract).
- KANNAN, R., MILLER, S. and SINGH, B. N., 1985, Tissue uptake and metabolism of amiodarone after chronic administration in rabbits. *Drug Metabolism and Disposition*, **13**, 646-650.
- KOOP, D. R., PERSSON, A. V. and COON, M. J., 1981, Properties of electrophoretically homogeneous constitutive forms of liver microsomal cytochrome P-450. *Journal of Biological Chemistry*, **256**, 10704-10711.
- MEIER, P. J., SPYCHER, M. A. and MEYER, U. A., 1981, Isolation and characterization of rough endoplasmic reticulum associated with mitochondria from normal rat liver. *Biochimica et Biophysica Acta*, **46**, 283-297.
- PICHARD, L., GILLET, G., FABRE, I., DALET-BELUCHE, I., BONLS, C., THENOT, J. P. and MAUREL, P., 1990, Identification of the rabbit and human cytochromes P-450 1A4 as the major enzymes involved in the *N*-demethylation of diltiazem. *Drug Metabolism and Disposition*, **18**, 711-719.
- PLOMP, T. A., 1991, Analytical profiles of amiodarone. In K. Florey (ed.), *Analytical Profiles of Drug Substances*, vol. 20 (San Diego: Academic Press), pp. 2-120.
- STOREY, G. C. A., ADAMS, P. C., CAMPBELL, R. W. F. and HOLT, D. W., 1969, High-performance liquid chromatographic measurement of amiodarone and desethylamiodarone in small tissue samples after enzymatic digestion. *Journal of Clinical and Pathology*, **177**, 340-352.
- VON MOLTKE, L. L., GREEBLATT, D. J., SCHMIDER, J., DUAN, S. X., WRIGHT, C. E., HARMATZ, J. S. and SHADER, R. I., 1996, Midazolam hydroxylation by human microsomes *in vitro*: inhibition by fluoxetine, norfluoxetine and by azole antifungal agents. *Journal of Clinical Pharmacology*, **36**, 783-791.
- WAXMAN, D. J., ATTISANO, C., GUENGERICH, F. P. and LAPENSON, D. P., 1988, Human liver microsomal steroid metabolism: identification of the major microsomal steroid hormone 6-hydroxylase cytochrome P450 enzyme. *Archives of Biochemistry and Biophysics*, **263**, 424-436.
- WRIGHTON, S. A., MAUREL, P., SCHUETZ, E. G., WATKINS, P. B., YOUNG, B. and GUZELIAN, P. S., 1985, Identification of the cytochrome P-450 induced by macrolide antibiotic in rat liver as the glucocorticoid responsive cytochrome P-450p. *Biochemistry*, **24**, 2171-2178.
- ZAREMBSKI, D. G., FISCHER, S. A., SANTUCCI, P. A., PORTER, M. T., COSTANZO, M. R. and TROHMAN, R. G., 1999, Impact of rifampicin on serum amiodarone concentrations in a patient with congenital heart disease. *Pharmacotherapy*, **19**, 249-251.

Role of endogenous nitric oxide in liver-specific functions and survival of cultured rat hepatocytes

M. T. DONATO[†], X. PONSODA[‡], E. O'CONNOR[§],
J. V. CASTELL[†] and M. J. GÓMEZ-LECHÓN^{†*}

[†] Unidad de Hepatología Experimental. Centro de Investigación. Hospital Universitario La Fe, Avda Campanar 21, E-46009 Valencia, Spain

[‡] Departament de Parasitologia i Biologia Cel·lular, Facultat de Ciències Biològiques, Avda Dr Moliner 50, E-46014 Burjassot, Spain

[§] Departament de Bioquímica i Biologia Molecular, Facultat de Medicina, Avda Blasco Ibáñez 20, E-46010 Valencia, Spain

Received 11 January 2001

1. The role of endogenous nitric oxide in rat hepatocyte functionality and survival in cell culture was examined. Towards this aim, cytochrome P450 activities (CYP1A1/2, 2B1, 2A1, 2C11, 2D1, 2E1 and 3A1), liver-specific metabolic functions and cell survival were comparatively evaluated in hepatocytes isolated from the male Sprague-Dawley rat and/or cultured in control conditions or in the presence of *N*-omega-nitro-L-arginine methyl ester (NAME), an inhibitor of nitric oxide synthesis.

2. Suppression of nitric oxide production by NAME paralleled a substantial preservation of hepatocyte phenotype in culture. The presence of NAME was particularly important during isolation and/or the 6-24 h culture. By 24 h, beneficial effects were evident in parameters particularly unstable in culture (glycogen content, P450), whereas no changes were produced in well-preserved functions (glucose, urea and albumin synthesis, glutathione, drug-conjugating enzymes).

3. Long-term treatment of hepatocytes with NAME also produced a reduction in caspase 3 activation and in the percentage of spontaneous apoptotic cells, and an increase in cell survival and transcriptional activity as shown by attached cellular protein content and the protein-DNA ratio respectively.

4. In conclusion, inhibition of early endogenous nitric oxide formation is an efficient procedure for obtaining hepatocyte cultures with stable expression of differentiated functions, high cell survival and few signs of cell senescence.

Introduction

In recent years, primary culture of hepatocytes has become a useful experimental approach for biochemical research and, in particular, for studying the metabolism and toxic effects of drugs. However, early spontaneous loss of cytochrome P450 (CYP), which precedes a gradual decrease in other differentiated cell functions, is the major limitation of primary cultured hepatocytes for biochemical research and, in particular, for studying the metabolism and toxic effects of drugs. Changes in hepatocyte phenotype have been interpreted in terms of the adaptative response of cells to a new culture environment and a lack of critical elements essential to hepatocellular functions (Paine 1990, Donato *et al.* 1992, Gómez-Lechón *et al.* 2000).

* Author for correspondence; e-mail: gomez_mjo@gva.es

Activation of nitric oxide (NO) biosynthesis during hepatocyte isolation with the classical two-step EGTA/collagenase method has been considered recently as the major factor responsible for the dramatic changes in CYP levels occurring during early culture stages (López-García 1998). NO is a signalling molecule synthesized from L-arginine and is involved in the regulation of a variety of cellular functions in different target cells, including hepatocytes (Harbrecht and Billiar 1995). Increased NO production in response to cytokines has been demonstrated in cultured hepatocytes (Nussler *et al.* 1995, Donato *et al.* 1997), and the decrease in CYP activities produced by cytokines was related to a functional inhibition of CYP by NO (Wink *et al.* 1993, Stadler *et al.* 1994, Osawa *et al.* 1995, Donato *et al.* 1997). Hepatocytes actively synthesize NO during the first 24 h after plating, even in the absence of cytokines (Nicholls-Grzemeski *et al.* 1999, López-García and Sanz-González 2000). This activation of NO biosynthesis shown in the initial culture period appeared to be directly related to the cell isolation procedure. Unknown contaminants of commercial collagenase, intra-extracellular calcium movements, oxidative/mechanical stress, ischaemia-reperfusion or hyperoxia have been proposed as possible stimuli for either constitutive NO synthase activation or inducible NO synthase induction (López-García 1998, Wang *et al.* 1998, Nicholls-Grzemeski *et al.* 1999, Rodríguez-Ariza and Paine 1999, López-García and Sanz-González 2000, Vargiu *et al.* 2000). It was recently observed that addition of *N*-omega-nitro-L-arginine methyl ester (NAME), a NO synthesis inhibitor, during liver perfusion can partially prevent rapid CYP and glycogen losses observed in culture (López-García 1998).

The present study includes an extensive characterization of the effects of NAME on the biochemical functionality of cultured hepatocytes. High maintenance of catalytic activity of most CYP isoforms, stabilization of intracellular glycogen content and longer cell survival were observed after inhibition of NO biosynthesis.

Materials and methods

Hepatocyte isolation and culture

Hepatocytes were isolated from male Sprague-Dawley rats (180–250 g) by reverse perfusion of the liver with collagenase (Boehringer Mannheim, Mannheim, Germany) (Gómez-Lechón *et al.* 1984). The studies were carried out in accordance with the *Guide for Care and Use of Laboratory Animals* published by the US National Institutes of Health. Hepatocytes were cultured on fibronectin-coated plates in Ham F-12/Leibovitz L-15 (1:1 v:v) medium (Flow, Irvine, UK) (containing 1.68 mM arginine) supplemented with 2% newborn calf serum (Gibco, Paisley, UK), 5 mM glucose, antibiotics, 0.2% bovine serum albumin and 10^{-8} M insulin. The medium was changed 1 h later to remove unattached hepatocytes. By 24 h, the cultures were shifted to serum-free medium containing 10^{-8} M dexamethasone. NAME (1.68 mM; Sigma, St. Louis, MO, USA) was added as indicated.

Measurement of CYP and conjugating activities

CYP activities were assayed by incubating hepatocytes with specific substrates: 7-ethoxycoumarin (various CYPs; Edwards *et al.* 1984), 7-ethoxyresorufin (CYP1A1/2; Burke *et al.* 1985), 7-pentoxoresorufin (CYP2B1; Burke *et al.* 1985), diclofenac (CYP2C11; Bort *et al.* 1999), dextromethorphan (CYP2D1; Kronbach *et al.* 1987), *p*-nitrophenol (CYP2E1; Tassaneeyakul *et al.* 1993) or testosterone (CYP2A1 and 3A1; Sonderfan *et al.* 1987). 7-Ethoxycoumarin *O*-deethylation (ECOD), 7-ethoxyresorufin *O*-deethylation and 7-pentoxoresorufin *O*-deethylation were determined fluorimetrically as described (Edwards *et al.* 1984, Donato *et al.* 1993). *p*-Nitrophenol hydroxylation was quantified spectrophotometrically (Gómez-Lechón *et al.* 1997). Testosterone hydroxylation, dextromethorphan *O*-demethylation and diclofenac 4'-hydroxylation were quantified by HPLC analysis (Kronbach *et al.* 1987, Gómez-Lechón *et al.* 1997, Bort *et al.* 1999). UDP-glucuronyltransferase and glutathione

S-transferase activities were determined fluorimetrically using 4-methylumbelliferone and 1-chloro-2,4-dinitrobenzene as substrates, respectively (Gómez-Lechón *et al.* 1997).

Biochemical measurements

The cellular glycogen content was enzymatically measured in microcultures by *in situ* hydrolysis with α -glucoamylase (Gómez-Lechón *et al.* 1996b). Gluconeogenesis was carried out in glucose-free Krebs-Ringer, using 10 mM lactate as gluconeogenic precursor (Gómez-Lechón *et al.* 1997). The ureogenic rate was measured in cells incubated with 3 mM ammonium chloride by measuring the appearance of urea in the culture medium and the synthesis of albumin was monitored by means of an ELISA (Castell *et al.* 1985). Intracellular ATP concentration was measured using the luciferin/luciferase assay according to the manufacturer's instructions. Bioluminescence was quantified in a Lumat LB luminescence photometer (Berthold GmbH, Wildbad, Germany). Total cellular protein content, intracellular glutathione levels, DNA and nitrite levels in culture medium were quantified as described (Lowry *et al.* 1951, Castell *et al.* 1985, Misko *et al.* 1993, Gómez-Lechón *et al.* 1996b).

Caspase 3 activity

Caspase 3 activation was measured using the fluorogenic substrate *N*-acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin (Ac-DEVD-AMC) as described (Nicholson *et al.* 1995) with some modifications. In brief, culture medium was centrifuged and floating cells pelleted. Pellet and cell monolayer were lysed (10 mM Tris-HCl, 10 mM NaH₂PO₄/NaHPO₄, pH 7.5, 130 mM NaCl, 1% Triton X-100, 10 mM sodium pyrophosphate) for 1 h on ice. A reaction mixture containing 20 mM Ac-DEVD-AMC, cell lysate and assay buffer (20 mM HEPES, pH 7.5, 10% glycerol, 2 mM DTT) was incubated for 1 h at 37°C. The 7-amino-4-methylcoumarin (AMC) liberated from Ac-DEVD-AMC was measured fluorimetrically (380 nm excitation and 460 nm emission).

Flow cytometric analysis

Cell monolayers were lysed (0.1% Na₃ citrate and 0.1% Triton X-100 in distilled water) and nuclei suspension was treated with propidium iodide (PI) and fluorescein isothiocyanate (FITC; all Sigma) for fluorescent staining of DNA (Vargas *et al.* 1987) and nuclear proteins (Darzynkiewicz 1990), respectively. Analyses were performed with an EPICS XL-MCL flow cytometer (Beckman-Coulter, Brea, CA, USA) equipped with an air-cooled, argon-ion laser emitting at 488 nm and 15 mW. For each run, 20 000 individual events were collected and stored in list-mode files for off-line mathematical analysis and graphic display using the EPICS XL System II v. 3.0 software (Beckman-Coulter). Relative nuclei size and complexity were estimated from laser light scatter measurements. Laser light scattered in the forward direction (FS) is mostly related to nucleus size (Papa *et al.* 1987), while laser light scattered sideways and collected at 90° from the laser beam (SS) is indicative of nuclear texture (granularity/complexity) (Papa *et al.* 1987, Darzynkiewicz 1990). DNA content in individual nuclei (i.e. nuclear ploidy) was estimated from the intensity of PI orange fluorescence (FL3, collected through a 610 DL dichroic filter plus a 575 BP band-pass filter). The degree of apoptosis was estimated from the percentage of nuclei with DNA content lower than the 2C (2n DNA content) peak in a single-parameter histogram of PI fluorescence distribution. In order to exclude debris from apoptotic nuclei, PI fluorescence measurements were gated on FS versus PI, and only particles with a size similar to diploid nuclei were considered. Nuclei aggregates were hardware-discriminated on the bases of the ratio of FL3 pulse versus integral signals. Total protein content in individual nuclei was estimated from FITC green fluorescence (FL1, collected through a 550 DL dichroic filter plus a 525 BP band-pass filter).

Statistical analysis

Experiments were performed in at least three cultures (two to three plates/variable). Statistical analysis was done by Student's *t*-test.

Results

Role of early endogenous NO production on hepatocyte functionality in short-term cultures

The kinetics of endogenous NO production (measured as nitrite release in culture medium) was examined (figure 1A). After 2–3 h of culture the accumulation of nitrite became detectable in hepatocytes incubated in medium without NAME, and by 24 h nitrite concentration reached 50 and 20 μ M respectively in

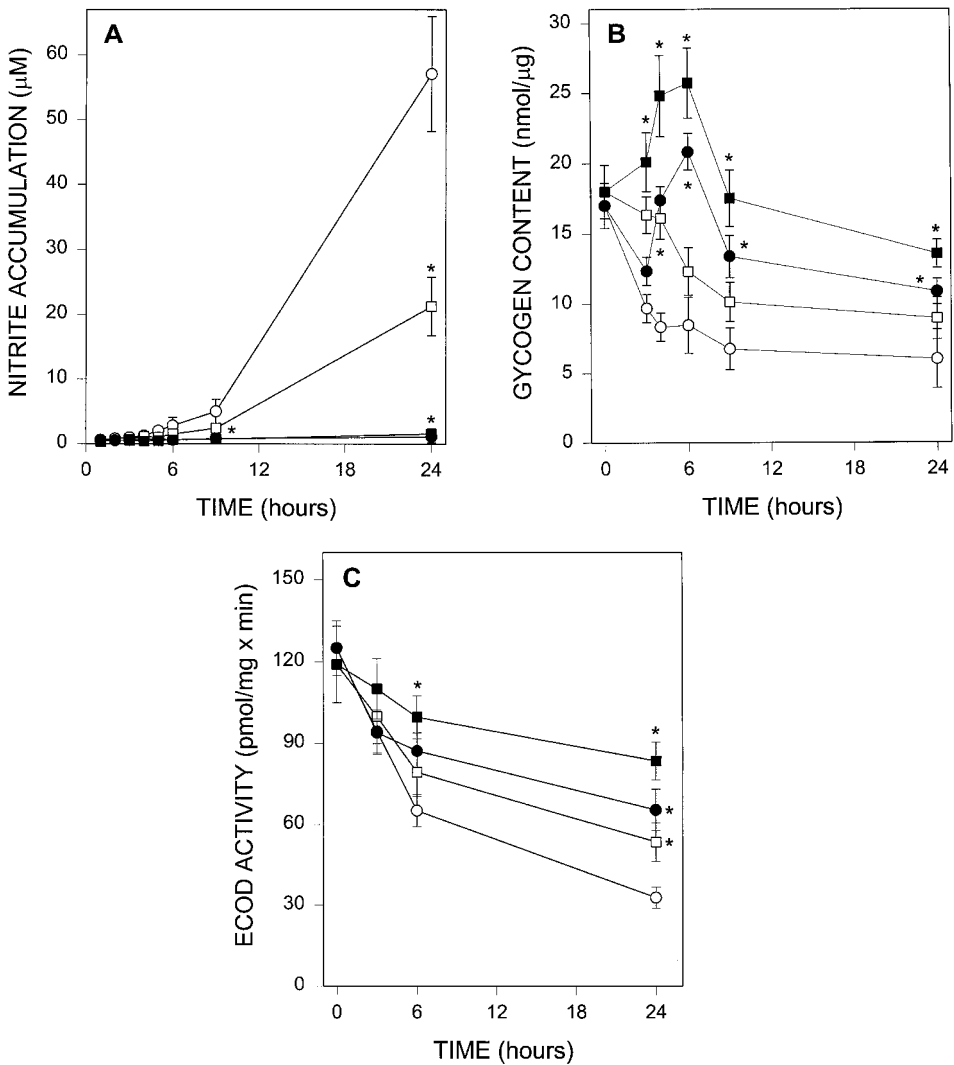


Figure 1. Time-course of endogenous NO formation, glycogen content and 7-ethoxycoumarin *O*-deethylase activity. Hepatocytes isolated under standard conditions (circles) or in the presence of 1.68 mM L-NAME (squares) were cultured in medium containing (closed symbols) or not (open symbols) 1.68 mM L-NAME. At different times in culture, nitrite accumulation in culture medium (indicative of NO formation) (A), glycogen content (B) and ECOD activity (C) were measured. Results are the mean $\pm$ SD, and correspond to three different cell preparations obtained under standard conditions or three other hepatocyte suspensions prepared in the presence L-NAME. * $p > 0.05$ with respect to hepatocytes isolated and cultured in the absence of L-NAME.

hepatocytes isolated in control conditions and in the presence of NAME. In contrast, no appreciable NO biosynthesis was observed during the same period in hepatocytes cultured with NAME.

Coincident with activation of NO biosynthesis, changes in hepatocyte functionality were observed. An early activation of glycogenolysis occurred in control hepatocytes. After 6 h of culture, initial glycogen content was reduced to 50% and thereafter a slower but continuous loss of glycogen was observed up to 24 h (figure

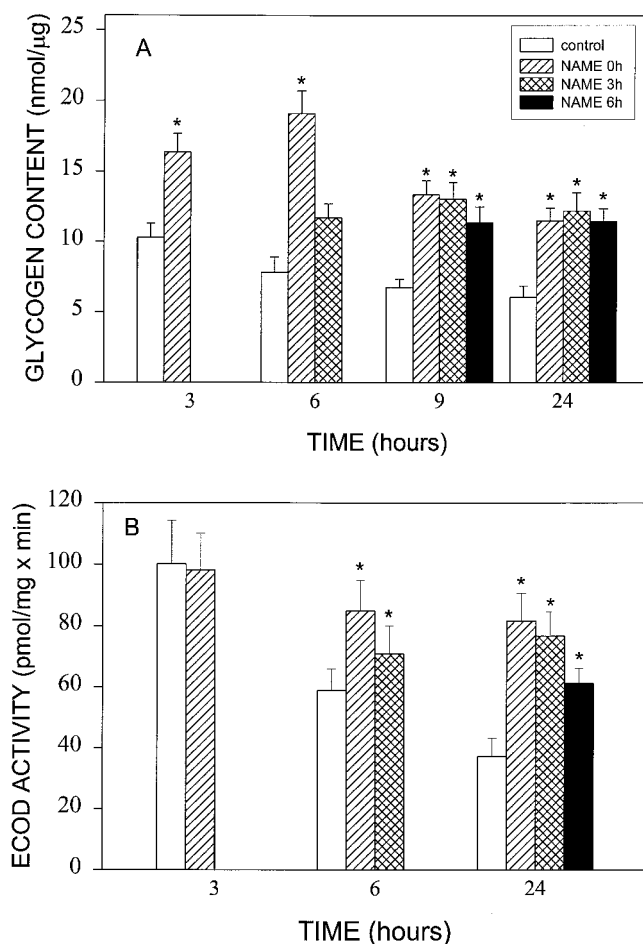


Figure 2. Glycogen content and 7-ethoxycoumarin *O*-deethylase activity of hepatocytes after L-NAME addition at different stages of culture. Hepatocytes isolated in standard conditions were cultured in control medium or in the presence of 1.68 mM L-NAME added at cell plating or 3 or 6 h later. At different times in culture, glycogen content (A) and ECOD activity (B) were determined. Results are the mean $\pm$ SD of three dishes from one representative culture. * $p > 0.05$ with respect to hepatocytes cultured in control medium.

1B). This glycogen depletion was partially prevented by NAME. After 24 h, hepatocytes obtained in the presence of NAME showed a higher glycogen content (about twofold) than cells isolated in control conditions. Regardless of the presence/absence of NAME during isolation, supplementation of culture medium with NAME resulted in an additional increase in glycogen stability. Similarly, the early loss of total CYP activity (measured as ECOD) was reduced by the addition of NAME to culture medium, and the best results were obtained when NAME was also present during the liver perfusion stage (figure 1C). Hepatocytes isolated and cultured with NAME maintained 75% of their initial activity by 24 h of culture.

These beneficial effects of NAME on hepatocyte functionality were not only observed when the inhibitor was added early (isolation period or at plating time). By 24 h, glycogen stores (figure 2A) and total CYP activity (figure 2B) were about twofold higher in NAME-treated hepatocytes than in control cells, regardless of

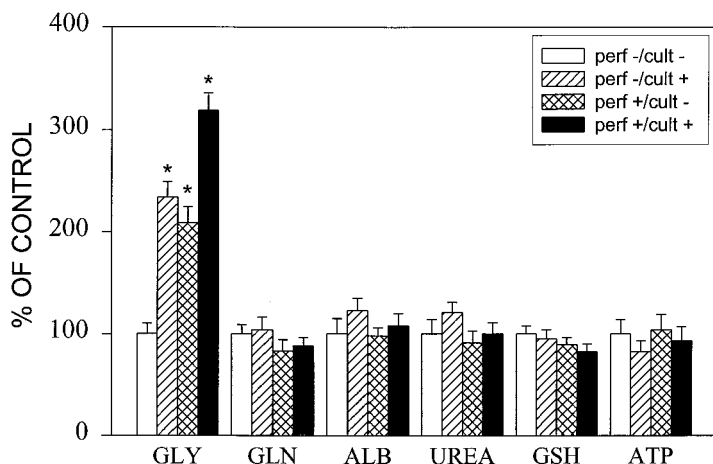


Figure 3. Effects of L-NAME on biochemical functionality of cultured hepatocytes. Hepatocytes isolated in standard conditions (perf $\rightarrow$) or in the presence of 1.68 mM L-NAME (perf $\rightarrow$) were cultured in medium containing (cult $\rightarrow$) or not (cult $\rightarrow$) 1.68 mM L-NAME. Glycogen content (GLY), gluconeogenesis rate (GLN), albumin synthesis (ALB), urea synthesis (UREA) and intracellular glutathione content (GSH) were determined in 24-h-cultured hepatocytes. Results are expressed as the percentage of corresponding values in control hepatocytes (cells isolated and culture in the absence of L-NAME). Data are the mean $\pm$ SD of three different cell preparations obtained without or with L-NAME. * $p > 0.05$ with respect to hepatocytes isolated and cultured in absence of L-NAME.

the time at which NAME was added (at plating, 3 and 6 h). Addition of NAME at any time during this culture period completely blocked NO production (data not shown for brevity).

Metabolic competence of cultured hepatocytes in the presence of NAME

The possible role of endogenous NO generation in changes of normal hepatocyte phenotype were extensively analysed. In contrast to the effect on glycogen content, the presence of NAME during perfusion and/or hepatocyte culture did not produce any changes in other biochemical functions. After 24 h of culture, gluconeogenesis, albumin synthesis, urea production and intracellular glutathione content of rat hepatocytes remained at similar levels in the presence or absence of NAME (figure 3). Analysis of ATP cellular content in the first 24 h of culture showed that treatment of hepatocytes with NAME did not alter cell energy status (figure 3).

Effects of NAME on CYP activities

In view of the beneficial action of NAME on total CYP preservation, its influence on individual CYP isozymes was also examined. CYP activities were evaluated by incubating cell monolayers with model substrates known to be specific for individual CYPs. Hepatocytes isolated and/or cultured with NAME showed, in general, higher levels of CYP activities than cells obtained and cultured in standard conditions. By 24 h, marked effects were observed on CYP1A1/2, 2A1, 2B1, 2D1, 2C11 and 3A1 activities, whereas little or no

Table 1. Effects of L-NAME on different CYP activities.

	CYP1A1/2	CYP2A1	CYP2B1	CYP2C11	CYP2D1	CYP2E1	CYP3A1
Isolated cells							
perf -	5.1 ± 0.7	47 ± 7	8.7 ± 9.2	69 ± 11	140 ± 21	314 ± 46	291 ± 37
perf +	4.7 ± 0.6	49 ± 7	8.6 ± 9.8	67 ± 10			302 ± 41
24 h cultured cells:							
perf -/cult -	1.9 ± 0.2	20 ± 2	2.6 ± 0.3	23 ± 3	49 ± 6	154 ± 18	121 ± 11
perf -/cult +	2.1 ± 0.3	26 ± 3	4.3 ± 0.5*	44 ± 6*	77 ± 9*	146 ± 17	150 ± 17
perf +/cult -	2.2 ± 0.3	23 ± 2	4.6 ± 0.4*	33 ± 3	87 ± 11*	144 ± 14	156 ± 18
perf +/cult +	3.5 ± 0.5*	30 ± 4*	6.5 ± 0.8*	51 ± 6*	98 ± 10*	146 ± 17	215 ± 22*

Hepatocytes were isolated in standard conditions (perf -) or in the presence of 1.68 mM L-NAME (perf +) and cultured in medium containing (cult +) or not (cult -) 1.68 mM L-NAME.

Activities are expressed as pmol product formed per min and per mg cellular protein. Data are the mean ± SD ($n = 3$).

* $p > 0.05$ with respect to hepatocytes isolated and cultured in absence of L-NAME.

changes were produced in CYP2E1 (table 1). The effects of NAME on CYP activities were less evident in longer periods of culture (data not shown). In contrast, the activities of UDP-glucuronyltransferase and glutathione *S*-transferase enzymes in cultured hepatocytes were not influenced by the inhibition of NO formation (data not shown).

Long-term hepatocyte survival in the presence of NAME

Since biochemical functionality was much better preserved when hepatocytes were isolated in the presence of NAME, survival and stability in long-term cultures of hepatocytes isolated under these conditions were examined. Hepatocyte survival was monitored by measuring cellular protein attached to culture plates as a function of time in culture. The results show longer survival and lower spontaneous detachment of hepatocytes cultured with NAME than in control cultures (figure 4A). Caspase 3 activation was evaluated as an apoptotic marker, and the results indicated that cell death by spontaneous apoptosis was lower in cells cultured with NAME (figure 4B). Similar results were observed when the degree of apoptosis was estimated from the percentage of nuclei with DNA content lower than the 2C peak as described above (figure 4C).

Figure 5 shows the most prominent features of the multiparametric analysis of isolated hepatocyte nuclei in which DNA content, protein content, size and granularity/complexity were simultaneously estimated. Based upon DNA content, two clear populations corresponding to 2C and 4C hepatocytes were distinguished (figure 5A). Two-dimensional displays of DNA versus FS (figure 5B), DNA versus SS (figure 5C) and DNA versus protein (figure 5D) made it possible to determine the relative distribution of these parameters among the DNA-ploidy classes by means of quantitative analysis regions enclosing 2C and 4C nuclei.

In order to assess those general nuclear characteristics as indicators of the observed protective effect of NAME, a series of parameters were compared in control and NAME-treated cultures, including the relative percentage of 2C and 4C nuclei (as an estimation of ploidy selective cell survival/loss); coefficient of variation of DNA distribution within 2C and 4C populations (an estimation of nuclear heterogeneity); ratio of DNA modal channel between 4C and 2C

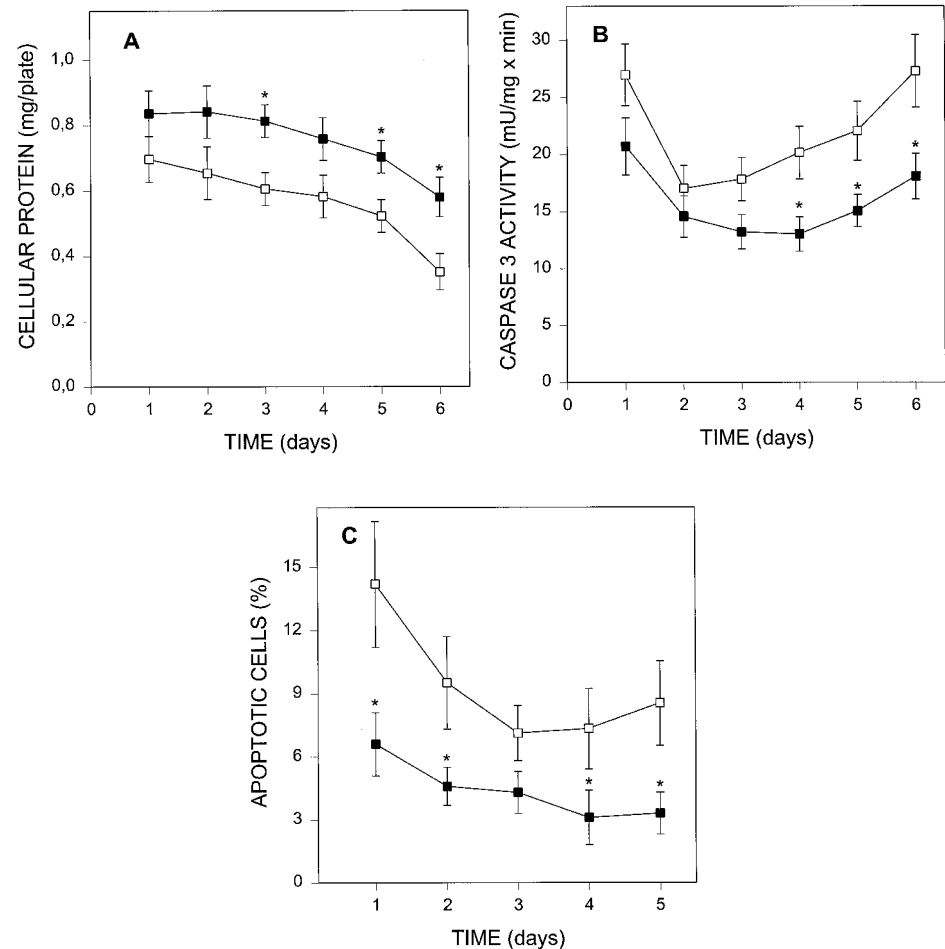


Figure 4. Long-term effects of L-NAME on hepatocyte survival. Hepatocytes isolated in the presence of 1.68 mM L-NAME were cultured in medium containing (closed symbols) or not (open symbols) 1.68 mM L-NAME. At different times in culture, total cellular protein content (A), caspase 3 activity (B) and degree of apoptosis (estimated by flow cytometric analysis from the percentage of nuclei with DNA content lower than the 2C peak) (C) were evaluated. Data are the mean $\pm$ SD of three different cultures. * $p > 0.05$ with respect to hepatocytes cultured in the absence of L-NAME.

populations (an estimation of PI accessibility to DNA); FS distribution within 2C and 4C populations (an estimation of nuclear size); SS distribution within 2C and 4C populations (an estimation of chromatin condensation); general nuclear protein in the whole hepatocyte population and within 2C and 4C populations (an estimation of nuclear transcriptional activity); and the calculated ratio of general protein content to DNA content in the whole hepatocyte population and within 2C and 4C populations (an estimation of nuclear transcriptional activity independent of the relative amount of chromatin/nucleus). Figure 6 shows the time-dependent change in nuclear complexity and protein-DNA ratio within the 2C fraction of hepatocyte nuclei. These are the only cytometric parameters that consistently differentiated controls and NAME-treated cultures.

Discussion

In the present study, it was observed that inhibition of early NO biosynthesis allowed a high preservation of liver phenotype and extended hepatocyte survival in culture. We have observed that the early addition of NAME, an inhibitor of NO synthesis, produced short-term beneficial effects in the metabolic functionality of hepatocytes (during the first 24 h in culture), and an improvement in cell survival and culture stability was also observed after longer periods of culture (up to 6 days). It seems evident that short-term beneficial effects of NAME are observed for those parameters which are less stable in culture, namely CYP activities and glycogen content. In contrast, hepatic functions highly preserved in culture (glucose, urea and albumin synthesis, drug-conjugating enzymes and GSH levels) (Gómez-Lechón *et al.* 1992) are not modified by NAME.

The fact that the presence of NAME during liver perfusion is enough to reduce biochemical changes in culture supports the idea that early cell signals related to NO biosynthesis are involved in hepatocyte functionality. The isolation procedure induced strong oxidative stress in hepatocytes which involves oxygen- and NO-derived species, that could contribute to the spontaneous, irreversible alteration of the biochemical status of cultured hepatocytes (Gómez-Lechón *et al.* 1996a, López-García and Sanz-González 2000). In particular, it has been pointed out that peroxynitrite released during liver perfusion with collagenase could account for profound changes in hepatocyte phenotype (López-García and Sanz-González 2000). In our opinion, culture conditions also play a role in the control of events responsible for induction of NO biosynthesis. Addition of NAME after 3 or 6 h of culture (at this time NO production is still low) resulted in a large inhibition of spontaneous losses of glycogen content and CYP activity (figure 2). These results suggest that regardless of the cellular signals released during hepatocyte isolation and the first 2–3 h of culture, other events occurring at a later culture period should be considered.

Although a general beneficial effect on CYP activities was derived from NAME treatment of hepatocytes, differences in the effects produced on each CYP isoforms were found (table 1). CYP2B1 was the most extensively stabilized isoform, whereas CYP2E1 seemed to be relatively insensitive to NAME. Since CYP2B1 is one of the CYPs most rapidly lost in standard culture conditions (Donato *et al.* 1994, Woodcroft and Novak 1998), the benefits of NAME for this isoform become more evident. The behaviour of CYP2E1 is not surprising because regulation of this isozyme is different from that of other CYPs (Kim *et al.* 1995, Woodcroft and Novak 1998). The physiological significance of NO biosynthesis in the liver is only beginning to be understood and the molecular basis of NO effects on CYPs and other proteins is not yet clear. Reactivity of NO with haem iron to yield nitrosyl-haem adducts may convert haemoproteins into a primary target for NO (Ignarro 1990, Chamulitrat *et al.* 1995). It has been postulated that NO inhibition of CYP-mediated oxidations in microsomal preparations involves both binding of NO to the prosthetic group in the catalytic centre and a destruction of the integrity of the primary structure of the haemoprotein, possibly resulting from the actions of species derived from the oxidation of NO by oxygen (Khatsenko *et al.* 1993, Wink *et al.* 1993). However, direct interaction of NO or NO-derived species may not be the only mechanism involved in functional enzyme inhibition. There is evidence that induction of endogenous NO synthesis after hepatocyte stimulation with cytokines leads to a decrease in CYP protein and mRNA expression, which can be

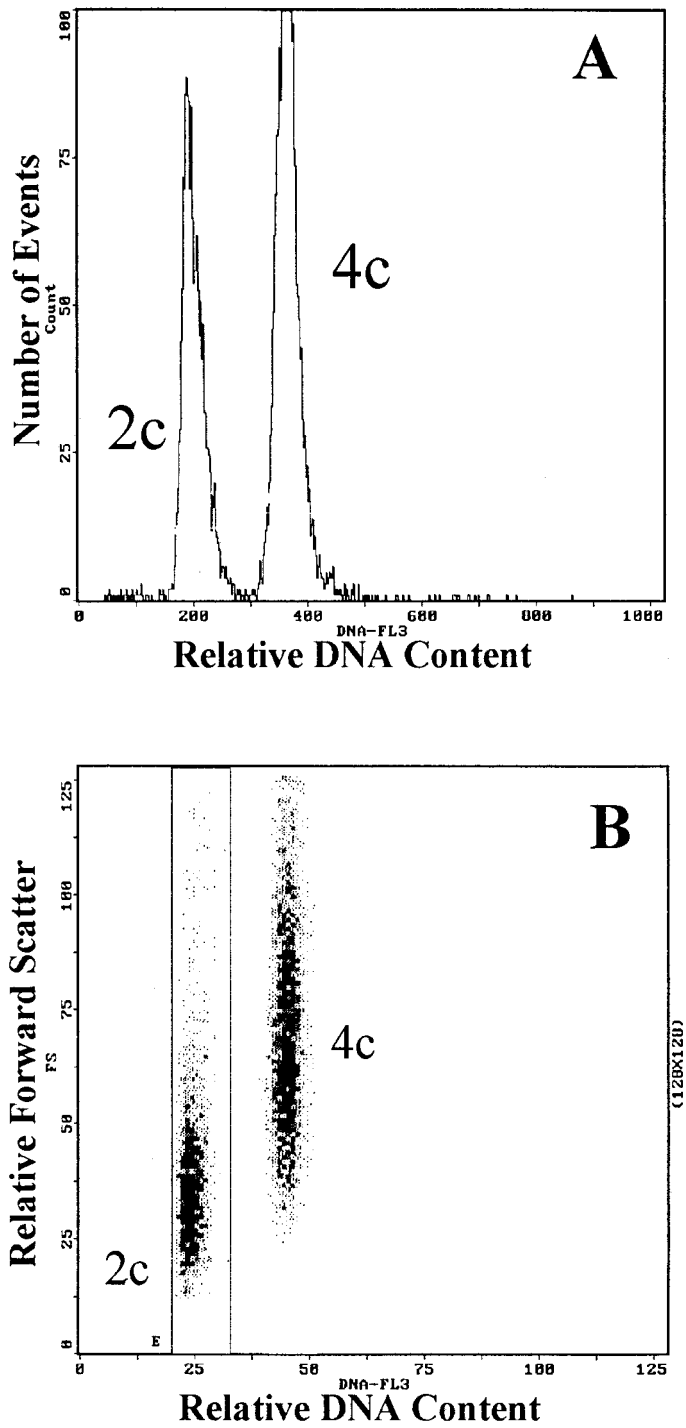


Figure 5. Representative results of the flow cytometric simultaneous analysis of biochemical and structural parameters in suspensions of isolated nuclei from rat cultured hepatocytes. (A) DNA content distribution, estimated from the fluorescence intensity of propidium iodide. Nuclei are diploid (2c) and tetraploid (4c). (B) Size of diploid and tetraploid nuclei estimated from forward-scatter measurements.

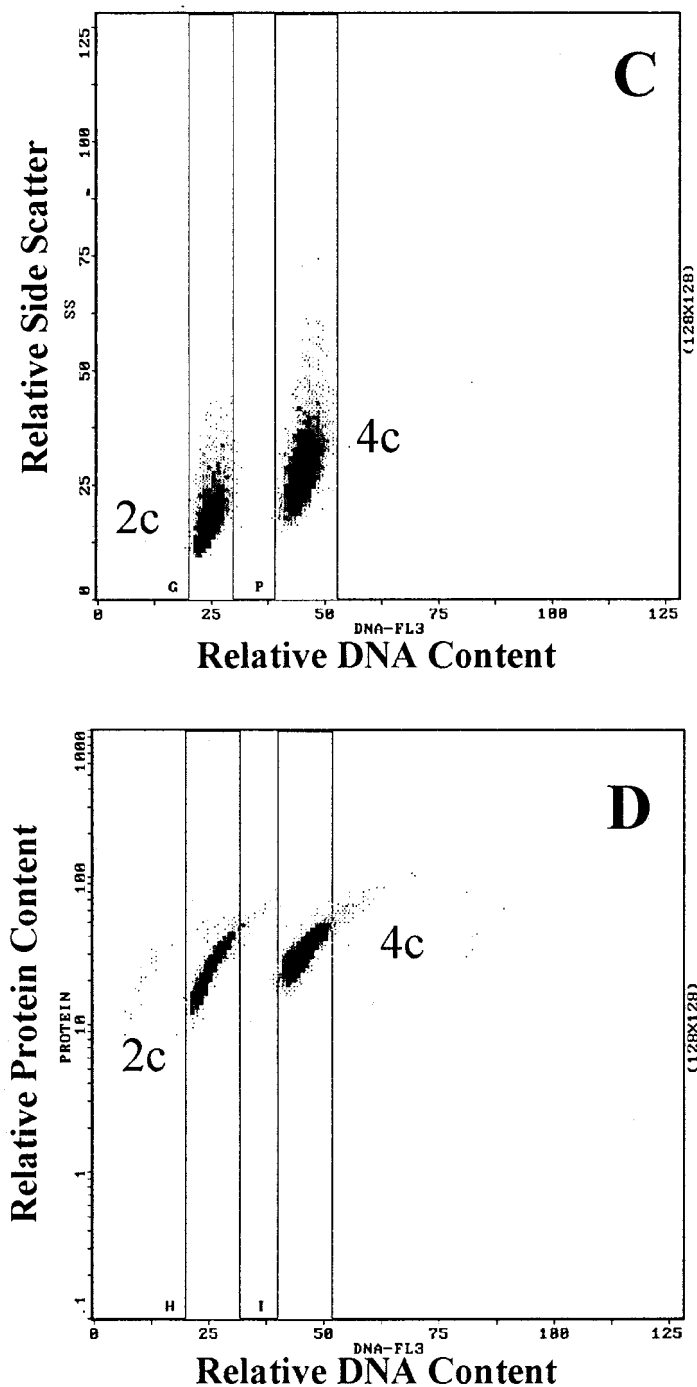


Figure 5 (continued). Representative results of the flow cytometric simultaneous analysis of biochemical and structural parameters in suspensions of isolated nuclei from rat cultured hepatocytes. (C) Complexity of diploid and tetraploid nuclei estimated from side-scatter measurements. (D) Protein content in diploid and tetraploid nuclei estimated from the fluorescence intensity of fluorescein isothiocyanate. The scale of corresponding axes in each graph indicate arbitrary units of light scatter or fluorescence intensity. Rectangular boxes in biparametric histograms are the analysis regions set up for the calculation of numerical data.

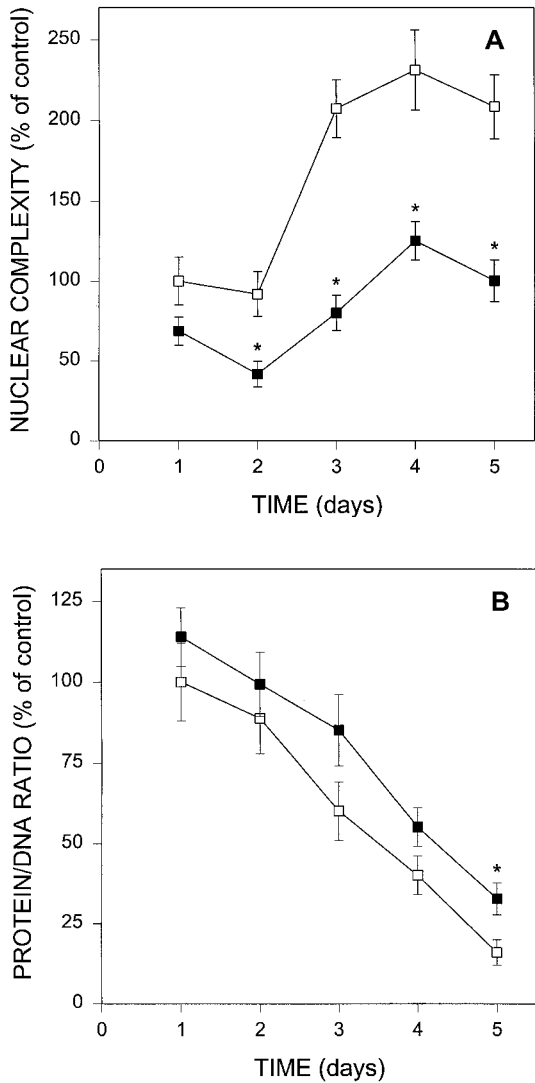


Figure 6. Cytometric parameters of hepatocytes cultured in the presence of L-NAME. Hepatocytes isolated in the presence of 1.68 mM L-NAME were cultured in medium containing (closed symbols) or not (open symbols) 1.68 mM L-NAME. At different time in culture, nuclear complexity (A) and protein-DNA ratio (B) were analysed. Results are the percentage of control value on day 1. Data are the mean $\pm$ SD of three different cultures. * $p > 0.05$ with respect to hepatocytes cultured in the absence of L-NAME.

up-regulated by NO-biosynthesis inhibitors (Stadler *et al.* 1994, Donato *et al.* 1997). Therefore, there is no reason to rule out the possibility that early production of NO negatively regulates specific hepatocyte genes, including CYP genes. This idea is consistent with the greater transcriptional activity found in NAME-treated hepatocytes (figure 6A).

Among the variety of biological effects attributed to NO, cytotoxicity induced by NO has been linked with the induction of apoptosis in certain cell types, however, it seems that NO suppresses apoptosis in other cells (Clemens 1999, Li

and Billiar 1999). The role of NO in apoptosis is particularly controversial in the case of hepatocytes. It has been reported that cytokine-induced NO formation or exogenous NO exposure initiates apoptosis in hepatocytes (Liu *et al.* 1996). More recently, it was suggested that modulation of caspase activation by NO results in an anti-apoptotic function (Li *et al.* 1999). The dual protective/injurious role of NO in the liver seems to be determined by different factors including the type of insult, the redox status, the localization and amount of NO production and the co-production of reactive oxygen species (Clemens 1999, Li and Billiar 1999). NO effects have most often been examined under apoptotic conditions induced by different external stimuli, however, spontaneous apoptosis also occurs in hepatocytes (Qiao and Farrel 1999). We have observed that the sole addition of NAME produced a significant reduction in spontaneous hepatocyte apoptosis, as evidenced by caspase 3 activation and the percentage of apoptosis estimated from cytometric analysis of nuclei DNA distribution (figure 4B and C). These results suggest that NO synthesized during hepatocyte isolation and culture under standard conditions may act as an inducer of cellular events responsible for programmed cell death. Cellular death signalling proceeds to an execution stage that involves the activation of the caspase cascade (Li *et al.* 1999) and an imbalance between cell survival and cell death signals in standard cell cultures may lead to the spontaneous apoptosis of a small subpopulation of sensitive hepatocytes. Oxidative stress has been suggested not only as responsible for the decay in biochemical functionality of cultured hepatocytes, but also as an inducer factor of spontaneous apoptosis in hepatocytes (Fau *et al.* 1997). Further study is needed to elucidate the molecular stimuli that trigger the apoptotic programme in hepatocyte cultures.

Nuclei obtained from hepatocyte cultures are used for studies on nuclear structure and function (Gómez-Lechón *et al.* 1995). DNA-ploidy and general protein content are traditionally related to cell growth and activation (Darzynkiewicz 1990), and morphological parameters of isolated nuclei (i.e. size, granularity/complexity) are indicators of the changes in chromatin structure which accompany cell activation and DNA transcription (Darzynkiewicz 1990). The time-course of nuclear complexity and protein-DNA ratio were different in control and NAME-treated hepatocytes (figure 6). Nuclear complexity increased during culture, but NAME-treated cells always exhibited lower intensity of nuclear granularity/complexity, suggesting a less condensed chromatin, compatible with more intense transcriptional activity (Darzynkiewicz 1990). Consistent with this is the fact that the protein-DNA ratio in 2C nuclei was always greater in the hepatocytes from NAME-treated cultures (figure 6B). These results were consistent with the other non-cytometric parameters exhibiting the protective effect of NAME. Our results confirm that simple, straightforward techniques involving intrinsic (i.e. FS, SS) or fluorochrome-revealed (i.e. DNA, protein) parameters may be useful in assessing metabolic competence, even in the absence of major morphological or functional alterations.

The present results support the idea that endogenous NO plays a role in the spontaneous loss of biochemical functionality and in the induction of apoptosis in cultured hepatocytes. NO seems to be particularly important at two different time-points: (1) during liver perfusion and (2) in the 6-24-h culture period, when hepatocytes actively synthesize NO. Inhibition of early NO formation not only led to a better hepatocyte functionality during the first 24 h in culture, but also prevented activation of caspase 3 and increased cellular survival up to 6 days,

thus suggesting that cell isolation and initial culture periods are crucial in the control of signalling pathways leading to apoptotic cell death at later times of culture. In conclusion, inhibition by NAME of early NO formation is an efficient procedure for obtaining hepatocytes with high cell survival, stable expression of differentiated functions, and few signs of early cell senescence.

Acknowledgements

The authors acknowledge the expert technical assistance of E. Belenchón and M. C. Lorenzo. The financial support of the European Union (Projects BMH4-CT96-0254 and BIO4-CT96-0052) is gratefully acknowledged.

References

- BORT, R., MACÉ, K., BOOBIS, A., GÓMEZ-LECHÓN, M. J., PFEIFER, A. and CASTELL, J. V., 1999, Hepatic metabolism of diclofenac: role of human CYP in the minor oxidative pathways. *Biochemical Pharmacology*, **58**, 787-796.
- BURKE, M. D., THOMPSON, S., ELCOMBE, C. R., HALPERT, J., HAAPARANTA, T. and MAYER, R. T., 1985, Ethoxy-, pentoxy-, and benzyloxyphenoxones and homologues: a series of substrates to distinguish between different induced cytochromes P-450. *Biochemical Pharmacology*, **34**, 3337-3345.
- CASTELL, J. V., LARRAURI, A. and GÓMEZ-LECHÓN, M. J., 1985, A study of the relative hepatotoxicity *in vitro* of the non-steroidal anti-inflammatory drugs ibuprofen, flurbiprofen and butibufen. *Xenobiotica*, **18**, 737-745.
- CHAMULITRAT, W., JORDAN, S. J., MASON, R. P., LITTON, A. L., WILSON, J. G., WOOD, E. R., WOLBERG, G. and MOLINA, L., 1995, Targets of nitric oxide in a mouse model of liver inflammation by *Corinebacterium parvum*. *Archives of Biochemistry and Biophysics*, **316**, 30-37.
- CLEMENS, M. G., 1999, Nitric oxide in liver injury. *Hepatology*, **30**, 1-5.
- DARZYŃKIEWICZ, Z., 1990, Probing nuclear chromatin by flow cytometry. In M. R. Melamed, T. Lindmo and M. L. Mortimer (eds), *Flow Cytometry and Sorting*, 2nd edn (New York: Wiley-Liss), pp. 315-340.
- DONATO, M. T., CASTELL, J. V. and GÓMEZ-LECHÓN, M. J., 1994, Cytochrome P450 activities in pure and co-cultured rat hepatocytes. Effects of model inducers. *In Vitro Cellular and Developmental Biology*, **30A**, 825-832.
- DONATO, M. T., GÓMEZ-LECHÓN, M. J. and CASTELL, J. V., 1992, Biotransformation of drug by cultured hepatocytes. In J. V. Castell and M. J. Gómez-Lechón (eds), *In Vitro Alternatives to Animal Pharmacotoxicology* (Madrid: Farmaindustria), pp. 149-178.
- DONATO, M. T., GÓMEZ-LECHÓN, M. J. and CASTELL, J. V., 1993, A microassay for measuring cytochrome P450IA1 and P450IIB1 activities in intact human and rat hepatocytes cultured on 96-well plates. *Analytical Biochemistry*, **213**, 29-33.
- DONATO, M. T., GUILLÉN, M. I., JOVER, R., CASTELL, J. V. and GÓMEZ-LECHÓN, M. J., 1997, Nitric oxide-mediated inhibition of cytochrome P450 by interferon-gamma in human hepatocytes. *Journal of Pharmacology and Experimental Therapeutics*, **281**, 484-490.
- EDWARDS, A. M., GLISTAK, M. L., LUCAS, C. M. and WILSON, P. A., 1984, 7-Ethoxycoumarin deethylase activity as a convenient measure of liver drug metabolizing enzymes: regulation in cultured rat hepatocytes. *Biochemical Pharmacology*, **33**, 1537-1546.
- FAU, D., LEKEHAL, M., FARRELL, G., MOREAU, A., MOULIS, C., FELDMANN, G., HAOUI, D. and PESSAYRE, D., 1997, Diterpenoids from germander, an herbal medicine, induce apoptosis prevented in isolated rat hepatocytes. *Gastroenterology*, **113**, 1334-1346.
- GÓMEZ-LECHÓN, M. J., CASTELL, J., GUILLÉN, I., O'CONNOR, J. E., NAKAMURA, T., FABRA, R. and TRULLENQUE, R., 1995, Effects of hepatocyte growth factor (HGF) on the growth and metabolism of human hepatocytes in primary culture. *Hepatology*, **21**, 1248-1254.
- GÓMEZ-LECHÓN, M. J., DONATO, M. T., PONSODA, X., JOVER, R. and CASTELL, J. V., 1992, Experimental *in vitro* models to evaluate hepatotoxicity. In J. V. Castell and M. J. Gómez-Lechón (eds), *In Vitro Alternatives to Animal Pharmacotoxicology* (Madrid: Farmaindustria), pp. 85-107.
- GÓMEZ-LECHÓN, M. J., DONATO, M. T., PONSODA, X., FABRA, R., TRULLENQUE, R. and CASTELL, J. V., 1997, Isolation, culture and use of human hepatocytes in drug research. In J. V. Castell and M. J. Gómez-Lechón (eds), *In Vitro Methods in Pharmaceutical Research* (London: Academic Press), pp. 129-153.

- GÓMEZ-LECHÓN, M. J., GUILLÉN, I., PONSODA, X., FABRA, R., TRULLENQUE, R., NAKAMURA, T. and CASTELL, J. V., 1996a, Cell cycle progression proteins (cyclins), oncogene expression, and signal transduction during the proliferative response of human hepatocytes to hepatocyte growth factor. *Hepatology*, **23**, 1012-1019.
- GÓMEZ-LECHÓN, M. J., JOVER, R., DONATO, T., PONSODA, X. and CASTELL, J. V., 2000, Expression of liver specific genes in hepatocytes cultured in collagen gel. In A. Marcieira-Coelho (ed.), *Matrix. Series Progress in Molecular and Subcellular Biology. Signalling Through the Cell Surface* (Berlin: Springer-Verlag), pp. 89-104.
- GÓMEZ-LECHÓN, M. J., LOPEZ, M. P. and CASTELL, J. V., 1984, Biochemical functionality and recovery of hepatocytes after deep-freezing storage. *In Vitro*, **20**, 826-832.
- GÓMEZ-LECHÓN, M. J., PONSODA, X. and CASTELL, J. V., 1996b, A microassay for measuring glycogen in 96-well-cultured cells. *Analytical Biochemistry*, **263**, 296-301.
- HARBRECHT, B. G. and BILLIAR, T. R., 1995, The role of nitric oxide in Kupffer cell-hepatocyte interactions. *Shock*, **3**, 79-87.
- IGNARRO, L. J., 1990, Biosynthesis and metabolism of endothelium-derived nitric oxide. *Annual Reviews of Pharmacology and Toxicology*, **30**, 535-560.
- KHATSENKO, O. G., GROSS, S. S., RIFKIND, A. B. and VANE, J. R., 1993, Nitric oxide is a mediator of the decrease in cytochrome P450-dependent metabolism caused by immunostimulants. *Proceedings of the National Academy of Sciences, USA*, **90**, 11147-11151.
- KIM, S. G., SHEHIN, S. E., STATES, C. and NOVAK, R. F., 1995, Evidence for increased translational efficiency in the induction of P450IIE1 by solvents: analysis of P450IIE1 mRNA polyribosomal distribution. *Biochemical and Biophysical Research Communications*, **172**, 767-774.
- KRONBACH, T., MATHYS, D., GUT, J., CATIN, T. and MEYERS, U. A., 1987, High-performance liquid chromatographic assays for bufuralol 1'-hydroxylase, debrisoquine 4-hydroxylase, and dextromethorphan o-demethylase in microsomes and purified cytochrome P-450 isozymes of human liver. *Analytical Biochemistry*, **162**, 24-32.
- LI, J. and BILLIAR, T. R., 1999, Nitric oxide IV. Determinants of nitric oxide protection and toxicity in liver. *American Journal of Physiology*, **276**, G1069-G1073.
- LI, J., BOMBECK, C. A., YANG, S., KIM, Y.-M. and BILLIAR, T. R., 1999, Nitric oxide suppresses apoptosis via interrupting caspase activation and mitochondrial dysfunction in cultured hepatocytes. *Journal of Biological Chemistry*, **274**, 17325-17333.
- LIU, Z.-Z., CUI, S., BILLIAR, T., DORKO, K., HALFER, W., GELLER, D. A., MICHALOPOULOS, G., BEGER, H.-G., ALGINA, J. and NUSSLER, A. K., 1996, Effects of hepatocellular mitogens on cytokine-induced nitric oxide synthesis in human hepatocytes. *Journal of Leukocyte Biology*, **60**, 382-388.
- LÓPEZ-GARCÍA, M. P., 1998, Endogenous nitric oxide is responsible for the early loss of P450 in cultured rat hepatocytes. *FEBS Letters*, **438**, 145-149.
- LÓPEZ-GARCÍA, M. P. and SANZ-GONZÁLEZ, S. M., 2000, Peroxynitrite generated from constitutive nitric oxide synthase mediates the early biochemical injury in short-term cultured hepatocytes. *FEBS Letters*, **466**, 187-191.
- LOWRY, O. H., ROSENBOUGH, N. J., FARR, A. L. and RANDALL, R. J., 1951, Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry*, **193**, 265-275.
- MISKO, T. P., SCHILLING, R. J., SALVEMINI, D., MOORE, W. M. and CURRIE, M. G., 1993, A fluorimetric assay for the measurement of nitrite in biological samples. *Analytical Biochemistry*, **214**, 11-16.
- NICHOLLS-GRZEMSKI, F. A., TIRMENSTEIN, M. A. and FARISS, M. W., 1999, Time-dependent production of nitric oxide by rat hepatocyte suspensions. *Biochemical Pharmacology*, **57**, 1223-1226.
- NICHOLSON, D. W., ALI, A. and THORNBERRY, N. A., 1995, Identification and inhibition of the ICE/CED-3 protease for mammalian apoptosis. *Nature*, **376**, 37-43.
- NUSSLER, A. K., DI SILVIO, M., LIU, Z. Z., GELLER, D. A., FREESWICK, P., DORKO, K., BARTOLI, F. and BILLIAR, T., 1995, Further characterization and comparison of inducible nitric oxide synthase in mouse, rat, and human hepatocytes. *Hepatology*, **21**, 1552-1560.
- OSAWA, Y., DAVILA, J. C., NAKATSUKA, M., MEYER, C. A. and DARBYSHIRE, J. F., 1995, Inhibition of P450 cytochromes by reactive intermediates. *Drug Metabolism Reviews*, **27**, 61-72.
- PAINE, A. J., 1990, The maintenance of cytochrome P-450 in rat hepatocyte culture: some applications of liver cell cultures to the study of drug metabolism, toxicity and the induction of the P-450 system. *Chemico-Biological Interactions*, **74**, 1-31.
- PAPA, S., CAPITANI, S., MATTEUCCI, A., VITALE, M., SANTI, P., MARTELLI, A. M. and MARALDI, N. M., 1987, Flow cytometric analysis of isolated rat liver nuclei during growth. *Cytometry*, **8**, 595-601.
- QIAO, L. and FARRELL, G. C., 1999, The effects of cell density, attachment substratum and dexamethasone on spontaneous apoptosis of rat hepatocytes in primary culture. *In Vitro Cellular and Developmental Biology*, **35**, 417-424.
- RODRÍGUEZ-ARIZA, A. and PAINE, A. J., 1999, Rapid induction of NF- κ B binding during liver cell isolation and culture: inhibition by L-NAME indicates a role for nitric oxide synthase. *Biochemical and Biophysical Research Communications*, **257**, 145-148.

- SONDERFAN, A. J., ARLOTTO, M. P., DUTTON, D. R., McMILLEN, S. K. and PARKINSON, A., 1987, Regulation of testosterone hydroxylation by rat liver microsomal cytochrome P-450. *Archives of Biochemistry and Biophysics*, **255**, 27-41.
- STADLER, J., TROCKFELD, J., SCHMALIX, W. A., BRILL, T., SIEWERT, J. R., GREIM, H. and DOEHMER, J., 1994, Inhibition of cytochromes P450A1 by nitric oxide. *Proceedings of the National Academy of Sciences, USA*, **91**, 3559-3563.
- TASSANEYAKUL, W., VERONESE, M. E., BIRKETT, D. J., GONZALEZ, F. J. and MINERS, J. O., 1993, Validation of 4-nitrophenol as an *in vitro* substrate probe for human liver CYP2E1 using cDNA expression and microsomal kinetic techniques. *Biochemical Pharmacology*, **46**, 1975-1981.
- VARGAS, J. L., O'CONNOR, J. E., ROCHE, E., KNECHT, E. and GRISOLIA, S., 1987, Analysis by flow cytometry of rat hepatocytes from different acinar zones. *Biochemical and Biophysical Research Communications*, **147**, 535-541.
- VARGIU, C., BELLIARDO, S., CRAVANZOLA, C., GRILLO, M. A. and COLOMBATTO, S., 2000, Oxygen regulation of rat hepatocyte iNOS gene expression. *Journal of Hepatology*, **32**, 567-573.
- WANG, H., GAO, X., FUKUMOTO, S., TADEMOTO, S., SATO, K. and HIRAI, K., 1998, Post-isolation inducible nitric oxide synthase gene expression due to collagenase buffer perfusion and characterization of the gene regulation in primary cultured murine hepatocytes. *Journal of Biochemistry*, **124**, 892-899.
- WINK, D. A., OSAWA, Y., DARBYSHIRE, J. F., JONES, C. R., ESHENAU, S. C. and NIMS, R. W., 1993, Inhibition of cytochromes P450 by nitric oxide and a nitric oxide-releasing agent. *Archives of Biochemistry and Biophysics*, **300**, 115-123.
- WOODCROFT, K. J. and NOVAK, R. F., 1998, Xenobiotic-enhanced expression of cytochromes P450 2E1 and 2B in primary cultured rat hepatocytes. *Drug Metabolism and Disposition*, **26**, 372-378.
- US National Institutes of Health, *Guide for Care and Use of Laboratory Animals*.

Cooperativity of α -naphthoflavone in cytochrome P450 3A-dependent drug oxidation activities in hepatic and intestinal microsomes from mouse and human

C. EMOTO[†], H. YAMAZAKI[†], H. IKETAKI[†], S. YAMASAKI[†],
T. SATOH[‡], R. SHIMIZU[‡], S. SUZUKI[‡], N. SHIMADA[§],
M. NAKAJIMA[†] and T. YOKOI^{†*}

[†] Faculty of Pharmaceutical Sciences, Kanazawa University, Kanazawa 920-0934, Japan

[‡] Biomedical Research Institute, Chiba 270-1402, Japan

[§] Daiichi Pure Chemicals, Ibaraki 319-1182, Japan

Received 6 July 2000

1. The effects of several CYP3A substrates (α -naphthoflavone (α NF), terfenadine, midazolam, erythromycin) on nifedipine oxidation and testosterone 6 β -hydroxylation activities were investigated in hepatic and intestinal microsomes from mouse and human.

2. α NF (10 μ M) and terfenadine (100 μ M) inhibited nifedipine oxidation activities (at substrate concentration of 100 μ M) in mouse hepatic microsomes to ~50%, but not in mouse intestinal microsomes. α NF (30 μ M) stimulated nifedipine oxidation activities in mouse and human intestinal microsomes and in human hepatic microsomes to ~1.3-1.8-fold. Inhibitory potencies (50% inhibition concentration, IC₅₀) of midazolam and erythromycin for nifedipine oxidations were calculated to be ~90 μ M in human intestinal microsomes. In contrast, testosterone (100 μ M) stimulated the nifedipine oxidation activities ~1.5-fold in hepatic and intestinal microsomes from mouse and human.

3. α NF showed different effects on the kinetic parameters including the Hill coefficients of nifedipine oxidation and testosterone 6 β -hydroxylation catalysed by hepatic and intestinal microsomes from mouse and human. Cooperativity in nifedipine oxidation was increased by the addition of α NF to pooled human hepatic microsomes, but little effects of α NF could be observed in individual human intestinal microsomes.

4. These results suggest that CYP3A enzymes in liver and intestine might have different characteristics and that observations from hepatic microsomes should not be directly applicable to intestine metabolism in some cases. Studies of drug-drug interactions of CYP3A substrates are recommended to be performed using intestinal samples.

Introduction

Cytochrome P450 (P450, CYP) consists of a superfamily of haem-containing monooxygenases associated with the metabolism of substrates, such as drugs, environmental pollutants and endogenous substrates (Guengerich 1991). P450s are microsomal enzymes mainly expressed in the liver as well as in extrahepatic tissues such as the intestine, lung and kidney (Krishna and Klotz 1994). In the adult human liver, CYP3A forms comprise on average ~30% of the total P450 present and the levels can reach ~60% (Shimada *et al.* 1994). It has also been reported that CYP3A4 is a dominant P450 form in the epithelial barrier of the small intestine (Watkins *et al.* 1987, Paine *et al.* 1997). The small intestine may be a major site for CYP3A-mediated metabolism, which would be an important factor in the

* Author for correspondence: e-mail: tyokoi@kenroku.kanazawa-u.ac.jp

interindividual variability in disposition after oral administration of CYP3A substrates, including felodipine (Edgar *et al.* 1990), midazolam (Smith *et al.* 1981) and terfenadine (Lalonde *et al.* 1996).

It has recently been pointed out that CYP3A enzymes have two interesting characteristics that are possibly related. First, CYP3A enzymes have the broadest substrate selectivity of the different P450 enzymes (Guengerich 1995). Second, the kinetic interactions are unusual (Korzekwa *et al.* 1998). The characteristics of the active sites have been determined indirectly from substrate specificities (Shou *et al.* 1994, Ma *et al.* 2000), site-directed mutagenesis studies (Domanski *et al.* 1998, Wang *et al.* 1998) and other approaches that are reviewed by Houston and Kenworthy (2000). For example, nifedipine has been shown to inhibit testosterone 6 β -hydroxylation, but testosterone does not inhibit nifedipine oxidation in human hepatic microsomes (Wang *et al.* 2000). Testosterone 6 β -hydroxylation activities catalysed by human hepatic microsomes and the recombinant CYP3A4 system have been demonstrated to show non-hyperbolic rate-substrate concentration profiles (homotropic cooperativity) (Houston and Kenworthy 2000). α -Naphthoflavone (α NF) is known to be an effector and substrate of CYP3A4 (Shou *et al.* 1994). The effects of α NF have been reported to vary depending on the reactions, yielding either stimulation (heterotropic cooperativity) or inhibition in hepatic microsomes and recombinant CYP3A4 systems (Ueng *et al.* 1997, Mäenpää *et al.* 1998). Studies using α NF as a heterotropic stimulator of polycyclic aromatic hydrocarbon metabolism have suggested that the effector and substrate are probably present in the active site at the same time (Shou *et al.* 1994). Interestingly, recent studies have suggested that substitution of larger amino acid side-chains in the wild-type CYP3A4 partially mimics the action of α NF by reducing the size of the active site (Harlow and Halpert 1998, Domanski *et al.* 2000).

The intestinal localization of CYP3A should be also significant in metabolism-based drug-drug interactions, as in the liver. Although the sequences of intestinal and hepatic CYP3A4 cDNAs have been reported to be identical (Lown *et al.* 1998) and the inhibitory effects of ketoconazole on intestinal and hepatic CYP3A4 are similar (Gibbs *et al.* 1999), the catalytic characteristics of CYP3A enzymes in the intestines are not well understood. It is necessary to clarify the similarities and differences between intestinal and hepatic microsomes in order to investigate the drug interactions of orally administered medicines.

In the present study, the enzymatic characteristics of CYP3A proteins were investigated by analysing the effects of CYP3A substrates including α NF on drug oxidation activities in hepatic and intestinal microsomes from the rifampicin-treated mouse and human. Nifedipine, terfenadine, midazolam and erythromycin, which are orally administered medicines, and testosterone and α NF were used as the CYP3A substrates to study *in vitro* substrate-substrate interactions with CYP3A enzymes. Kinetic parameters in the cooperativity for nifedipine oxidation and testosterone 6 β -hydroxylation activities in hepatic and intestinal microsomes were investigated in the absence and presence of α NF.

Materials and methods

Chemicals

Nifedipine, terfenadine, erythromycin, α NF, testosterone and (*p*-amidinophenyl) methanesulphonyl fluoride (APMSF) were from Wako Pure Chemicals (Osaka, Japan). Oxidized nifedipine,

rifampicin, aprotinin, bestatin, leupeptin and trypsin inhibitor (Type II-S: soybean) were from Sigma (St Louis, MO, USA) and 6 β -hydroxytestosterone was from Ultrafine Chemicals (Manchester, UK). Midazolam was kindly provided by Yamanouchi Pharmaceuticals (Tokyo, Japan). Other reagents were of the highest grade commercially available.

Intestinal and hepatic microsomes from mouse and human

Male C57BL/6CrSlc mice, 8 weeks old, were from Japan SLC, Inc. (Hamamatsu, Japan). In order to induce Cyp3a enzymes, five mice were treated intraperitoneally with rifampicin (100 mg kg⁻¹) for 4 days (Yanagimoto *et al.* 1997). Pooled microsomes from mouse small intestines were prepared according to Iatsimirskaia *et al.* (1997) with slight modifications. Briefly, immediately after excision of the small intestine, a 10 cm segment of the upper part of the small intestine was placed on a stainless steel dish kept on ice and cut longitudinally, then washed in ice-cold washing buffer (0.9% NaCl, 1 mM APMSF, 1 mM EDTA) by gentle swirling for 1 min. The mucosal cells were gently scraped off with a micro-cover glass. The scraped off sample was dipped in 3 vols ice-cold buffer A (50 mM Tris-HCl buffer, pH 7.4, containing 150 mM KCl, 1 mM APMSF, 1 mM EDTA, 1 mg ml⁻¹ trypsin inhibitor, 10 μ M leupeptin, 0.04 unit ml⁻¹ aprotinin, 1 μ M bestatin, and 20% v/v glycerol) and was homogenized using a motor-driven Teflon-tipped pestle (three strokes). The homogenates were centrifuged at 9000g for 20 min at 4°C and then the supernatant was centrifuged at 105 000g for 60 min at 4°C. The fatty layer on the surface was removed carefully and the microsomal pellets were resuspended in ice-cold buffer A.

The pooled hepatic microsomes from mouse were prepared by the same methods above except for the use of 0.9% NaCl instead of the washing buffer. Protein concentrations were determined according to the method of Lowry *et al.* (1951). The effects of treatment of rifampicin on the levels of Cyp3a mRNA and CYP3a proteins in hepatic and intestinal microsomes from mouse were confirmed by Northern blot analysis and Western blot analysis, respectively (data not shown). $V_{\max}$ for nifedipine oxidation activities of mouse hepatic and intestinal microsomes were increased to 2.1- and 1.4-fold by the rifampicin treatment, respectively. Hill coefficient n was also changed from 1.0 to 2.0 and from 1.9 to 1.5 in hepatic and intestinal microsomes by rifampicin, respectively, but S_{50} was similar in these microsomes (~30 μ M).

Pooled human hepatic microsomes designated as HLM161 (lot 12) were obtained from Gentest (Woburn, MA, USA). Information concerning these microsomes was provided in the data sheet by the manufacturer (20 mg protein ml⁻¹; 0.5 nmol P450 mg protein⁻¹). Individual human intestinal microsomes from Caucasians (designated as HIM51, HIM52, and HIM53 from a 55-year-old male, 55-year-old female and 58-year-old male, respectively) were prepared by Biomedical Research Institute (Chiba, Japan). Protein concentrations of HIM51, HIM52, and HIM53 were 6.0, 13 and 7.5 mg ml⁻¹, respectively.

Enzyme assays

Nifedipine oxidation and testosterone 6 β -hydroxylation activities were determined as described by Yamazaki and Shimada (1997). The standard incubation mixture (final volume of 0.25 ml) contained 0.1 and 0.3 mg protein of hepatic and intestinal microsomes, respectively, 100 mM potassium phosphate buffer, pH 7.4, an NADPH-generating system consisting of 0.5 mM NADP⁺, 5 mM glucose 6-phosphate, 0.5 unit ml⁻¹ glucose 6-phosphate dehydrogenase, and 100 μ M nifedipine or testosterone. The reaction mixtures were incubated at 37°C for 10 min and then terminated by the addition of 1.5 ml dichloromethane and 0.2 M NaCl-0.1 M sodium carbonate for nifedipine oxidation or of 1.5 ml dichloromethane and 0.3 M NaCl for testosterone 6 β -hydroxylation. After mixing by using a vortex device, the mixtures were centrifuged at 900g for 10 min. Organic phases were evaporated at 40°C under a gentle nitrogen stream. The residue was dissolved in 200 μ l of 64% methanol, and a 100 μ l portion was subjected to HPLC. HPLC analyses were performed using a C₁₈ 5- μ m analytical column (Mightysil, 150 $\times$ 4.6 mm i.d.; Kanto Chemicals, Tokyo, Japan). The column temperature was 35°C. The elution of both testosterone and nifedipine metabolites was conducted with a 64% methanol (v/v) at a flow rate of 1 ml min⁻¹ (testosterone) and 0.75 ml min⁻¹ (nifedipine). The eluate was monitored at 240 and 254 nm, respectively.

The substrates and inhibitors (dissolved in methanol) except for α NF, which was dissolved in dimethyl sulphoxide, were added simultaneously to the P450 enzymes. The final concentration of solvent in the incubation mixture was <1%.

Data analysis

Non-linear regression analysis by the computer program Prism (Graphpad Software, San Diego, CA, USA) was used to determine $V_{\max}$, K_m or S_{50} (for substrates showing sigmoidicity), and the Hill coefficient using the equation:

$$v = (V_{\max} \cdot S^n) / (S_{50}^n + S^n)$$

according to the method of Ueng *et al.* (1997), where S_{50} is the substrate concentration showing the half-maximal velocity, n is a measure of cooperativity and V_{max} is the maximal velocity. Estimates of variance (SE, denoted by $\pm$) are presented from the analysis of individual sets of data.

Results

Effects of several CYP3A4 substrates on nifedipine oxidation activities in hepatic and intestinal microsomes from mouse and human

The effects of several CYP3A4 substrates, testosterone, terfenadine, midazolam, erythromycin and α NF on the nifedipine oxidation activities in hepatic and intestinal microsomes from mouse and human were investigated (figure 1). The substrate concentration of nifedipine ($100\mu\text{M}$) chosen in this study was ~ 3 -fold over the mean S_{50} ($32\mu\text{M}$) in the hepatic and intestinal microsomes from mouse and human (see below). Testosterone enhanced the nifedipine oxidation activities

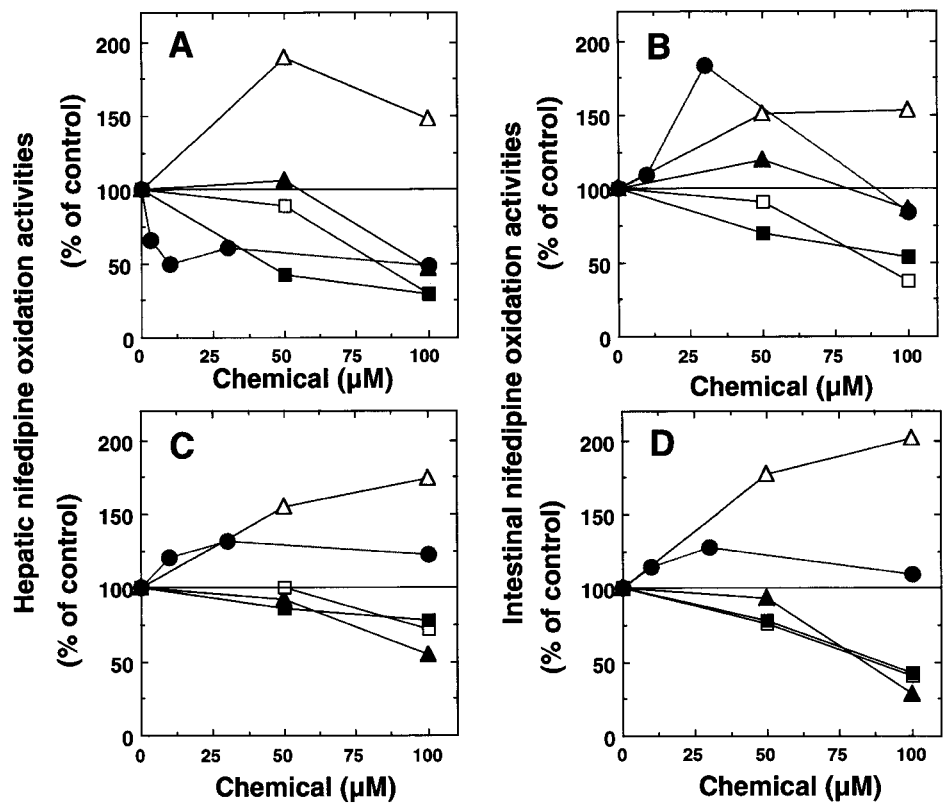


Figure 1. Effects of CYP3A substrates on nifedipine oxidation activities catalysed by hepatic and intestinal microsomes from the rifampicin-treated mouse and human. Nifedipine ($100\mu\text{M}$) was incubated at 37°C for 10 min with pooled hepatic (A) and intestinal (B) microsomes from the rifampicin-treated mouse, a pooled human liver microsomal sample, HLM161 (C), and a human intestinal microsomal sample, HIM51 (D), in the presence of testosterone (Δ), terfenadine ($\blacktriangle$), midazolam ($\square$), erythromycin ($\blacksquare$) and α NF ($\bullet$). Control activities (without chemicals) of nifedipine oxidation by hepatic and intestinal microsomes from mouse and by hepatic and intestinal microsomes from human were 7.2 , 0.23 , 8.4 and $0.60\text{ nmol min}^{-1}\text{ mg protein}^{-1}$, respectively. Results are averages of duplicate determinations.

in all hepatic and intestinal microsomes from mouse and human (figure 1). Terfenadine showed no effects on the nifedipine oxidation activities in mouse intestinal microsomes (figure 1B), although terfenadine (100 μM) inhibited the activities in mouse hepatic microsomes and in human hepatic and intestinal microsomes to 47, 55 and 28%, respectively. Midazolam (100 μM) moderately inhibited the nifedipine oxidation activities in mouse hepatic and intestinal microsomes and in human intestinal microsomes, although it weakly inhibited the activity in human hepatic microsomes (figure 1C). Erythromycin (50–100 μM) strongly inhibited the nifedipine oxidation activities in mouse hepatic microsomes and moderately inhibited the activities in mouse intestinal microsomes. On the other hand, the inhibitory effects of erythromycin (100 μM) in human hepatic and intestinal microsomes appeared to be opposite those in compared with mouse hepatic and intestinal microsomes (figure 1C and D). αNF inhibited the nifedipine oxidation activities in mouse hepatic microsomes (figure 1A), but stimulated the activities in mouse intestinal microsomes and in human hepatic and intestinal microsomes.

From these data, the 50% inhibition concentrations (IC_{50}) of terfenadine, midazolam and erythromycin for nifedipine oxidation activities were calculated to be $\sim 90 \mu\text{M}$ in human intestinal microsomes. However, the IC_{50} of these CYP3A substrates were not obtained ($>100 \mu\text{M}$) in human hepatic microsomes under the conditions used.

Effects of αNF on kinetic parameters for nifedipine oxidation and testosterone 6 β -hydroxylation catalysed by hepatic and intestinal microsomes from mouse

Since different effects of αNF on nifedipine oxidation were observed in hepatic and intestinal microsomes from mouse and human (figure 1), the effects of αNF on the kinetic parameters for nifedipine oxidation were investigated (figure 2 and table 1). In the absence of αNF , the nifedipine oxidation activities of hepatic microsomes from the rifampicin-treated mouse showed sigmoidal rate-substrate concentration profiles, but the curve became hyperbolic by the addition of αNF (figure 2A). A non-linear Eadie-Hofstee plot was also changed to a linear plot by the addition of αNF (figure 2B). The apparent S_{50} and V_{max} for the nifedipine

Table 1. Kinetic parameters for nifedipine oxidation and testosterone 6 β -hydroxylation catalysed by hepatic and intestinal microsomes from the rifampicin-treated mouse.

Microsomes	αNF (30 μM)	Nifedipine oxidation			Testosterone 6 β -hydroxylation		
		S_{50} (μM)	V_{max} (nmol min ⁻¹ mg protein ⁻¹)	n	S_{50} (μM)	V_{max} (nmol min ⁻¹ mg protein ⁻¹)	n
Liver	—	32.2 $\pm$ 1.0	9.45 $\pm$ 0.27	2.0 $\pm$ 0.1	68.9 $\pm$ 1.3	48.1 $\pm$ 6.26	1.2 $\pm$ 0.2
	+	23.3 $\pm$ 1.1	7.45 $\pm$ 0.21	1.4 $\pm$ 0.1	62.4 $\pm$ 1.6	35.2 $\pm$ 7.45	1.4 $\pm$ 0.1
Intestine	—	30.8 $\pm$ 1.1	0.30 $\pm$ 0.01	1.5 $\pm$ 0.2	72.8 $\pm$ 1.1	0.52 $\pm$ 0.02	1.4 $\pm$ 0.2
	+	51.1 $\pm$ 1.1	0.67 $\pm$ 0.05	1.3 $\pm$ 0.1	48.0 $\pm$ 1.2	0.56 $\pm$ 0.04	1.4 $\pm$ 0.1

Assays were performed by using nine to 12 different substrate concentrations ranging from 0.3 to 150 μM nifedipine and 0.5 to 500 μM testosterone. Each value was derived from a single assay and several data points were confirmed by independent determinations. Kinetic parameters were calculated from the fitted curves by non-linear regression (mean $\pm$ SE).

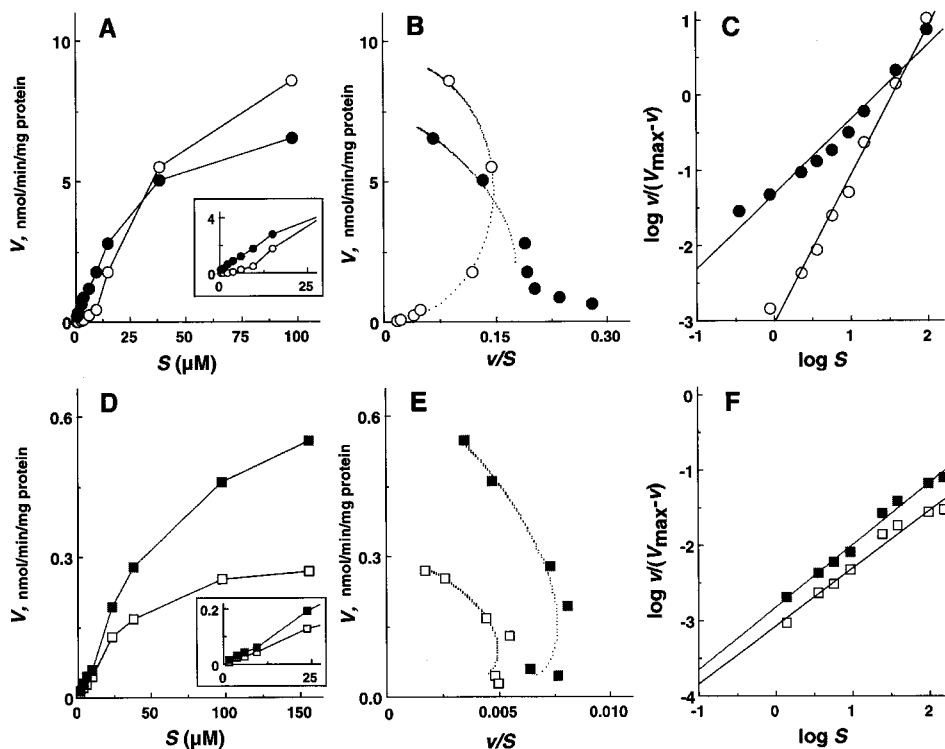


Figure 2. Effects of α NF on steady-state kinetics of nifedipine oxidation activities by hepatic and intestinal microsomes from the rifampicin-treated mouse. Nifedipine (1–150 μ M) was incubated at 37°C for 10 min with hepatic ($\circ$, $\bullet$) and intestinal ($\square$, $\blacksquare$) microsomes in the absence ($\circ$, $\square$) or presence ($\bullet$, $\blacksquare$) of α NF (30 μ M). Plots of v (nmol min⁻¹ mg protein⁻¹) versus S (substrate concentration, μ M) (A, D), v versus v/S (B, E), and $\log v/(V_{\max} - v)$ versus $\log S$ (C, F) are presented from a single assay and several data points were confirmed by independent determinations. The inserts (A, D) indicate the velocity at low substrate concentrations. Kinetic parameters were calculated from the fitted curves using the computer program Prism.

oxidation activities (table 1) were decreased to 72 and 79% by the addition of α NF. The Hill coefficient n , which was calculated as the slope of plots of $\log v/(V_{\max} - v)$ versus $\log S$ (figure 2C), was reduced from 2.0 to 1.4 in hepatic microsomes from the rifampicin-treated mouse (table 1). In contrast, nifedipine oxidation activities in intestinal microsomes from the rifampicin-treated mouse were increased by the addition of α NF (figure 2DF), with increased S_{50} and $V_{\max}$ and an unchanged n (table 1).

The effects of α NF on the testosterone 6 β -hydroxylation activities catalysed by hepatic and intestinal microsomes from mouse were also investigated (table 1). The addition of α NF resulted in a 27% decrease in $V_{\max}$ and in an unchanged S_{50} for testosterone 6 β -hydroxylation activities catalysed by hepatic microsomes from the rifampicin-treated mouse. In intestinal microsomes, however, α NF decreased S_{50} and showed no effects on $V_{\max}$ for testosterone 6 β -hydroxylation. The Hill coefficient n of testosterone 6 β -hydroxylation was unchanged in hepatic and intestinal microsomes.

Table 2. Kinetic parameters for nifedipine oxidation and testosterone 6 β -hydroxylation catalysed by hepatic and intestinal microsomes from human.

Microsomes	Sample	α NF (30 μ M)	Nifedipine oxidation				Testosterone 6 β -hydroxylation			
			S_{50} (μ M)	$V_{\max}$ (nmol min ⁻¹ mg protein ⁻¹)	n		S_{50} (μ M)	$V_{\max}$ (nmol min ⁻¹ mg protein ⁻¹)	n	
Liver	HLM161	—	16.8 $\pm$ 1.3	9.86 $\pm$ 1.08	1.1 $\pm$ 0.4		47.6 $\pm$ 1.1	10.5 $\pm$ 0.43	1.3 $\pm$ 0.1	
		+	35.2 $\pm$ 1.2	13.0 $\pm$ 1.06	2.5 $\pm$ 0.8		112 $\pm$ 1.4	10.2 $\pm$ 1.55	1.1 $\pm$ 0.2	
Intestine	HIM51	—	44.9 $\pm$ 1.1	0.89 $\pm$ 0.07	1.3 $\pm$ 0.2		68.8 $\pm$ 1.1	1.85 $\pm$ 0.05	1.3 $\pm$ 0.1	
		+	30.8 $\pm$ 1.2	1.03 $\pm$ 0.11	1.2 $\pm$ 0.3		66.1 $\pm$ 1.1	1.98 $\pm$ 1.10	1.6 $\pm$ 0.3	
	HIM52	—	37.5 $\pm$ 1.1	0.71 $\pm$ 0.04	1.6 $\pm$ 0.2		58.9 $\pm$ 1.2	1.50 $\pm$ 0.10	1.3 $\pm$ 0.2	
		+	29.9 $\pm$ 1.1	0.73 $\pm$ 0.05	1.3 $\pm$ 0.3		90.8 $\pm$ 1.5	2.00 $\pm$ 0.38	1.1 $\pm$ 0.4	
	HIM53	—	30.6 $\pm$ 1.1	0.77 $\pm$ 0.02	1.4 $\pm$ 0.1		53.7 $\pm$ 1.1	1.83 $\pm$ 0.06	1.3 $\pm$ 0.1	
		+	31.5 $\pm$ 1.2	1.04 $\pm$ 0.10	0.9 $\pm$ 0.2		70.8 $\pm$ 1.1	1.95 $\pm$ 0.08	1.3 $\pm$ 0.1	

Assays were performed by using nine to 12 different substrate concentrations ranging from 0.3 to 100 μ M nifedipine and 0.5 to 500 μ M testosterone. Each value was derived from a single assay and several data points were confirmed by independent determinations. Kinetic parameters were calculated from the fitted curves by non-linear regression (mean $\pm$ SE).

Effects of α NF on kinetic parameters for nifedipine oxidation and testosterone 6 β -hydroxylation catalysed by hepatic and intestinal microsomes from human

The apparent S_{50} , $V_{\max}$ and n for nifedipine oxidation and testosterone 6 β -hydroxylation activities in human hepatic and intestinal microsomes are summarized in table 2. S_{50} and n for the nifedipine oxidation activities in pooled human hepatic microsomes (HLM161) were increased $\sim$ 2-fold by α NF. S_{50} for the nifedipine oxidation activities in individual human intestinal microsomes HIM51 and HIM52 and n for those in HIM53 were decreased in the presence of α NF. $V_{\max}$ in HLM161 and HIM53 was increased by the addition of α NF to 132 and 135%, respectively.

When testosterone was used as a substrate, α NF did not affect the kinetic parameters in human hepatic microsomes, except for a 2.4-fold increase in S_{50} for testosterone 6 β -hydroxylation. In intestinal microsomes, $V_{\max}$ and S_{50} in HIM52 were increased by the addition of α NF to 133 and 154%, respectively.

Discussion

The intestine has been recognized as a potential site for drug oxidations because of its early exposure to orally administered xenobiotics. The upper part of the small intestine has been reported to serve as the major site for intestinal CYP3A-mediated first-pass metabolism of midazolam in human (Paine *et al.* 1996, 1997). It is generally accepted that CYP3A enzymes are major enzymes in the human intestine as well as in the liver (Watkins *et al.* 1987, Paine *et al.* 1997). The sequences of intestinal and hepatic CYP3A4 cDNAs have been reported to be identical (Lown *et al.* 1998); however, the catalytic characteristics of CYP3A enzymes in the intestines are not well understood. An analysis of human CYP3A4 cooperativity by Harlow and Halpert (1998) showed that increasing the size of residues 211 and 214 by site-directed mutagenesis decreases the size of the effector site and mimics the effector-bound wild-type enzyme. This interesting finding suggests that substrate binding to CYP3A4 affects its enzymatic characteristics.

Accordingly, we investigated the effects of α NF on the CYP3A-dependent drug oxidation to obtain information on the differences in enzymatic characteristics between intestine and liver using microsomal CYP3A enzymes in mouse and human.

CYP3A substrates such as erythromycin and testosterone have been reported to have little or no effect on the metabolism of each other by recombinant CYP3A4 (Shet *et al.* 1993). Kenworthy *et al.* (1999) have reported four distinct categorized groups for CYP3A4 substrates based on their mutual inhibitory potencies: erythromycin and testosterone form the first group, and diazepam and midazolam form the second group; terfenadine can be well placed in either group, while nifedipine has a different relationship and makes a fourth group. The four typical CYP3A substrates and an effector used in this study had different effects on the nifedipine oxidation activities in hepatic and intestinal microsomes from mouse and human (figure 1). The present results on species differences with regard to the effects of α NF on the nifedipine oxidation activities in hepatic microsomes were consistent with the reported findings concerning quinine 3-hydroxylation activities, showing an inhibition in mouse and an activation in human (Zhao and Ishizaki 1997). In the present study, inhibition and activation were observed in the nifedipine- α NF interactions in hepatic and intestinal microsomes from mouse, respectively, although testosterone- α NF interactions did not markedly change the kinetic parameters in mouse hepatic and intestinal microsomes. This is the first report, to our knowledge, indicating that the interaction patterns of α NF with CYP3A enzymes were also tissue- and substrate-dependent.

α NF increased the cooperativity in nifedipine oxidation in human hepatic microsomes. On the other hand, its effects were little in human intestinal microsomes (table 2). The present results showing the slight inhibitory effects of α NF on testosterone 6 β -hydroxylation (at a substrate concentration of 100 μ M) by human hepatic microsomes were consistent with the reported findings using purified CYP3A4 and CYP3A4-NADPH P450 reductase fusion protein (Ueng *et al.* 1997). Interestingly, α NF stimulated testosterone 6 β -hydroxylation in individual human intestinal microsomes (table 2), with inconsistent findings from recombinant CYP3A4 or hepatic microsomes (Ueng *et al.* 1997). The action of α NF as an effector on CYP3A enzymes would differ in intestinal and hepatic microsomes. These results in combination with a homology model of CYP3A4 (Szkwarz and Halpert 1997) would suggest that the active site of intestinal CYP3A proteins might be very large and probably capable of accommodating more than one substrate and/or effector at a time in the area of the active site (Cupp-Vickery *et al.* 2000, Hosea *et al.* 2000). The different cooperativities of α NF in intestinal and hepatic microsomes might be clarified by a structural analysis of intestinal CYP3A enzymes.

The present results suggest that CYP3A enzymes in the liver and intestine might have different characteristics with regard to substrate-substrate interactions. The classification of CYP3A4 substrates according to the mutual inhibitory effects using hepatic microsomes or recombinant CYP3A4 systems may be reconsidered with respect to intestinal metabolism. In general, microsomal P450 enzymes require electrons from NADPH-P450 reductase and cytochrome b_5 for catalysing drug oxidations. Although it has been reported that both of the electron-transferring components are abundantly expressed in rabbit intestinal microsomes (Nakamura *et al.* 2000), different characteristics based on the catalytic activities of

CYP3A enzymes would be affected by the CYP3A protein structures, NADPH-P450 reductase levels, cytochrome b_5 contents and/or phospholipid environment between intestinal and hepatic microsomes.

In conclusion, the cooperativity of nifedipine oxidation activities in hepatic microsomes was dramatically affected by α NF, but the cooperativity in intestinal microsomes-mediated drug oxidations was still retained after the addition of α NF. The substrate concentrations in mucosal cells of the intestine may also be an important factor for intestinal drug metabolisms *in vivo*. Thus, it is recommended that one use intestinal samples for the investigation of drug interactions in the intestines *in vitro*. Recently, we proposed two enzyme sources for studies of intestinal metabolism (Emoto *et al.* 2000a, b); one is intestinal microsomes prepared with several kinds of protease inhibitors and glycerol and the other is everted sacs of mouse small intestine. Further studies on the function of intestinal CYP3A enzymes are necessary for understanding substrate-substrate interactions in the human intestine.

Acknowledgements

The work was supported in part by grants from the Ministry of Education, Science, Sports, and Culture of Japan, and by a Pharmaceutical and Medical Safety Research Grant from the Ministry of Health and Welfare of Japan. The authors thank Brent Bell for critical reading the manuscript.

References

- CUPP-VICKERY, J., ANDERSON, R. and HATZIRIS, Z., 2000, Crystal structures of ligand complexes of P450eryF exhibiting homotropic cooperativity. *Proceedings of National Academy of Sciences, USA*, **97**, 3050-3055.
- DOMANSKI, T. L., HE, Y. A., HARLOW, G. R. and HALPERT, J. R., 2000, Dual role of human cytochrome P450 3A4 residue Phe-304 in substrate specificity and cooperativity. *Journal of Pharmacology and Experimental Therapeutics*, **293**, 585-591.
- DOMANSKI, T. L., LIU, J., HARLOW, G. R. and HALPERT, J. R., 1998, Analysis of four residues within substrate recognition site 4 of human cytochrome P450 3A4: Role in steroid hydroxylase activity and α -naphthoflavone stimulation. *Archives of Biochemistry and Biophysics*, **350**, 223-232.
- EDGAR, B., BAILEY, D. G., BERGSTRAND, R., JOHNSON, G. and LURJE, L., 1990, Formulation dependent interaction between felodipine and grapefruit juice. *Clinical Pharmacology and Therapeutics*, **47**, 181.
- EMOTO, C., YAMAZAKI, H., YAMASAKI, S., SHIMADA, N., NAKAJIMA, M. and YOKOI, T., 2000a, Use of everted sacs of mouse small intestine as enzyme sources for the study of drug oxidation activities *in vitro*. *Xenobiotica*, **30**, 971-982.
- EMOTO, C., YAMAZAKI, H., YAMASAKI, S., SHIMADA, N., NAKAJIMA, M. and YOKOI, T., 2000b, Characterization of cytochrome P450 enzymes involved in drug oxidations in mouse intestinal microsomes. *Xenobiotica*, **30**, 943-953.
- GIBBS, M. A., THUMMEL, K. E., SHEN, D. D. and KUNZE, K. L., 1999, Inhibition of cytochrome P-450 3A (CYP3A) in human intestinal and liver microsomes: comparison of K_i values and impact of CYP3A5 expression. *Drug Metabolism and Disposition*, **27**, 180-187.
- GUENGERICH, F. P., 1991, Reactions and significance of cytochrome P-450 enzymes. *Journal of Biological Chemistry*, **266**, 10019-10022.
- GUENGERICH, F. P., 1995, Human cytochrome P450 enzymes. In P. R. Ortiz de Montellano (ed), *Cytochrome P450* (New York: Plenum), pp. 473-535.
- HARLOW, G. R. and HALPERT, J. R., 1998, Analysis of human cytochrome P450 3A4 cooperativity: construction and characterization of a site-directed mutant that displays hyperbolic steroid hydroxylation kinetics. *Proceedings of National Academy of Sciences, USA*, **95**, 6636-6641.
- HOSEA, N. A., MILLER, G. P. and GUENGERICH, F. P., 2000, Elucidation of distinct ligand binding sites for cytochrome P450 3A4. *Biochemistry*, **39**, 5929-5939.

- HOUSTON, J. B. and KENWORTHY, K. E., 2000, *In vitro in vivo* scaling of CYP kinetic data not consistent with the classical Michaelis-Menten model. *Drug Metabolism and Disposition*, **28**, 246-254.
- IATSIMIRSKAIA, E., TULEBAEV, S., STOROZHUK, E., UTKIN, I., SMITH, D., GERBER, N. and KOUDRIAKOVA, T., 1997, Metabolism of rifabutin in human enterocyte and liver microsomes: kinetic parameters, identification of enzyme systems, and drug interactions with macrolides and antifungal agents. *Clinical Pharmacology and Therapeutics*, **61**, 554-562.
- KENWORTHY, K. E., BLOOMER, J. C., CLARKE, S. E. and HOUSTON, J. B., 1999, CYP3A4 drug interactions: correlation of 10 *in vitro* probe substrates. *British Journal of Clinical Pharmacology*, **48**, 716-727.
- KORZEKWA, K. R., KRISHNAMACHARY, N., SHOU, M., OGAI, A., PARISE, R. A., RETTIE, A. E., GONZALEZ, F. J. and TRACY, T. S., 1998, Evaluation of atypical cytochrome P450 kinetics with two-substrate models: evidence that multiple substrates can simultaneously bind to cytochrome P450 active sites. *Biochemistry*, **37**, 4137-4147.
- KRISHNA, D. R. and KLOTZ, U., 1994, Extrahepatic metabolism of drugs in humans. *Clinical Pharmacokinetics*, **26**, 144-160.
- LALONDE, R. L., LESSARD, D. and GAUDREAU, J., 1996, Population pharmacokinetics of terfenadine. *Pharmaceutical Research*, **13**, 832-838.
- LOWN, K. S., GHOSH, M. and WATKINS, P. B., 1998, Sequences of intestinal and hepatic cytochrome P450 3A4 cDNAs are identical. *Drug Metabolism and Disposition*, **26**, 185-187.
- LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. and RANDALL, R. J., 1951, Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry*, **193**, 265-275.
- MA, B., PRUEKSARITANONT, T. and LIN, J. H., 2000, Drug interactions with calcium channel blockers: possible involvement of metabolite/intermediate complexation with CYP3A. *Drug Metabolism and Disposition*, **28**, 125-130.
- MAENPÄÄ, J., HALL, S. D., RING, B. J., STROM, S. C. and WRIGHTON, S. A., 1998, Human cytochrome P450 3A (CYP3A) mediated midazolam metabolism: the effect of assay conditions and regioselective stimulation by α -naphthoflavone, terfenadine and testosterone. *Pharmacogenetics*, **8**, 137-155.
- NAKAMURA, T., OKADA, K., NAGATA, K. and YAMAZOE, Y., 2000, Intestinal cytochrome P450 and response to rifampicin in rabbits. *Japanese Journal of Pharmacology*, **82**, 232-239.
- PAINE, M. F., KHALIGHI, M., FISHER, J. M., SHEN, D. D., KUNZE, K. L., MARSH, C. L., PARKINS, J. D. and THUMMEL, K. E., 1997, Characterization of interintestinal and intrainestinal variations in human CYP3A-dependent metabolism. *Journal of Pharmacology and Experimental Therapeutics*, **283**, 1552-1562.
- PAINE, M. F., SHEN, D. D., KUNZE, K. L., PERKINS, J. D., MARSH, C. L., MCVICAR, J. P., BARR, D. M., GILLIES, B. S. and THUMMEL, K. E., 1996, First-pass metabolism of midazolam by the human intestine. *Clinical Pharmacology and Therapeutics*, **60**, 14-24.
- SHET, M. S., FISHER, C. W., HOLMANS, P. L. and ESTABROOK, R. W., 1993, Human cytochrome P450 3A4: enzymatic properties of a purified recombinant fusion protein containing NADPH-P450 reductase. *Proceedings of National Academy of Sciences, USA*, **90**, 11748-11752.
- SHIMADA, T., YAMAZAKI, H., MIMURA, M., INUI, Y. and GUENGERICH, F. P., 1994, Interindividual variations in human liver cytochrome P-450 enzymes involved in the oxidation of drugs, carcinogens and toxic chemicals: studies with liver microsomes of 30 Japanese and 30 Caucasians. *Journal of Pharmacology and Experimental Therapeutics*, **270**, 414-423.
- SHOU, M., GROGAN, J., MANCEWICW, J. A., KRAUSZ, K. W., GONZALEZ, F. J., GELBOIN, H. V. and KORZEKWA, K. R., 1994, Activation of CYP3A4: Evidence for the simultaneous binding of substrates in a cytochrome P450 active site. *Biochemistry*, **33**, 6450-6455.
- SMITH, M. T., EADIE, M. J. and BROPHY, T. O. R., 1981, The pharmacokinetics of midazolam in man. *European Journal of Clinical Pharmacology*, **19**, 271-278.
- SZKLARZ, G. D. and HALPERT, J. R., 1997, Molecular modeling of cytochrome P450 3A4. *Journal of Computer-Aided Molecular Design*, **11**, 265-272.
- UENG, Y.-F., KUWABARA, T., CHUN, Y.-J. and GUENGERICH, F. P., 1997, Cooperativity in oxidations catalysed by cytochrome P450 3A4. *Biochemistry*, **36**, 370-381.
- WANG, H. F., DICK, R., YIN, H. Q., LICAD-COLES, E., KROETZ, D. L., SZKLARZ, G., HARLOW, G., HALPERT, J. R. and CORREIA, M. A., 1998, Structure/function relationships of human liver cytochromes P450 3A: aflatoxin B₁ metabolism as a probe. *Biochemistry*, **37**, 12536-12545.
- WANG, R. W., NEWTON, D. J., LIU, N., ATKINS, W. M. and LU, A. Y. H., 2000, Human cytochrome P-450 3A4: *in vitro* drug-drug interaction patterns are substrate-dependent. *Drug Metabolism and Disposition*, **28**, 360-366.
- WATKINS, P. B., WRIGHTON, S. A., SCHUETZ, E. G., MOLOWA, D. T. and GUZELIAN, P. S., 1987, Identification of glucocorticoid-inducible cytochromes P-450 in the intestinal mucosa of rats and man. *Journal of Clinical Investigation*, **80**, 1029-1036.
- YAMAZAKI, H. and SHIMADA, T., 1997, Progesterone and testosterone hydroxylation by cytochromes P450 2C19, 2C9, and 3A4 in human liver microsomes. *Archives of Biochemistry and Biophysics*, **346**, 161-169.

- YANAGIMOTO, T., ITOH, S., SAWADA, M. and KAMATAKI, T., 1997, Mouse cytochrome P450 (Cyp3a11): predominant expression in liver and capacity to activate aflatoxin B₁. *Archives of Biochemistry and Biophysics*, **340**, 215-218.
- ZHAO, X.-J. and ISHIZAKI, T., 1997, The *in vitro* hepatic metabolism of quinine in mice, rats and dogs: comparison with human liver microsomes. *Journal of Pharmacology and Experimental Therapeutics*, **283**, 1168-1176.

Identification and reactivity of the major metabolite (β -1-glucuronide) of the anti-tumour agent 5,6-dimethylxanthenone-4-acetic acid (DMXAA) in humans

S. F. ZHOU[†], J. W. PAXTON^{†*}, M. D. TINGLE[†], P. KESTELL[‡],
M. B. JAMESON[‡], P. I. THOMPSON[‡] and B. C. BAGULEY[‡]

[†] Department of Pharmacology and Clinical Pharmacology, [‡] Auckland Cancer Society Research Centre, University of Auckland, Auckland, New Zealand

Received 26 October 2000

1. The novel anti-tumour agent 5,6-dimethylxanthenone-4-acetic acid (DMXAA) is extensively metabolized by glucuronidation and 6-methylhydroxylation, resulting in DMXAA acyl glucuronide (DMXAA-G) and 6-hydroxymethyl-5-methylxanthenone-4-acetic acid (6-OH-MXAA).

2. The major human urinary metabolite of DMXAA was isolated and purified by a solid-phase extraction (SPE) method. The isolated metabolite was hydrolysed to free DMXAA by strong base, and by β -glucuronidase. Liquid chromatography-mass spectrometry (LC-MS) and spectral data indicated the presence of a molecular ion $[M + 1]^+$ at m/z 459, which was consistent with the molecular weight of protonated DMXAA-G.

3. The glucuronide was unstable in buffer at physiological pH, plasma and blood with species variability in half-life. Hydrolysis and intramolecular migration were major degradation pathways.

4. *In vitro* and *in vivo* formation of DMXAA-protein adducts was observed. The formation of DMXAA-protein adducts in cancer patients receiving DMXAA was significantly correlated with plasma DMXAA-G concentration and maximum plasma DMXAA concentration.

5. At least five metabolites of DMXAA were observed in patient urine, with up to 60% of the total dose excreted as DMXAA-G, 5.5% as 6-OH-MXAA and 4.5% as the glucuronide of 6-OH-MXAA.

6. These data suggest that the major metabolite in patients' urine is DMXAA β -1-glucuronide, which may undergo hydrolysis, molecular rearrangement and covalent binding to plasma protein. The reactive properties of DMXAA-G may have important implications for the pharmacokinetics, pharmacodynamics and toxicity of DMXAA.

Introduction

The novel anti-tumour agent 5,6-dimethylxanthenone-4-acetic acid (DMXAA), developed by the Auckland Cancer Society Research Centre (ACSRC), has recently completed Phase I clinical trials in New Zealand and the UK under the direction of the Cancer Research Campaign's Phase I/II Clinical Trials Committee. Unlike conventional cytotoxic anti-tumour agents, DMXAA induces rapid vascular collapse and necrosis in transplantable murine tumours, which is thought to be due to immune modulation by induction of cytokines, in particular tumour necrosis factor, interferons, serotonin and nitric

* Author for correspondence; e-mail: j.paxton@auckland.ac.nz

oxide (Thomsen *et al.* 1991, Philpott *et al.* 1995, Baguley *et al.* 1997). The disposition of DMXAA has been studied extensively using rodent models and *in vitro* human liver microsomes, which indicated that DMXAA is extensively metabolized by conjugation to glucuronic acid and 6-methyhydroxylation, resulting in two major metabolites, DMXAA 1-*O*-acyl glucuronide (DMXAA-G) and 6-hydroxymethyl-5-methylxanthenone-4-acetic acid (6-OH-MXAA) (Webster *et al.* 1995, Miners *et al.* 1997, Kestell *et al.* 1999, Zhou *et al.* 2000). There were significant species differences in the major urinary metabolites of DMXAA, with $2.9 \pm 0.1\%$ and $60.0 \pm 0.2\%$ excreted as DMXAA-G in the rat and rabbit respectively (Kestell *et al.* 1999). Using the isolated perfused rat liver model, seven DMXAA metabolites were observed in the bile. The major biliary metabolite was identified by mass spectrometry as the acyl glucuronide accounting for $> 50\%$ of the DMXAA dose (Webster *et al.* 1995). A second compound resistant to alkaline hydrolysis was characterized as a hydroxylated DMXAA metabolite. A total of 28% of DMXAA was recovered unchanged in the perfusate, liver and bile.

Glucuronidation of a variety of drugs is catalysed by a family of uridine diphosphate glucuronosyltransferase (UGT) (EC.2.4.1.17) isoforms (Burchell *et al.* 1998). Accumulating evidence has indicated that acyl glucuronides of many drugs are chemically reactive, electrophilic metabolites that undergo hydrolysis, intramolecular rearrangement, and covalent binding to tissue proteins (Faed 1984, Spahn-Lugguth and Benet 1992). The hydrolysis of the acyl glucuronide results in a conjugation-deconjugation cycling, and may lead to enterohepatic recycling; whereas intramolecular rearrangement is a process whereby the xenobiotic moiety migrates from the 1- β -position to the C-2, C-3 or C-4 position of the glucuronic acid ring (Faed 1984, Spahn-Lugguth and Benet 1992, Fenselau 1994). Both β -1-glucuronides and isomers formed by intramolecular migration may be involved in the covalent binding to proteins, leading to the formation of protein adducts, which have been associated with immune-mediated adverse effects for drugs such as diclofenac, diflunizal, zomepirac and tolmetin (Ojingwa *et al.* 1988, McKinnon and Dickinson 1989, Smith *et al.* 1992, Spahn-Lugguth and Benet 1992, Bischer *et al.* 1995, Ding *et al.* 1995, Sallustio *et al.* 1997a).

The aims of the present study were to investigate the urinary metabolite profiles in patients receiving DMXAA treatment; to identify and quantitate the major urinary metabolite of DMXAA; to characterize and compare the degradation kinetics of this metabolite in aqueous buffers, plasma and blood; to investigate the irreversible binding of DMXAA to plasma proteins via 1- β -acyl glucuronide *in vitro* and in patients; and to examine the influence of albumin site I and II ligands and other drugs on the degradation of DMXAA-G and its covalent binding to plasma proteins.

Materials and methods

Chemicals and reagents

The sodium salts of DMXAA (98% purity as determined by thin-layer chromatography) and the internal standard (IS), 2,5-dimethylxanthenone-4-acetic acid (SN24350) were synthesized in the ACSRC by the method of Rewcastle *et al.* (1989). DMXAA was protected from light exposure to avoid degradation (Rewcastle *et al.* 1990). Authentic DMXAA-G and 6-OH-MXAA were isolated and purified from the bile and urine of rat respectively by a solid-phase extraction method (both metabolites had a purity of 99% as determined by HPLC), and their structure identified by liquid chromatography-mass spectrometry (LC-MS) and $[^1\text{H}]$ -nuclear magnetic resonance (Kestell *et al.* 1999). β -Glucuroni-

dase (EC.3.2.1.31 Helix pomatia, 19700 units ml⁻¹, sulphatase free), warfarin, diclofenac, diazepam, phenylbutazone, cyproheptadine, sodium cyanide (NaCN), sodium cyanoborohydride (NaCNBH₃), essentially fatty acid-free human serum albumin, and D-saccharic acid 1,4-lactone were purchased from Sigma-Aldrich Chemicals Co. (Auckland, New Zealand). All other reagents were of analytical or HPLC grade as appropriate.

Blood and plasma

Male Wistar Kyoto rats (200–250 g, $n = 20$), male C57Bl/6 mice (25–32 g, $n = 50$) and male white New Zealand rabbits (3–3.4 kg, $n = 3$) were housed under constant temperature (20–25°C) and humidity (40–60%) with access to water and a commercial diet (NRM Co., Auckland, New Zealand) ad libitum according to institutional guidelines. All animal procedures were approved by the Animal Ethics Committee of the University of Auckland. Animals were anaesthetized with halothane and blood was obtained by cardiac puncture using heparinized disposable plastic syringes in rat and rabbit, and through the ocular sinus in mouse. Fresh heparinized blood was obtained from healthy human volunteers ($n = 6$) and cancer patients ($n = 10$) in a Phase I DMXAA trial. Plasma was separated by centrifugation at 1000g for 15 min. Ethical approval was obtained from the Northern New Zealand Research Ethics Committee, and all subjects gave written informed consent. Total protein concentrations in plasma were determined using the bicinchoninic acid (BCA) method (Smith *et al.* 1985). Plasma albumin concentration was measured using the bromocresol green method (Doumas and Biggs 1972).

Patient urine

The urine from three cancer patients administered DMXAA (240–850 mg m⁻²) by intravenous (i.v.) infusion over 20 min was collected for 24 h in vessels containing 0.1 M ammonium acetate buffer (pH 5.0) with 40% methanol and stored at –20°C until used.

Mass spectrometry

The metabolite isolated from patients' urine was characterized by LC-MS with electrospray (ESP) or atmospheric pressure chemical ionization (APCI) interface (Hewlett Packard, Avondale, PA, USA). ESP was carried out in the positive-ion mode and the ion-spray interface was operated at a needle voltage of ± 4000 V. The gas temperature, drying gas flow rate and nebulizer pressure were set at 350°C, 101 min⁻¹ and 25 psi. Fragmentation of analyte ions during full-scanning acquisition was achieved by increasing the fragmentor voltage from 60 to 100 V. The mobile phase flow rate was 0.5 ml min⁻¹. The mobile phase was the same as used for HPLC. The method for APCI has already been described (Zhou *et al.* 2000).

High-performance liquid chromatography

Two HPLC systems (I and II) were used in this study. The HPLC system I was used to quantitate DMXAA and DMXAA-G in buffer and biological matrices from *in vitro* studies as described (Zhou *et al.* 1999, 2000). Briefly, buffer, HSA solutions or plasma (100 μ l) containing DMXAA-G was mixed with 50 μ l methanol containing 20 μ M IS, followed by 0.4 ml ice-cold acetonitrile: methanol (3:1 v/v). After centrifugation at 2500g for 15 min to remove precipitated proteins, the supernatant was removed and evaporated to dryness under nitrogen using a Speedvac concentrator (Savant Instruments Co., Farmingdale, NY, USA). The residue was dissolved in 200 μ l mobile phase, and 50 μ l was injected into the HPLC system I. The isomers of DMXAA-G were determined under similar HPLC conditions as for DMXAA-G, except that the mobile phase was acetonitrile: 10 μ M ammonium acetate buffer (19:81 v/v, pH 5.0) at a flow rate of 2.0 ml min⁻¹. The same calibration curves for DMXAA-G were used, assuming that all isomers have the same extinction coefficient. For the quantitation of DMXAA in plasma, HSA, whole blood or released from protein adducts, samples were treated with the same procedure as for DMXAA-G. Calibration curves were set up over the concentration range 0.5–100 μ M by adding known amounts of DMXAA to plasma, albumin solution, whole blood or 0.1 M phosphate buffer (pH 7.4). To determine the concentration of DMXAA released from albumin or plasma protein adducts, calibration curves were constructed with DMXAA by adding known amounts of DMXAA to washed blank HSA or human plasma prior to base hydrolysis. The peak area ratio of DMXAA or DMXAA-G to internal standard was used to determine the concentration of unknown by linear least-squares regression analysis.

The HPLC system II was used to quantitate the major DMXAA metabolites (DMXAA-G, 6-OH-MXAA and the glucuronide of 6-OH-MXAA) in urine from patients receiving DMXAA (Kestell *et al.* 1999). The isolation of patients' urinary DMXAA-G, 6-OH-MXAA and the glucuronide of 6-OH-MXAA by semi-preparative chromatography has been described (Webster *et al.* 1995, Kestell *et al.* 1999).

All HPLC methods had acceptable accuracy (85–115% of true values) and precision (intra- and interassay coefficients of variation < 15%).

Urinary metabolite profile and quantitation of major urinary DMXAA metabolites in patients

The urine from patients receiving 850 mg m⁻² DMXAA was collected and an aliquot (200 µl) was mixed with a volume of ice-cold acetonitrile, then centrifuged at 1500g for 10 min. Supernatant (40 µl) was injected into the HPLC and LC-MS for analysis.

Isolation and purification of DMXAA-G from patient urine

The SPE method (with some modifications) was used to isolate and purify DMXAA-G from patient urine (Webster *et al.* 1995, Kestell *et al.* 1999). Briefly, 5 ml diluted patient urine was loaded onto a preconditioned 6 ml C18 Bond Elute cartridge (Varian Co. Harbor City, CA, USA), followed by a 10 and 15% methanol (in Milli-Q water) wash. The cartridge was then eluted with 25% methanol. Eluents were pooled, loaded onto two of the same cartridges as above (newly prewashed with methanol, followed with Milli-Q water), then eluted with methanol. Each eluent was checked for its contents by HPLC. Those eluents containing only DMXAA-G were combined and evaporated using a Speedvac concentrator under a stream of nitrogen. About 4.5 mg yellowish white residue was harvested. The purity of the concentrated metabolite residue was determined by HPLC.

Chemical and β -glucuronidase-catalysed hydrolysis of DMXAA-G

Total DMXAA content in the metabolite isolated from patients' urine was determined by incubation with 1 M KOH at 70°C for 30 min. DMXAA was shown to be stable under these conditions. The metabolite at a concentration of 5.6 mg ml⁻¹ (DMXAA equivalents) was also incubated at 37°C in 0.1 M sodium phosphate buffer (pH 5.0) in a total volume of 300 µl for 30 min with either 2000 units ml⁻¹ β -glucuronidase, β -glucuronidase plus 20 mM D-saccharic acid 1,4-lactone or β -glucuronidase previously boiled at 90°C for 10 min. The reaction was initiated by addition of β -glucuronidase, and stopped by addition of 1 vol. acetonitrile containing 0.5% acetic acid (v/v). The mixture was centrifuged at 3000g for 10 min to remove precipitated protein. The supernatant was removed and evaporated under nitrogen. The residue was reconstituted with mobile phase and 50 µl was injected into the HPLC to determine the susceptibility of the metabolite to β -glucuronidase. The percentage of β -1-conjugate present relative to total conjugates of DMXAA was determined by the comparison of DMXAA released by β -glucuronidase relative to hydrolysis with strong base.

Stability of DMXAA-G in buffer, plasma and blood

In vitro studies of degradation of DMXAA-G were performed by incubations containing 20 µg ml⁻¹ (71 µM) DMXAA-G, either in 0.1 M phosphate buffer at pH 6.8 or 7.4, in 0.1 M phosphate buffer (pH 7.4) with 40 mg ml⁻¹ HSA, or human plasma, at 37°C in a water bath for up to 48 h. Aliquots (200 µl) of the incubation mixture were taken at the following time points: 0, 0.5, 1, 2, 4, 6, 8, 24 and 48 h. The reactions were stopped by the addition of 2 vols of an ice-cold acetonitrile:methanol mixture (3:1 v/v) containing 0.5% acetic acid (v/v) and 10 µM IS and cooling on ice. All mixtures were centrifuged at 3500g for 10 min, the supernatant removed and evaporated to dryness under nitrogen flow using a Speedvac. The residue was reconstituted with 200 µl mobile phase and injected into the HPLC to estimate acyl migration and hydrolysis. All incubations were conducted in triplicate.

The stability of DMXAA-G in fresh plasma and blood from mouse, rat, rabbit and human was performed by incubations at 37°C for up to 120 min with 2.8 µg ml⁻¹ (10 µM) DMXAA-G. The effect of D-saccharic acid 1,4-lactone on the degradation of DMXAA-G in plasma and blood from the mouse, rat, rabbit and human was examined by incubation of plasma containing 10 µM DMXAA-G and 1 mg ml⁻¹ D-saccharic acid 1,4-lactone at 37°C for up to 120 min. DMXAA-G was quantitated as described above.

In vitro DMXAA-protein adduct formation

The covalent binding of DMXAA via its acyl glucuronide to both HSA and human plasma was performed by adding 0.178 mM DMXAA-G (50 µg ml⁻¹, DMXAA equivalents) to HSA (40 g l⁻¹) in 0.1 M sodium phosphate buffer at pH 7.4, or human plasma. After incubation at 37°C for up to 48 h, aliquots (200 µl) were taken at various times to estimate the total DMXAA equivalents bound to HSA as described below. Kinetic studies of the covalent binding were performed by incubating DMXAA-G (8.9–712 µM; 2.5–200 µg ml⁻¹ DMXAA equivalents) and 40 g l⁻¹ HSA in 0.1 M phosphate buffer (pH 7.4) at 37°C for up to 6 h. Further covalent binding studies were performed in the presence of imine-trapping reagents, sodium cyanide or sodium cyanoborohydride (10 mM).

Determination of DMXAA liberated from protein adducts

Plasma or albumin solutions (200 µl) containing the DMXAA adducts were mixed with 2 vols ice-cold acetonitrile:methanol (3:1 v/v) containing 1% (v/v) acetic acid and 10 µM IS. After cooling on ice to precipitate the proteins, the pH was adjusted to ~5 with fresh 1 M KOH and centrifuged at 1500g for 10 min. The precipitated protein pellet was washed $\times 12$ with 6 vols methanol:diethylether (3:1 v/v), and

dried at 40°C under nitrogen flow using a Speedvac. HPLC analysis of the final washes ensured that there was no unbound DMXAA or DMXAA-G remaining in the pellet. The dried protein was hydrolysed by addition of 1 M KOH (400 µl) and incubation at 70°C for 2.5 h. After hydrolysis, aliquots (20 µl) of the incubate were added to test tubes with 380 µl 1 M NaOH and stored at -20°C for protein assay by the BCA method (Smith *et al.* 1985). The remaining incubate was acidified to pH ~3 by the addition of 85% phosphoric acid (38 µl), followed by 500 µM IS in 0.1 M phosphate buffer (pH 5.0) (10 µl), and the mixture extracted with 2 ml dichloromethane to remove the DMXAA released by hydrolysis. After gentle shaking for 5 min, the tubes were centrifuged at 3000g for 5 min. The aqueous phase was then discarded and the organic phase was evaporated to dryness at 40°C under nitrogen. The residue was reconstituted with 200 µl mobile phase and the DMXAA determined by HPLC. The extent of covalent binding was normalized to protein concentration and expressed as the amount of DMXAA (ng) bound mg⁻¹ protein. Control studies with DMXAA, as well as HPLC analysis of washes, indicated that the washing procedure completely removed adsorbed and reversibly bound DMXAA from protein precipitates. DMXAA, which was not removed by the washing procedure but released only on treatment of the protein precipitate with strong base, was defined as 'covalently bound' (Pohl *et al.* 1988).

Effects of drugs on the degradation of DMXAA-G

The effect of various drugs, especially potential albumin binding ligands, on DMXAA-G degradation and covalent binding in HSA solution was determined in incubations in 0.1 M phosphate buffer at pH 7.4 containing 50 µg ml⁻¹ DMXAA-G (DMXAA equivalents), 40 mg ml⁻¹ HSA and 1 µM ligand (diazepam, phenylbutazone, warfarin, ibuprofen and DMXAA). All incubations were performed in triplicate. All potential ligands except DMXAA were dissolved in ethanol at a final concentration of 1% (v/v). DMXAA was dissolved in 0.1 M phosphate buffer, pH 7.4. Each potential ligand was also incubated with HSA in the absence of DMXAA-G to determine whether there are any interfering chromatographic peaks. Preliminary experiments demonstrated that the addition of an aliquot of ethanol at a final concentration of 1% (v/v) had no significant effect on the degradation of DMXAA-G.

Determination of DMXAA-protein adduct formation in cancer patients

The DMXAA-protein adducts in the plasma from 10 cancer patients receiving DMXAA (850-2600 mg m⁻²) by i.v. infusion over 20 min were quantitated as described above. Blood samples taken at 0, 0.083, 0.5, 0.75, 1, 1.5, 2, 4, 6, 8, 12 and 24 h from two patients, and at 4 h from the other eight patients after the commencement of dosage were immediately centrifuged, the plasma separated and stabilized by the addition of orthophosphoric acid (7.5 µl/1 ml plasma), and stored at -20°C until analysis.

Data analysis

All data were expressed as mean ± SD. The Prism 3.0 program (Graphpad Software Co., CA, USA) was used for all data analysis. Single- and two-binding site models (equations 1 and 2) and a modified two-binding site model (equation 3) (Semmes and Shen 1990) were used to describe the kinetics of *in vitro* irreversible binding of DMXAA to HSA:

$$C_b = N \cdot Cu / (K_d + Cu) \quad (1)$$

$$C_b = N_1 \cdot Cu / (K_{d1} + Cu) + N^2 \cdot Cu / (K_{d2} + Cu) \quad (2)$$

$$C_b = N_1 \cdot Cu / (K_{d1} + Cu) + (N^2 / K_{d2}) \cdot Cu, \quad (3)$$

where C_b is the concentration of bound DMXAA obtained from subtracting unbound (C_u) from total DMXAA-G concentration; K_d is the apparent dissociation constant for the binding site; N is the concentration of binding sites on plasma protein; and subscripts 1 and 2 are the first and the second type of sites. The choice of model was confirmed by comparing and reviewing the relative residuals and the standard error of the parameter estimates from the non-linear regression analysis. Akaike's information criterion (AIC) was also used as the criterion for comparing the models (Yamaoka *et al.* 1978). It is given by:

$$AIC = N_d \ln Re + 2N_p, \quad (4)$$

where N_d is the number of experimental data points, N_p is the number of parameters in an estimated model and Re is the residue sum of squares. The equation with minimum AIC is regarded as the best representation of the model.

Calculation of kinetic data of degradation of DMXAA-G in aqueous buffers, plasma and blood were performed by iterative non-linear regression analysis of the measured data using the equation for first order reaction kinetics:

$$C_t = C_{(0)} e^{-kt}, \quad (5)$$

where C_t is the concentration of DMXAA-G at time after incubation (t); $C_{(0)}$ is the initial concentration of DMXAA-G; and k is the first order degradation constant. Half-life ($T_{1/2}$) is calculated as $0.693/k$.

To analyse *in vivo* DMXAA-protein adduct formation data, it was assumed that the rate of adduct formation would approximate a zero-order process at the concentrations of DMXAA-G achieved. Therefore, the *in vivo* DMXAA-protein adduct formation was fitted to the simple one-compartment pharmacokinetic model assuming zero-order input and first-order elimination:

$$C't = C_{ss}[1 - e^{-k't'}], \quad (6)$$

where $C't$ is the concentration of adducts at time (t') after commencement of dosing; C_{ss} is the concentration of adducts at steady-state; and k' is the apparent elimination rate constant. Statistical comparisons were made with the Student's t -test for data analysis. Results are statistically different at a significance of $p < 0.05$.

Results

Urinary Metabolic profile of DMXAA in humans

HPLC and LC-MS analysis indicated similar metabolite profiles in both patients' urine with at least five DMXAA metabolites present (figure 1). M1 showed signals at m/z 475, which was identical to the molecular weight of protonated 6-OH-MXAA glucuronide or its structural isomers (figure 2). M2 was a metabolite ion showing signals at a m/z 299, which had identical retention time and m/z as that of the authentic 6-OH-MXAA, indicating M2 being 6-OH-

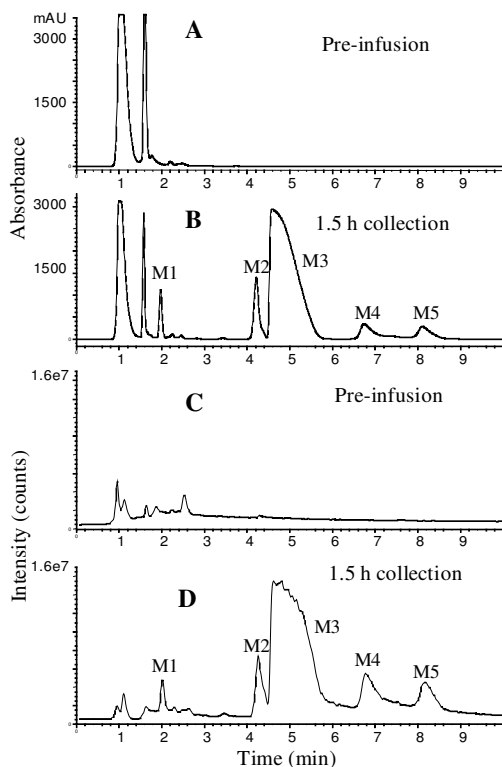


Figure 1. HPLC (A, B) and LC-MS (C, D) metabolite profiles from the non-hydrolysed urine of a patient sample during the 1.5 h before (A) and after (B) i.v. infusion of DMXAA (850 mg m^{-2}). There were at least five DMXAA metabolites present in the patient's urine after DMXAA treatment. The m/z range scanned for data acquisition was 100–700.

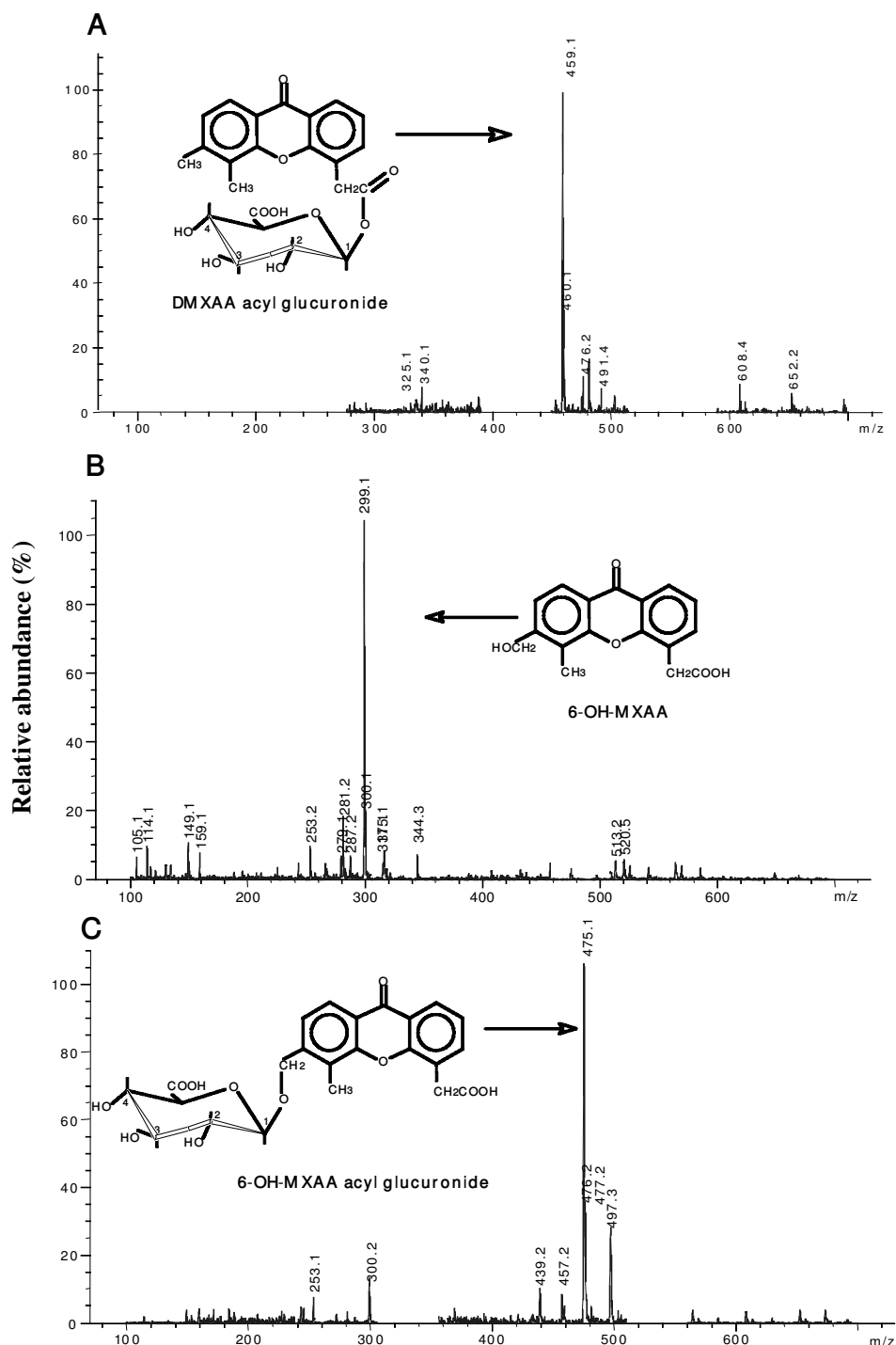


Figure 2. Mass spectra for the major DMXAA metabolites in a patient's urine. LC-MS indicated the presence of a molecular ion $[M + H]^+$ at m/z 459, 298 and 475, which was consistent with the molecular weight of protonated DMXAA acyl glucuronide (A), 6-OH-MXAA (B) and the glucuronide of 6-OH-MXAA (C).

MXAA (figure 2). The most predominant peak of metabolite ion (M3) showed signals at m/z 459, which was consistent with the molecular weight of protonated DMXAA-G. M4 and M5 showed signals at m/z 459, which were considered to be the isomers of DMXAA-G.

The major metabolite (M3) was then isolated and purified by SPE. HPLC analysis of the 25% methanol SPE eluate indicated the presence of only the major DMXAA metabolite. The concentrated metabolite residue exhibited one major peak on HPLC with a purity of 98.6%, with the remaining 1.4% as polar impurities and DMXAA. Incubation of this metabolite with sodium hydroxide or β -glucuronidase resulted in the disappearance of the metabolite peak, with the concomitant appearance of a peak with the spectral characteristic of DMXAA. The percentage of β -1-conjugate (relative to possible total conjugates of DMXAA) was 99%, when determined by the comparison of DMXAA released by β -glucuronidase relative to alkaline hydrolysis. The metabolite could not be cleaved with boiled β -glucuronidase, or β -glucuronidase in the presence of the inhibitor, D-saccharic acid 1,4-lactone. Further investigation of the isolated metabolite by LC-MS indicated the presence of a molecular ion $[M + H]^+$ at m/z 459, which was consistent with the molecular weight of protonated DMXAA-G. The protonated molecule at m/z 459 underwent typical fragmentations attributable to the loss of the glucuronic acid moiety (molecular weight 176), with the formation of parent molecule ions of DMXAA $[M + 1]^+$ at m/z 283.

Quantitation of the major urinary DMXAA metabolites in humans

Two patients receiving DMXAA at 240 mg m^{-2} excreted 1.8 and 1.3% of the total dose as unchanged DMXAA and 24.2 and 28.8% as DMXAA-G respectively in urine within 24 h. When the same dose was repeated in one of these patients 3 weeks later, this increased to 6 and 60% DMXAA and DMXAA-G respectively. Another patient treated with DMXAA at 850 mg m^{-2} excreted 2.4% as parent DMXAA, 35.9% as DMXAA-G, 5.5% as 6-OH-MXAA, and 4.5% as glucuronide of 6-OH-MXAA.

Degradation of DMXAA-G in buffer, plasma and blood

The degradation of DMXAA-G in aqueous buffers at pH 6.8 and 7.4, HSA solution at pH 7.4, plasma and blood from various species appeared to follow first-order kinetics over the initial 8 h with different estimated first order degradation constant and apparent half-lives (table 1). The degradation of DMXAA-G was dependent on pH in buffer solutions with a shorter half-life at pH 7.4 (1.30 h) than at pH 6.8 (3.06 h) (figure 3A and B). Addition of HSA at pH 7.4 accelerated the intramolecular rearrangement and hydrolysis of the acyl glucuronide, resulting in a shorter half-life (0.42 h) (figure 3C). In aqueous buffers, HSA or human plasma, intramolecular rearrangement and hydrolysis were the major degradation pathways (figure 3). The first-order degradation half-lives of DMXAA-G in plasma and blood from various species are shown in table 1. The degradation $T_{1/2}$ of DMXAA-G in plasma was shorter compared with blood in each species. D-Saccharic acid 1,4-lactone (1 mg ml^{-1}) also significantly inhibited the degradation of DMXAA-G in plasma and blood for up to 2 h.

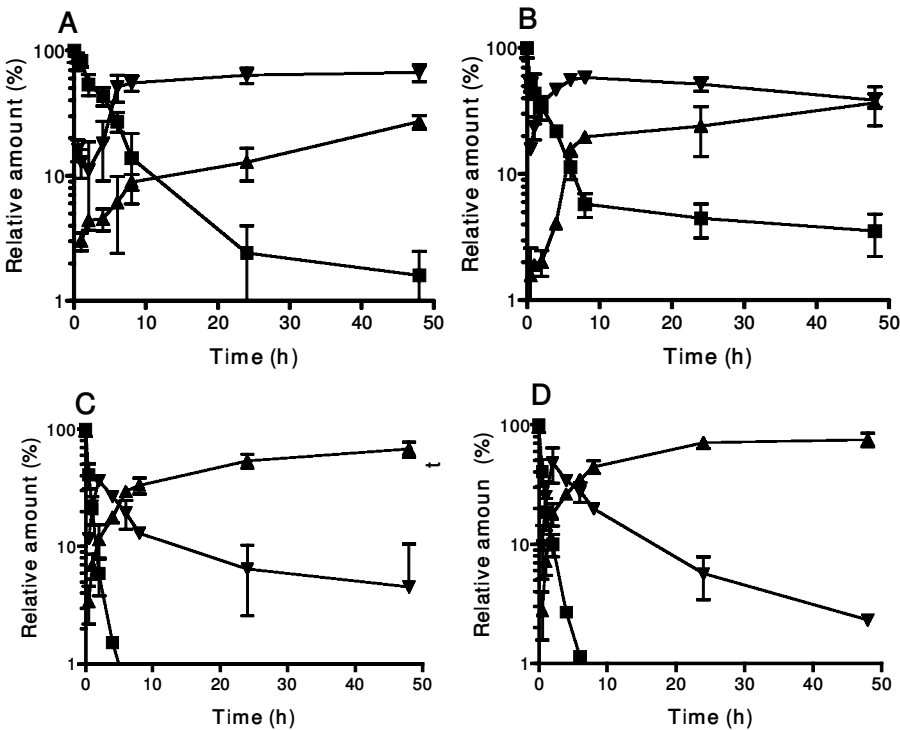


Figure 3. Stability of DMXAA acyl glucuronide in 0.1 M phosphate buffer at pH 6.8 (A) or 7.4 (B), 40 mg ml⁻¹ human serum albumin at pH 7.4 (C) and human plasma (D). DMXAA acyl glucuronide, DMXAA and isomers are represented by ■, ▲ and ▼ respectively. DMXAA acyl glucuronide at 20 mg ml⁻¹ (71 μM) was incubated in buffer, human serum albumin or plasma at 37°C in a water bath for up to 48 h. Data are the mean ± SD of three determinations.

Table 1. Apparent first-order degradation constant and half-lives of DMXAA 1-*O*-acylglucuronide in aqueous buffer, plasma and blood.

Matrix	$-k^*$	$T_{1/2}$ (h)*	r^{2*}
Phosphate buffer			
pH 6.8	0.2264	3.06	0.9716
pH 7.4	0.5330	1.30	0.8623
HSA at pH 7.4	1.652	0.42	0.9976
Plasma			
Mouse	1.5120	0.46	0.9844
Rat	2.0796	0.33	0.9751
Rabbit	1.3188	0.53	0.9954
Human	1.4712	0.47	0.9888
Blood			
Mouse	0.7434	0.93	0.8367
Rat	1.4478	0.48	0.9104
Rabbit	0.9540	0.73	0.9661
Human	0.7110	0.97	0.9412

* k , $T_{1/2}$ and r are the apparent first-order degradation constant, half-life and correlation coefficient respectively.

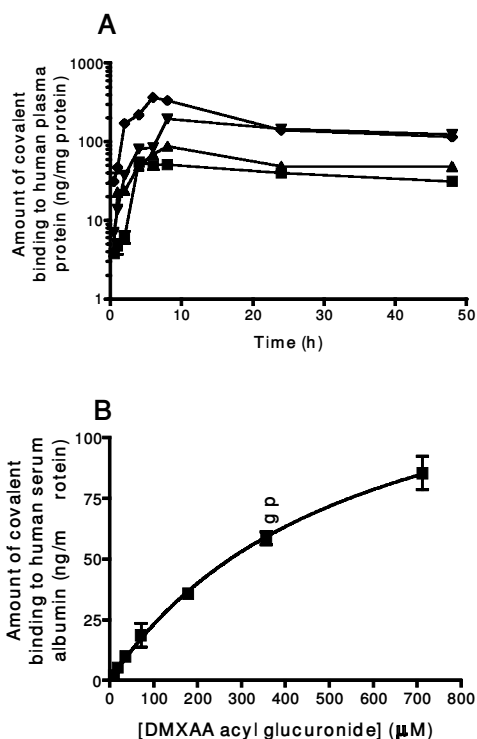


Figure 4. Time- and concentration-dependent formation of the DMXAA-protein adduct in human plasma (■) and human serum albumin (▲). Either sodium cyanoborohydride (▼) or sodium cyanide (◆) increased the covalent binding of DMXAA acyl glucuronide to human serum albumin (A). Kinetic studies of the covalent binding were performed by incubating DMXAA-G (8.9–712 μM) and 40 g l^{-1} HSA in 0.1 M phosphate buffer at pH 7.4 at 37°C for up to 6 h. The curve in (B) represents the fitting line of one binding site model. Data are the mean $\pm$ SD of three determinations.

In vitro covalent binding of DMXAA-G to human plasma protein

The formation of DMXAA-protein (albumin) adducts in HSA and human plasma showed time- and concentration-dependence (figure 4A and B). Maximum covalent binding to HSA and plasma protein occurred at 6–8 h. Thereafter, a slow degradation of the adducts was apparent. The addition of imine-trapping reagents sodium cyanide and sodium cyanoborohydride to incubations significantly increased the covalent binding of DMXAA-G to human albumin over a 48 h incubation. At 6 h, a 4- and 2-fold increase in covalent binding was obtained in the presence of sodium cyanide and sodium cyanoborohydride respectively. No covalent binding was demonstrated in similar incubations with DMXAA as control.

The formation of DMXAA-protein adducts followed Michaelis-Menten kinetics. Model fitting showed that one binding site model was the best one with $K_d = 553 \pm 37 \mu\text{M}$ and $N = 151 \pm 6 \mu\text{M}$ (figure 4B).

Effects of drugs on the degradation of DMXAA-G in HSA

The effects of warfarin, phenylbutazone, diazepam, ibuprofen and DMXAA on the degradation of DMXAA-G incubated for 6 h with HSA at 37°C are shown in

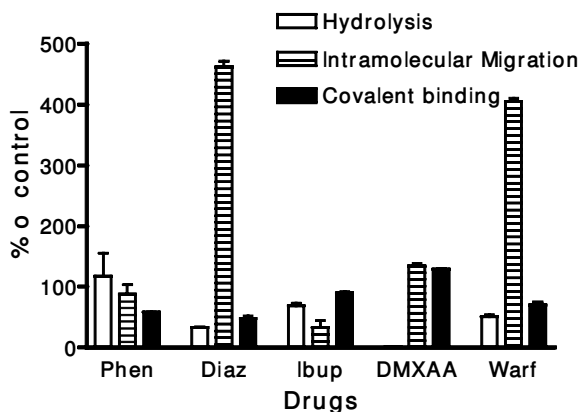


Figure 5. Effects of warfarin (Warf), phenylbutazone (Phen), diazepam (Diaz), ibuprofen (Ibup) and DMXAA on the hydrolysis, intramolecular migration and covalent binding of DMXAA acyl glucuronide in human serum albumin solution. Values (mean $\pm$ SD from three determinations) are expressed as percentage of a control in the absence of ligand.

figure 5. Warfarin and diazepam decreased the release of DMXAA (51 and 34% of controls, respectively) ($p < 0.05$), and increased the concentration of DMXAA-G isomers ~ 4 -5-fold. Ibuprofen decreased the isomer formation of DMXAA-G to 33 and 40% of controls, respectively ($p < 0.05$). Ibuprofen decreased the liberation of DMXAA to 69% of controls ($p < 0.05$). DMXAA increased the covalent binding and acyl migration to 129 and 134% of controls, respectively ($p < 0.05$). Its effect on the hydrolysis of DMXAA-G could not be determined. In contrast, warfarin, phenylbutazone and diazepam decreased the formation of covalent bound adducts by 30, 41 and 52% of controls, respectively ($p < 0.05$).

In vivo formation of DMXAA-protein adduct in cancer patients

DMXAA-protein adduct formation in the plasma of two cancer patients receiving DMXAA was detectable in both patients 10 min after DMXAA administration, reaching maximal concentrations of 129 and 154 ng DMXAA covalently bound mg^{-1} plasma proteins, with corresponding formation half-lives of 0.92 and 0.56 h respectively (figure 6A). A one-compartment model with zero-order input and first-order decay was fitted. The formation of DMXAA-protein adducts was significantly correlated with plasma DMXAA-G concentration ($r^2 = 0.96$, $p < 0.001$) or maximum plasma DMXAA concentration ($r^2 = 0.91$, $p < 0.001$) in 10 cancer patients receiving DMXAA (figure 6B).

Discussion

This study has demonstrated that humans can metabolize DMXAA to DMXAA-G and 6-OH-MXAA, and the latter was conjugated to glucuronic acid. Our *in vivo* results are in agreement with previous studies using human liver microsomes and cDNA-expressed isoforms of P450 and UGT (Miners *et al.* 1997, Zhou *et al.* 2000). The human urinary DMXAA metabolite profile was more similar to that observed in the rabbit (with DMXAA-G as the predominant metabolite) than the mouse or rat (Kestell *et al.* 1999). As the molecular weight

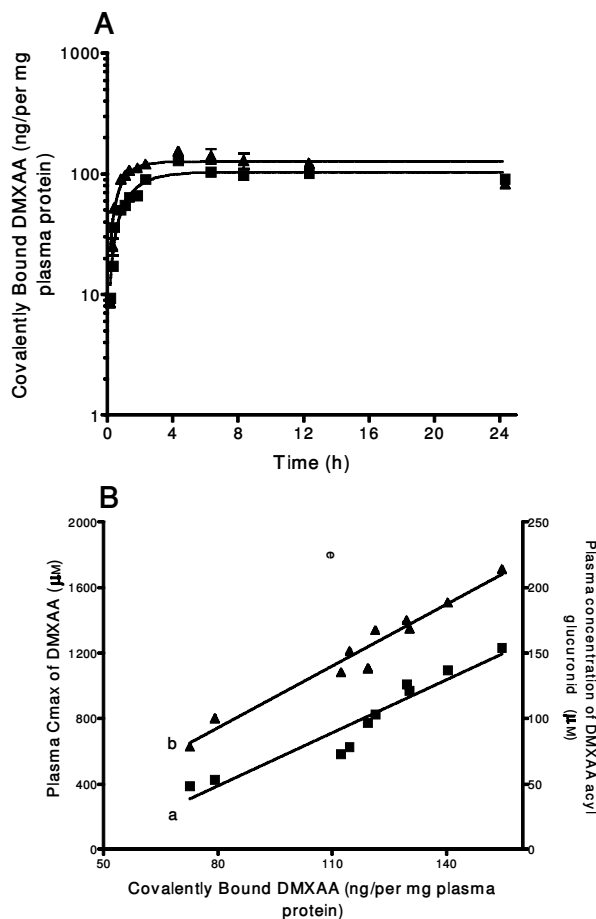


Figure 6. Plasma concentration-time profile for DMXAA-protein adduct in two cancer patients receiving DMXAA treatment (A), and the relationship between DMXAA-protein adduct formation and the maximum plasma concentration of DMXAA (■, line a) or the plasma DMXAA-G concentration at 4 h time point (▼, line b) in 10 cancer patients receiving DMXAA treatment (B). Each point represents the mean ± SD of at least two determinations.

of the DMXAA-G is > 450, biliary excretion of DMXAA-G may also be a major route in humans. These studies also suggest that acyl glucuronidation of DMXAA is the predominant metabolic pathway, with 6-methylhydroxylation playing a lesser role. This is consistent with our *in vitro* studies using human liver microsomes where intrinsic clearance by glucuronidation was 2.6-fold that of 6-methylhydroxylation.

The results indicate that the β -1-glucuronide of DMXAA is chemically unstable under physiological conditions, undergoing enzymatic and non-enzymatic hydrolysis, plus intramolecular rearrangement as outlined in figure 7. DMXAA β -1-glucuronide was unstable in buffer solution, with a degradation half-life of 1.30 h at pH 7.4, which is identical to the acyl glucuronide of salicylic acid, but longer than those of the acyl glucuronides of various drugs such as tolmetin (0.26 h), diclofenac (0.51 h) and diflunizal (0.67 h) (William and Dickson 1994, Ebner *et al.* 1999). A 2-fold variation in the degradation half-life of

DMXAA-G in the blood was observed with the longest (0.97 h) in humans and the shortest (0.48 h) in the rat, and a rank order of human > mouse > rabbit > rat. These results were not in agreement with the rank order for *in vivo* plasma clearance (human > rabbit > rat > mouse) (Kestell *et al.* 1999, Jameson *et al.* 2000), suggesting that the species differences in the degradation rate of DMXAA-G in plasma or blood could not explain the differences in the systemic clearance of DMXAA across species. D-Saccharic acid 1,4-lactone significantly inhibited the degradation of DMXAA-G in the plasma and blood from all species investigated, indicating that β -glucuronidase may play an important role in the hydrolysis of DMXAA-G and may be an influencing factor for the disposition of DMXAA *in vivo*. It has been suggested that β -glucuronidase activity may contribute to the systemic disposition of drugs undergoing glucuronidation (Sperker *et al.* 1997). In addition, the net *in vivo* glucuronidation rate of bilirubin has been reported to be dependent on the hepatic microsomal β -glucuronidase activity in mouse and rat (Dwivedi *et al.* 1987, Whiting *et al.* 1993). The unstable nature of DMXAA-G in biological matrices may lead to conjugation-deconjugation cycling via its hydrolysis, which may contribute to the non-linear pharmacokinetics observed *in vivo* in animals and humans. Therefore, it is expected that the plasma clearance of DMXAA may be a function of the production, hydrolysis, and renal clearance of DMXAA-G. Any factors affecting these processes may lead to changes in the clearance of DMXAA.

The formation of DMXAA-protein adducts *in vitro* was time and concentration dependent. Albumin appears to be a target for covalent adduct formation by DMXAA-G, but it is not known whether other tissue proteins may be involved. Warfarin, diazepam and phenylbutazone significantly inhibited the covalent binding of DMXAA-G, suggesting that the involvement of both the site I and II binding domains on albumin. Alternatively, conformational changes induced by the binding of ligand may have an impact on the covalent binding to albumin. Further studies are required to elucidate which amino acid residues on albumin are involved in the irreversible binding of DMXAA-G. Site II tyrosine-411, lysine-541, lysine-525 and serine-480 residues are covalently modified by acyl glucuronides of some drugs (Presle *et al.* 1996). The imine-trapping reagents NaCN and NaCNBH₃ increased the formation of DMXAA-albumin adducts, indicating the involvement of an imine formation mechanism (glycation). The cyanide anion is used as a selective agent for trapping imine intermediates, and cyanoborohydride salts at neutral pH selectively reduce imines in preference to aldehydes or ketones (Smith *et al.* 1990, Mayer *et al.* 1993). Glycation involves intramolecular rearrangement, allowing formation of an open-chain conjugate with a free aldehyde group able to react with an amino group on proteins, resulting in adduct formation (figure 7). Another mechanism for the covalent binding of an acyl glucuronide to plasma protein is transacylation (van Breemen and Fensel 1985, Ding *et al.* 1993, Fenselau 1994). Transacylation involves the nucleophilic attack of functional groups (-NH₂, -SH, -OH) on proteins at the acyl carbonyl of the β -1-glucuronide, or its rearrangement isomers.

In cancer patients, the formation of DMXAA-protein adducts significantly correlated with the concentration of DMXAA-G and maximum plasma DMXAA concentration. This is in agreement with Benet *et al.* (1993), who have reported that the amount of *in vivo* adduct formation for a number of carboxylic acid drugs in humans was directly related to the plasma AUC of their acyl glucuronides.

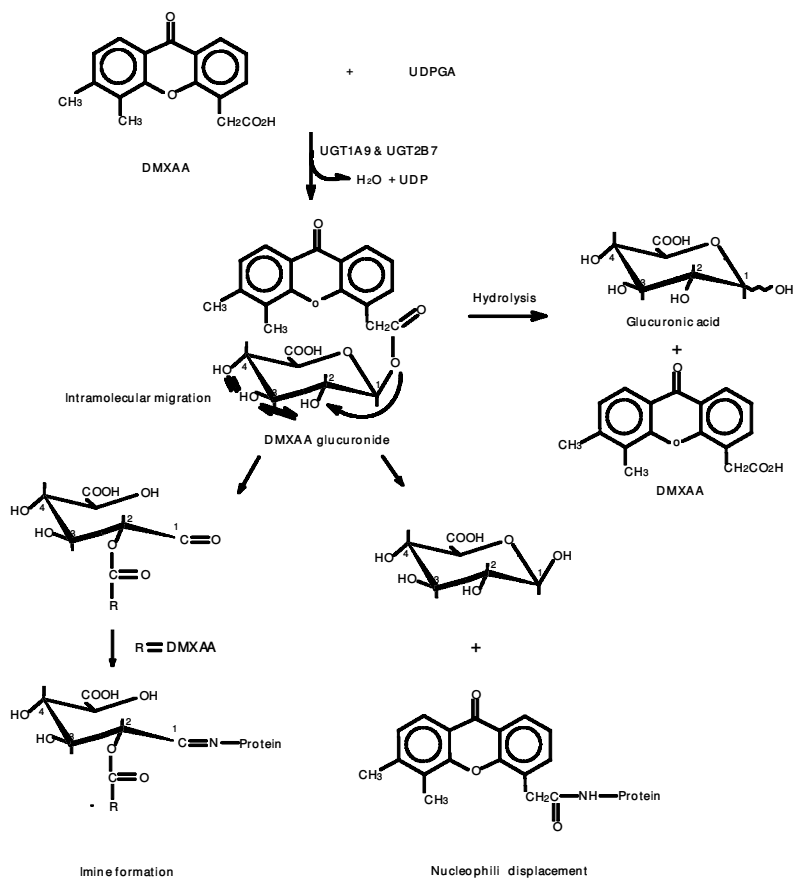


Figure 7. Possible degradation pathways for DMXAA acyl glucuronide *in vitro* and *in vivo*: (1) hydrolysis catalysed by β -glucuronidase in biological matrixes or non-enzymatically, resulting in parent molecule DMXAA; (2) intramolecular rearrangement of acyl residue at C1, C2, C3 and C4, which is postulated to occur at physiological pH; the rearrangement of isomers are reversible except C2 to C1; (3) covalent binding to proteins possibly two mechanisms: (a) nucleophilic attack of functional groups ($-\text{NH}_2$, $-\text{SH}$, $-\text{OH}$) on proteins at the acyl carbonyl of the β -1-glucuronide or its rearrangement isomers, (b) intramolecular rearrangement allowing formation of an open-chain conjugate with a free aldehyde group to react with amino group on proteins resulting in adduct formation.

Similarly, the formation of tolmetin-plasma adducts was increased in humans after multiple dosing (Zia-amirhosseini *et al.* 1994); the concentrations of clofibrac-protein adducts were correlated with renal function in patients (Sallustio *et al.* 1991); and the concentrations of zomepirac-protein adducts increased with co-administration of probenecid, which is known to decrease the renal excretion of acyl glucuronides (Meffin *et al.* 1983, Smith *et al.* 1986). Thus, it could be predicted that any conditions that increase the concentration of glucuronide, such as impairment of renal function, repeated or multiple administration of parent drug, escalation of dosage, or co-administration of various drugs, may increase adduct formation.

The toxicological implications of DMXAA covalent binding to proteins in humans are not clear. The reactivity of acyl glucuronides of certain drugs may lead to toxicity through the formation of protein adducts, resulting in cancer, hyper-

sensitivity and/or cellular dysfunction. It has been reported that the acyl glucuronides of both clofibric acid and gemfibrozil produced significant concentration-related DNA strand nicking and exhibited over 10-fold greater reactivity than the endogenous glycosylation agent glucose 6-phosphate, which is supposed to be associated with the hepatocarcinogenicity of the parent aglycones (Sallustio *et al.* 1997b). However, covalent binding of acyl glucuronides to tissue proteins is not always associated with toxicity. It is suggested that the manifestation of toxicity may depend on whether the proteins are essential to cellular function, and how efficiently they are repaired or regenerated. There may be a threshold of covalent binding below which no toxicity is detectable. If this is true, it is possible that the protein adducts of DMXAA may initiate an unfavourable immune response in susceptible individuals especially with repetitive dosing. The covalent binding of acyl glucuronides to albumin may provide a protective function, as albumin is not only a good scavenger of reactive acyl glucuronide metabolites, but also a promoter of their degradation. To understand the consequences of acyl glucuronide reactivity, it is necessary to identify the target protein of covalent binding, their subcellular localization and kinetics of adduct formation and clearance. The clinical significance of these results for the treatment of patients with DMXAA has yet to be determined. There were no obvious signs of hypersensitivity in patients in the Phase I trial of DMXAA (Jameson *et al.* 2000).

In summary, the results of the present study provide evidence that there are at least five metabolites present in the urine of patients receiving DMXAA, with DMXAA- β -1-glucuronide as the predominant metabolite excreted in urine. The latter undergoes hydrolysis, intramolecular rearrangement and irreversible binding to plasma protein. The reactive properties of DMXAA acyl glucuronide may have important implications for the pharmacokinetics and pharmacodynamics of DMXAA.

Acknowledgements

The work was supported by the Maurice and Phyllis Paykel Trust, and the University of Auckland Research Fund. S. F. Z. was supported by an Auckland Medical Research Foundation Senior Scholarship.

References

- BAGULEY, B. C., ZHUANG, L. and KESTELL, P., 1997, Increased plasma serotonin following treatment with flavone-8-acetic acid, 5,6-dimethylxanthenone-4-acetic acid, vinblastine, and colchicine: relation to vascular effects. *Oncology Research*, **9**, 55-60.
- BENET, L. Z., SPAHN-LANGGUTH, H., IWAKAW, S., C., V., MIZUMA, T., MAYER, S., MUTSCHLER, E. and LIN, E. T., 1993, Predictability of the covalent binding of acidic drugs in man. *Life Science*, **53**, 141-146.
- BISCHER, A., ZIAAMIRHOSSEINI, P., IWAKI, M., McDONAGH, A. F. and BENET, L. Z., 1995, Stereoselective binding properties of naproxen glucuronide diastereomers to proteins. *Journal of Pharmacokinetics and Biopharmaceutics*, **23**, 379-395.
- BURCHELL, B., BRIELEY, C. H., MONAGHAN, G. and CLARKE, D. J., 1998, The structure and function of the UDP-glucuronosyltransferase gene family. *Advances in Pharmacology*, **42**, 335-338.
- DING, A., OJINGWA, J. C., McDONAGH, A. F., BURLINGAME, A. L. and BENET, L. Z., 1993, Evidence for covalent binding of acyl glucuronides to serum albumin via an amine mechanism as revealed by tandem mass spectrometry. *Proceedings of the National Academy of Sciences, USA*, **90**, 3797-3801.

- DING, A., ZIA-AMIRHOSSEINI, P., MCDONAGH, A. F., BURLINGAME, A. L. and BENET, L. Z., 1995, Reactivity of tolmetin glucuronide with human serum albumin - identification of binding sites and mechanisms of reaction by tandem mass spectrometry. *Drug Metabolism and Disposition*, **23**, 369-376.
- DOUMAS, B. T. and BIGGS, H. G., 1972, Determination of serum albumin. *Standard Methods in Clinical Chemistry*, **7**, 175-188.
- DWIVEDI, C., DOWNIE, A. A. and WEBB, T. E., 1987, Net glucuronidation in different rat strains: importance of microsomal beta-glucuronidase. *FASEB Journal*, **1**, 303-307.
- EBNER, T., HEINZEL, G., PROX, A., BESCHKE, K. and WACHSMUTH, H., 1999, Disposition and chemical stability of telmisartan 1-O-acylglucuronide. *Drug Metabolism and Disposition*, **27**, 1143-1149.
- FAED, E. M., 1984, Properties of acyl glucuronide: implications for studies of the pharmacokinetics and metabolism of acidic drugs. *Drug Metabolism Reviews*, **15**, 1213-1249.
- FENSELAU, C., 1994, Acyl glucuronide as chemical reactive intermediates. In F. C. Kauffman (ed) *Conjugation-Deconjugation Reactions in Drug Metabolism and Toxicity* (Berlin: Springer-Verlag), pp. 367-389.
- JAMESON, M. B., THOMSON, P. I., BAGULEY, B. C., EVANS, B. D., HARVEY, V. J., MCCRYSTAL, M. R. and KESTELL, P., 2000, Phase I pharmacokinetic and pharmacodynamic study of 5,6-dimethylxanthene-4-acetic acid (DMXAA), a novel antivascular agent. In *Proceedings of the Annual Meeting of American Society of Clinical Oncologists*, p. 705.
- KESTELL, P., PAXTON, J. M., REWCASTLE, G. W., DUNLOP, I. and BAGULEY, B. C., 1999, Plasma disposition, metabolism and excretion of the experimental antitumour agent 5,6-dimethylxanthene-4-acetic acid in the mouse, rat and rabbit. *Cancer Chemotherapy and Pharmacology*, **43**, 323-330.
- MAYER, S., MUTSCHLER, E., BENET, L. Z. and SPAHNLUUGUTH, H., 1993, *In vitro* and *in vivo* irreversible plasma protein binding of becloric acid enantiomers. *Chirality*, **5**, 120-125.
- McKINNON, G. E. and DICKINSON, R. G., 1989, Covalent binding of diflunisal and probenecid to plasma protein in humans: persistence of the adducts in the circulation. *Research Communications in Chemistry, Pathology, and Pharmacology*, **66**, 339-354.
- MEFFIN, P. J., ZILM, D. M. and VEENENDAAL, J. R., 1983, A renal mechanism for the clofibrilic acid-probenecid interaction. *Journal of Pharmacology and Experimental Therapeutics*, **227**, 739-742.
- MINERS, J. O., VALENTE, L., LILLYWHITE, K. J., MACKENZIE, P. I., BURCHELL, B., BAGULEY, B. C. and KESTELL, P., 1997, Preclinical prediction of factors influencing the elimination of 5,6-dimethylxanthene-4-acetic acid, a new anticancer drug. *Cancer Research*, **57**, 284-289.
- OJINGWA, J., SPAHNLUUGUTH, H. and BENET, L. Z., 1988, Studies of tolmetin reversible and irreversible binding to proteins via its glucuronide conjugate. *Pharmaceutical Research*, **5(suppl.)**, S214.
- PHILPOTT, M., BAGULEY, B. C. and CHING, L.-M., 1995, Induction of tumour necrosis factor-alpha by single and repeated doses of the antitumour agent 5,6-dimethylxanthene-4-acetic acid. *Cancer Chemotherapy and Pharmacology*, **36**, 143-148.
- POHL, L. R., SATOH, H., CHRIST, D. D. and KENNA, J. G., 1988, The immunological and metabolic basis of drug hypersensitivities. *Annual Review in Pharmacology*, **28**, 367-387.
- PRESLE, N., LAPICQUE, F., FOURNEL-GIGLEUX, S., MAGDALOU, J. and NETTER, P., 1996, Stereoselective irreversible binding of ketoprofen glucuronides to albumin: characterization of the site and the mechanism. *Drug Metabolism and Disposition*, **24**, 1050-1057.
- REWCASTLE, G. W., ATWELL, G. J., BAGULEY, B. C., CALVELEY, S. B. and DENNY, W. A., 1989, Potential antitumour agents. 58. Synthesis and structure-activity relationships of substituted xanthene-4-acetic acids active against the colon 38 tumour *in vivo*. *Journal Medicinal Chemistry*, **32**, 793-799.
- REWCASTLE, G. W., KESTELL, P., BAGULEY, B. C. and DENNY, W. A., 1990, Light-induced breakdown of flavone acetic acid and xanthene analogues in solution. *Journal of the National Cancer Institute*, **82**, 528-529.
- SALLUSTIO, B. C., FAIRCHILD, B. A. and PANNALL, P. R., 1997a, Interaction of human serum albumin with the electrophilic metabolite 1-O-gemfibrozil-b-D-glucuronide. *Drug Metabolism and Disposition*, **25**, 55-60.
- SALLUSTIO, B. C., HARKIN, L. A., MANN, M. C., KRIVICKAS, S. J. and BURCHAM, P. C., 1997b, Genotoxicity of acyl glucuronide metabolites formed from clofibrilic acid and gemfibrozil: a novel role for phase II-mediated bioactivation in the hepatocarcinogenicity of the parent aglycone. *Toxicology and Applied Pharmacology*, **147**, 459-464.
- SALLUSTIO, B. C., KNIGHTS, K. M., ROBERTS, B. J. and ZACEST, R., 1991, *In vivo* covalent binding of clofibrilic acid to human plasma proteins and rat liver proteins. *Biochemical Pharmacology*, **42**, 1421-1425.
- SEMMES, R. L. O. and SHEN, D. D., 1990, Nonlinear binding of valproic acid (VPA) and E-D2-valproic acid to rat plasma proteins. *Pharmaceutical Research*, **7**, 461-467.
- SMITH, P. C., BENET, L. Z. and MCDONAGH, A. F., 1986, Irreversible binding of zomepirac to plasma protein *in vitro* and *in vivo*. *Journal of Clinical Investigation*, **77**, 934-939.

- SMITH, P. C., BENET, L. Z. and McDONAGH, A. F., 1990, Covalent binding of zomepirac glucuronide to proteins: evidence for a Schiff base mechanism. *Drug Metabolism and Disposition*, **18**, 639-644.
- SMITH, P. C., SONG, W. Q. and RODRIGUEZ, R. J., 1992, Covalent binding of etodolac acyl glucuronide to albumin *in vitro*. *Drug Metabolism and Disposition*, **20**, 962-965.
- SMITH, P. K., KROHN, R. I., HERMANSON, G. T., MALLIA, A. K., GARTER, F. H., PROVENZANO, M. D., FUJIMOTO, E. K., GOEKE, N. M., OLSON, B. J. and KLENK, D. C., 1985, Measurement of protein using bicinchoninic acid. *Annals of Biochemistry*, **150**, 76-85.
- SPAHN-LUGGUTH, H. and BENET, L. Z., 1992, Acyl glucuronide revisited: is the glucuronidation process a toxification as well as a detoxification mechanism? *Drug Metabolism Review*, **24**, 5-48.
- SPECKER, B., BACKMAN, J. T. and KROEMER, H. K., 1997, The role of β -glucuronidase in drug disposition and drug targeting in humans. *Clinical Pharmacokinetics*, **33**, 18-31.
- THOMSEN, L. L., CHING, L. M., ZHUANG, L., GAVIN, J. B. and BAGULEY, B. C., 1991, Tumor-dependent increased plasma nitrate concentrations as an indication of the antitumor effect of flavone-8-acetic acid and analogues in mice. *Cancer Research*, **51**, 77-81.
- VAN BREEMEN, R. B. and FENSEL, A. U., 1985, Acylation of albumin by 1-O-acyl glucuronide. *Drug Metabolism and Disposition*, **13**, 318-320.
- WEBSTER, L. K., ELLIS, A. G., KESTELL, P. and REWCASTLE, G. W., 1995, Metabolism and elimination of 5,6-dimethylxanthene-4-acetic acid in the isolated perfused rat liver. *Drug Metabolism and Disposition*, **23**, 363-368.
- WHITING, J. F., NARCISO, J. P., CHAPMAN, V., RANSIL, B. J., SWANK, R. T. and GOLLAN, J. L., 1993, Deconjugation of bilirubin-IXa glucuronides: a physiologic role of hepatic microsomal β -glucuronidase. *Journal of Biological Chemistry*, **268**, 23197-23201.
- WILLIAM, A. M. and DICKSON, R. G., 1994, Modulation of reversible and covalent interaction of diflunisal acyl glucuronide and its isomers with human plasma protein *in vitro*. *Biochemical Pharmacology*, **47**, 457-467.
- YAMAOKA, K., NAKAGAWA, T. and UNO, T., 1978, Application of Akaike's information criterion (AIC) in the evaluation of linear pharmacokinetic equations. *Journal of Pharmacokinetics and Biopharmaceutics*, **6**, 165-167.
- ZHOU, S. F., PAXTON, J. W., TINGLE, M. D. and KESTELL, P., 2000, Identification of the human liver cytochrome P450 isozyme responsible for the 6-methylhydroxylation of the novel anticancer drug 5,6-dimethylxanthene-4-acetic acid. *Drug Metabolism and Disposition*, **28**, 1449-1456.
- ZHOU, S. F., PAXTON, J. W., TINGLE, M. D., MCCALL, J. and KESTELL, P., 1999, Determination of two major metabolites of the novel anti-tumour agent 5,6-dimethylxanthene-4-acetic acid in hepatic microsomal incubations by high-performance liquid chromatography with fluorescence detection. *Journal of Chromatography (B)*, **734**, 129-136.
- ZIA-AMIRHOSSEINI, P., OJINGWA, J. C., SPAHN-LUGGUTH, H., McDONAGH, A. F. and BENET, L. Z., 1994, Enhanced covalent binding of tolmetin to proteins in humans after multiple dosing. *Clinical Pharmacology and Therapeutics*, **55**, 21-27.

Glutathione depletion in rat hepatocytes: a mixture toxicity study with α,β -unsaturated esters

A. FREIDIG*, M. HOFHUIS, I. VAN HOLSTIJN and J. HERMENS

TNO Food and Nutrition Research, Division Explanatory Toxicology, PO Box 360, 3700 AJ Zeist, The Netherlands

Received 27 July 2000

1. Glutathione (GSH) depletion is often reported as an early cytotoxic effect, caused by many reactive organic chemicals. In the present study, GSH depletion in primary rat hepatocytes was used as an *in vitro* effect-equivalent to measure the toxic potency of α,β -unsaturated esters (acrylates and methacrylates).

2. When these compounds were administered as a mixture, GSH depletion was dose additive. The result of the mixture study shows that GSH depletion may be a useful effect-equivalent for the risk assessment of mixtures of α,β -unsaturated esters.

3. To get more insight in the underlying mechanisms of GSH depletion, the metabolism of two esters was investigated in greater detail. One of them, allyl methacrylate, was metabolized to acrolein. This metabolic pathway can explain the high potency of allyl methacrylate to deplete GSH despite its low intrinsic chemical reactivity.

Introduction

Risk assessment of mixtures is recognized as an important issue in environmental and human toxicology (Feron *et al.* 1998, Yang *et al.* 1998) because most exposures are complex in nature. Typically hundreds of xenobiotic chemicals are isolated from air or water samples (Feron *et al.* 1998, Hendriks *et al.* 1994). For many compounds in such complex mixtures, the chemical structure remains unknown. On the other hand, many commercially available substances are in fact complex mixtures (e.g. petroleum products or polymer products like paints or adhesives) and consist of mixtures of structurally related chemicals with different physico-chemical and toxicological properties. Often, only a tentative characterization is available for such mixtures, like the range of boiling point or the average chain length. To provide a scientific framework for effect assessment of mixtures, sum-parameters for toxic effects of similar acting compounds have been proposed. A well-known example of this strategy is the use of toxic equivalent factors (TEF) for the assessment of the effect of dioxin-like compounds (Safe 1987, Safe *et al.* 1987). Other effect equivalents are the inhibition percentage of acetylcholinesterase, which is used for pesticide exposure assessment (Carlock *et al.* 1999) or the critical body burden which is used in aquatic toxicology as a measure for narcosis potency (Könemann 1981a, McCarty *et al.* 1992). Effect equivalents are generally based on internal target concentrations. To link a sum-parameter with an external

* Author for correspondence; e-mail: freidig@voeding.tno.nl

exposure concentration, a physiologically based pharmacokinetic (PBPK) model can be used, which can account for differences in kinetics of each compound in a mixture. This combination was used by Verhaar *et al.* (1997, Yang *et al.* 1998) for the risk assessment of jet fuel in a workplace exposure scenario. Frederick *et al.* (1992) established a PBPK model that uses time-integrated glutathione (GSH) depletion as an effect equivalent for toxic effects of ethyl acrylate, an α,β -unsaturated ester. Because GSH depletion is well established as a cytotoxic effect it may be worthwhile to test whether GSH depletion can be used as an effect equivalent for mixtures of reactive chemicals.

α,β -Unsaturated esters are used in various combinations in polymer chemistry (Greim *et al.* 1995), and a mixture toxicity model for these compounds may improve their risk assessment. Many of these chemicals are Michael acceptors and react easily with biological thiols to form a covalent bond (Boyland and Chasseaud 1969, Hashimoto and Aldridge 1970, Potter and Tran 1992, McCarthy *et al.* 1994, Freidig *et al.* 1999a). Metabolism of α,β -unsaturated esters has been shown to occur by hydrolysis and by conjugation with GSH (Elovaara *et al.* 1983, Vodicka *et al.* 1990, Frederick *et al.* 1992, 1994, Greim *et al.* 1995, McCarthy and Witz 1997). Toxicity tests in rodents and fish have demonstrated that toxic effects can be organ specific (Hashimoto and Aldridge 1970, Tanii and Hashimoto 1982, Elovaara *et al.* 1983, Ghanayem *et al.* 1985, Ghanayem *et al.* 1986, Vodicka *et al.* 1990, Frederick *et al.* 1992, Lomax *et al.* 1997) and that differences in chemical reactivity alone is not enough to explain differences in potency (Russom *et al.* 1988, Freidig *et al.* 1999b).

In the present study, primary rat hepatocytes were used to measure GSH depletion caused by 11 individual α,β -unsaturated esters and two mixtures. The objective of the present study was to test if effect data from individual chemicals could be used to predict the effects of mixtures and if GSH depletion may serve as an effect equivalent for these compounds in further studies. Hepatocytes were chosen as a model system because the liver is the primary producer of GSH and because hepatocytes contain many enzymes that are related to GSH metabolism (Kretzschmar 1996). It should be noted, however, that because of their high metabolic activity and their high GSH concentration, hepatocytes may be more resistant to GSH depletion than cells from target organs, like e.g. gills, gastrointestinal tract or respiratory tract. The effect of two mixtures was tested for dose additivity (Brown 1968) and for response additivity (Plackett and Hewlett 1952).

The concept of an effect equivalent on the basis of GSH depletion might be applicable across different mechanisms. Based on the experimental EC_{50} data in hepatocytes, we sought more insight in the underlying mechanistic aspects of GSH depletion. In particular, the low EC_{50} of allyl methacrylate, which is in contrast to the low chemical reactivity of this compound suggested that different modes of action were present within our test set. To investigate alternative mechanisms of GSH depletion, allyl methacrylate was compared with the strongest Michael acceptor in the set, diethyl fumarate using a number of biochemical parameters. Dose-response curves for cellular GSH levels were compared with the concentration of a lipid peroxidation marker (malondialdehyde) and with the production of acrolein and acetaldehyde, both being probable metabolites of allyl methacrylate and diethyl fumarate, respectively.

Materials and methods

Chemicals

The following chemicals were used as received (pro-analysis grade): *o*-phthalaldehyde (OPA), from Arcos ('s Hertogenbosch, The Netherlands), reduced glutathione (GSH), pentafluorobenzyl-hydroxylamine (PFB), acetaldehyde, acrolein, 1,1,3,3-tetraethoxypropane, NaWO_4 , 3,4-dichlorotoluene, ethyl acrylate, 2-hydroxyethyl acrylate, isobutyl acrylate, diethyl fumarate, allyl methacrylate, benzyl methacrylate, 2-ethoxyethyl methacrylate, tetrahydrofurfuryl methacrylate, isobutyl methacrylate and methyl methacrylate from Fluka Sigma-Aldrich (Zwijndrecht, The Netherlands) and isopropyl methacrylate from Pfalz&Bauer (Waterbury, CT, USA).

Animals

Male Wistar (U:Wu) rats were fed *ad libitum* with a grain-based diet and had free access to drinking water. The animals weighted ~ 300 g at the time of killing.

Cell culture and exposure

Hepatocytes were isolated by whole liver perfusion using the two-step collagenase technique as described by Seglen (1976). The cells were incubated at 37°C in air-tight 50 ml tissue culture flasks (Greiner, Alphen a/d Rijn, The Netherlands) at a density of 8×10^5 cells ml^{-1} . Initial culture medium consisted of Williams' E medium, supplemented with 0.1 M HEPES, 26 mM NaHCO_3 , 2 mM L-glutamine, 1 μM insulin, 10 μM hydrocortisone, 70 μM gentamycin and 3% newborn calf serum (NCS). After 3 h, a cell monolayer was formed and the initial culture medium was removed and replaced by culture medium without NCS. Non-attached and dead cells were thereby washed off. Exposure of hepatocytes started 24 h after isolation. The old medium was removed and replaced by culture medium without NCS in which the tested chemical had been dissolved. Geometric dilution series with a factor of two and five concentration steps were used. The hepatocytes were harvested after 4 h of exposure. Culture medium was removed and an aliquot was used to determine LDH activity. For some chemicals, samples of culture medium were frozen and stored at -20°C for subsequent analysis of aldehyde production. The cell monolayer was suspended with a Triton X-100 solution (0.5%) and homogenized by vortexing the cell suspension for 10 min. Aliquots of the homogenate were used to determine LDH activity, reduced GSH concentration and protein content.

Protein content

Protein content of the homogenate was measured according to Bradford (1976).

Cell viability

The viability of the cells was assessed by lactate dehydrogenase (LDH) leakage. The percentage of LDH leakage was determined by comparing LDH activity in the culture medium with total LDH activity in the culture flask (medium + cell homogenate). LDH activity was measured according to Bergmeyer *et al.* (1965).

GSH measurement

Cell homogenates were precipitated with 5% trichloroacetic acid, centrifuged at 10000g and the supernatant kept frozen at -20°C until analysis (maximum 48 h). The supernatant was diluted 1:10 with distilled water and reduced GSH was measured by RP-HPLC with post-column OPA derivatization as described by Fujita *et al.* (1993) and modified by Freidig *et al.* (1999a, b). Cellular GSH concentrations were calculated as nmol mg^{-1} protein.

Analysis of aldehydes in culture medium

Three aldehyde products from the metabolism of unsaturated esters as well as from endogenous metabolism of the hepatocytes were analysed in the culture medium of exposed cells. Extraction of the medium, derivatization with PFB and analysis of the aldehydes on a gas chromatograph with electron capture detector was performed according to DeZwart *et al.* (1997), with the following minor adjustments: PFB-derivates were extracted from aqueous solutions with cyclohexane using 3,4-trichlorotoluene as internal standard. Standard solutions of acrolein and acetaldehyde were prepared in water. Standard aqueous solutions of malondialdehyde were prepared from 1,1,3,3-tetraethoxypropane according to DeZwart (1997). Aqueous standards were derivatized along with the samples and tentative identification and quantification of the three aldehydes in the culture medium were achieved

using retention times and responses of the authentic compounds. Because we did not correct for losses of aldehydes during sampling and extraction of the medium, the actual concentrations in the cell culture medium may have been underestimated.

Calculations

Two dose-response curves for GSH depletion and LDH leakage (each concentration in duplicate) were measured for each tested ester using hepatocytes from different isolations. For GSH depletion, EC_{50} and slopes were fitted from each dose-response curve using the sigmoidal dose-response algorithm of Prism software (GraphPad Software, San Diego, CA, USA) given in equation (1) and C being the nominal exposure concentration. Because many compounds caused only a partial LDH leakage at the highest tested concentration, LOECs ($p < 0.05$, one tailed) were used to assess changes in cell viability.

$$\text{GSH}(\% \text{ of control}) = \frac{100}{1 + 10^{-\text{slope}(\log(EC_{50}) - \log(C))}}. \quad (1)$$

Mixture toxicity

Two different models, dose addition and independent response addition were used to test for a possible additivity of GSH depletion. Two equitoxic mixtures of six esters were prepared based on their individual EC_{50} for GSH depletion. Concentrations of the compounds were transformed to toxic units (TU) according to equation (2) and each of the six compounds was added at equal TU(i) (equitoxic mixtures) to the mixture. The potency of a mixture is thereby given by the $\sum \text{TU}$, defined in equation (3) (Brown 1968, Könemann 1981b).

$$\text{TU}(i) = \frac{\text{conc}(i)}{EC_{50}(i)} \quad (2)$$

$$\sum \text{TU} = \sum_{i=1}^n \text{TU}(i). \quad (3)$$

Each mixture was tested in a geometric dilution series starting with a $\sum \text{TU} = 6$. Dose-response curves were recorded for GSH depletion and LDH leakage as described for the individual compounds. If a mixture is dose additive, a $\sum \text{TU} = 1$ is expected to cause 50% GSH depletion. Furthermore, a model for response additivity was applied to the experimental data to compare the dose addition model. The probabilistic addition model describing independent joint action (Plackett and Hewlett 1952, Musch 1996) was chosen to describe a mixture situation where each compound would deplete GSH by an independent pathway. Percentages of GSH depletion, caused by individual compounds below their EC_{50} were estimated from fitted sigmoidal dose-response curves and used as effect probabilities, P_i . To calculate the effect probability of the mixture, P_{mix} the P_i 's of each compound in the mixture were added according to equation (4).

$$P_{\text{mix}} = 1 - [(1 - P_1)(1 - P_2) \dots (1 - P_n)]. \quad (4)$$

Results

Single chemical tests

A series of α,β -unsaturated esters, which were known to reach chemically with GSH (Freidig *et al.* 1999a), were tested for their potency of inducing GSH depletion *in vitro*. EC_{50} for GSH depletion of single chemicals after 4 h are presented in table 1. They range from 0.14 mM for allyl methacrylate to 7.42 mM for methyl methacrylate. Slope factors for the dose-response curves (table 1) varied between 0.8 and 4.0. Cell viability, as determined by LDH leakage, was not affected by a concentration causing 50% depletion of GSH, except for two compounds (allyl- and isobutyl methacrylate). However, at higher exposure concentrations more esters were found to decrease the cell viability during the 4-h assay.

Table 1. α,β -Unsaturated esters tested for induction of GSH depletion in hepatocytes, EC_{50} and slope factors were fitted using a sigmoidal dose-response curve (equation 1).

Substance	$kGSH^a$ ($mmin^{-1}$)	GSH depletion				LDH-leakage included in	
		EC_{50}^b (mM)	(SE) ^c (mM)	Slope ^b	(SE) ^c	LOEC ^c (mM)	Mixture
Allyl methacrylate	0.51	0.14	0.02	1.5	0.30	0.14	1
2-Hydroxyethyl acrylate	50.00	0.28	0.18	2.2	0.80	1.2	-
Diethyl fumarate	112.00	1.44	0.65	2.0	0.60	>7.3	2
Isobutyl acrylate	42.00	1.70	0.38	3.4	2.60	6.5	2
Ethyl acrylate	40.00	1.85	1.21 ^d	2.9	0.70 ^d	15.0	2
Benzyl methacrylate	0.33	2.36	0.49	0.8	0.60	>2.1	1
2-Ethoxyethyl methacrylate	0.25	2.73	1.11 ^d	2.0	0.30 ^d	>15.9	1,2
Tetrahydrofurfuryl methacrylate	0.30	3.05	0.24	1.7	0.36	>17.3	1,2
Isobutyl methacrylate	0.19	5.00	1.93	1.3	1.90	4.0	1
Isopropyl methacrylate	0.00	5.35	1.08 ^d	3.4	1.10 ^d	14.5	-
Methyl methacrylate	0.20	7.42	1.21 ^d	4.0	1.80 ^d	>15.5	1,2

^a Chemical reaction rate constant of the α,β -unsaturated esters with GSH, taken from Freidig *et al.* (1999a).

^b Fitted dose-response curve parameters, according to equation (1) in the text. Values represent the average of two determinations unless indicated otherwise.

^c Average standard error of two dose-response curves.

^d Fitted parameters and standard errors were derived from one dose-response curve.

^e Lowest observed effect concentration, where the leakage of LDH was significantly ($p > 0.05$) above the LDH leakage in non-exposed cell cultures.

Mixture tests

Two mixtures, each containing six esters, were tested under identical conditions as the single substances. Their compositions are given in table 1. Dose-effect relations for both mixtures are presented in figure 1. $\sum TU$ was used as dose-equivalent on the x -axis. Mixture 1 was dose additive for GSH depletion ($EC_{50} = 1.00 \sum TU$, 95% confidence interval (CI) = 0.49-2.01 $\sum TU$), whereas mixture 2 was more potent than predicted by dose additivity ($EC_{50} = 0.58 \sum TU$, 95% CI = 0.39-0.86 $\sum TU$). The model for response addition underestimated the potency for both mixtures (figure 1). Cell viability was only affected at the highest tested concentration for both mixtures. LOECs for LDH-leakage were 5.3 $\sum TU$ for mix 1 and 6.6 $\sum TU$ for mix 2, respectively.

Comparison of two esters

Allyl methacrylate (AMA) and diethyl fumarate (DF) differ in chemical reactivity toward GSH, DF being 200 times more reactive than AMA (table 1). However, AMA was 10 times more potent than DF depleting GSH *in vitro*. We chose to investigate the effect of these two compounds on primary rat hepatocytes in greater detail to see whether different modes of action could be identified that cause GSH depletion. Figure 2 shows the dose-dependent GSH depletion and cell viability of hepatocytes exposed to both substances. For AMA, loss of cell integrity measured by LDH leakage was paralleled by a decrease in cellular GSH. Loss of GSH might therefore be caused by an increasing damage of cell membranes. At the lowest exposure concentration of DF, GSH concentration was increased by a factor 2.5 above levels of unexposed hepatocytes ($32 \pm 7 \text{ nmol mg}^{-1} \text{ protein}$).

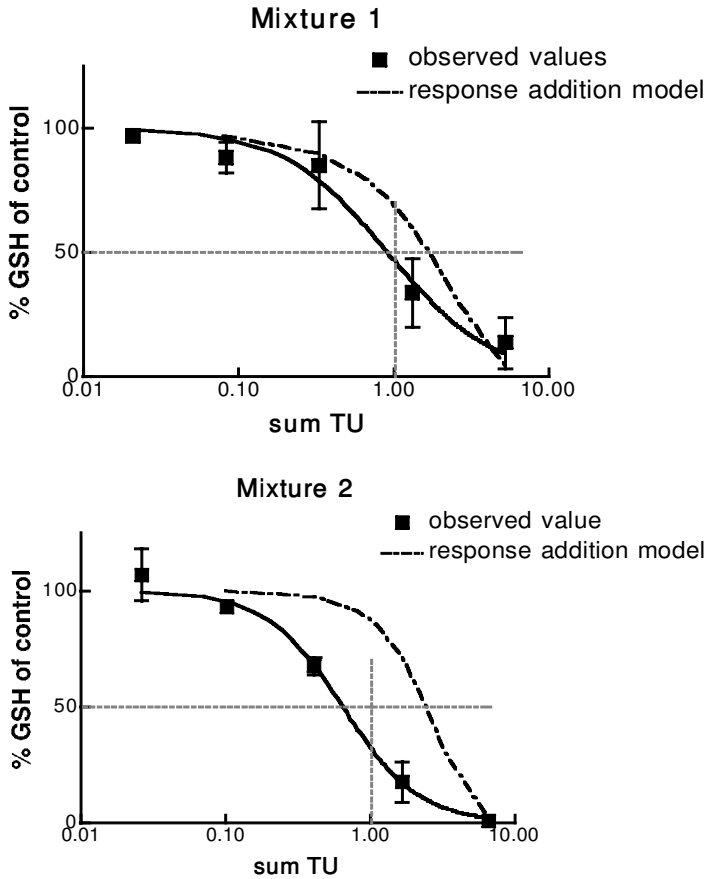


Figure 1. Dose-response curves for two mixtures of α,β -unsaturated esters. The composition of the mixtures is given in table 1. Concentration of the mixtures was expressed as the sum of toxic units ($\sum TU$). For dose addition, a 50% depletion is expected at $\sum TU=1$. An alternative mixture model (response addition) was less accurate and underestimated the observed effect of the mixtures.

Increasing DF concentrations depleted GSH but there was no effect on cell viability.

Three aldehydes were determined in the culture media of exposed cells. Malondialdehyde (MDA) was used as a marker of lipid peroxidation in AMA and DF exposed cells, whereas acetaldehyde and acrolein were used as an indicator for the metabolism pathways of DF and AMA, respectively. Figure 2c shows that in AMA exposed cultures, MDA concentration increases together with LDH leakage. For D-exposed cells no increase of MDA-production was detected for the tested concentrations. Acrolein, which was suspected to be formed by alcohol dehydrogenase after hydrolysis of AMA, was detected above background levels at the two highest exposure concentrations (Figure 2d). Because cell integrity was partially lost at these two concentrations, acrolein could have been formed by cytosolic enzymes that leaked into the medium. In the medium of DF exposed cells, acetaldehyde concentrations up to 50 μM were detected (figure 2e). This oxidative metabolic product of DF was most probably formed in the cells

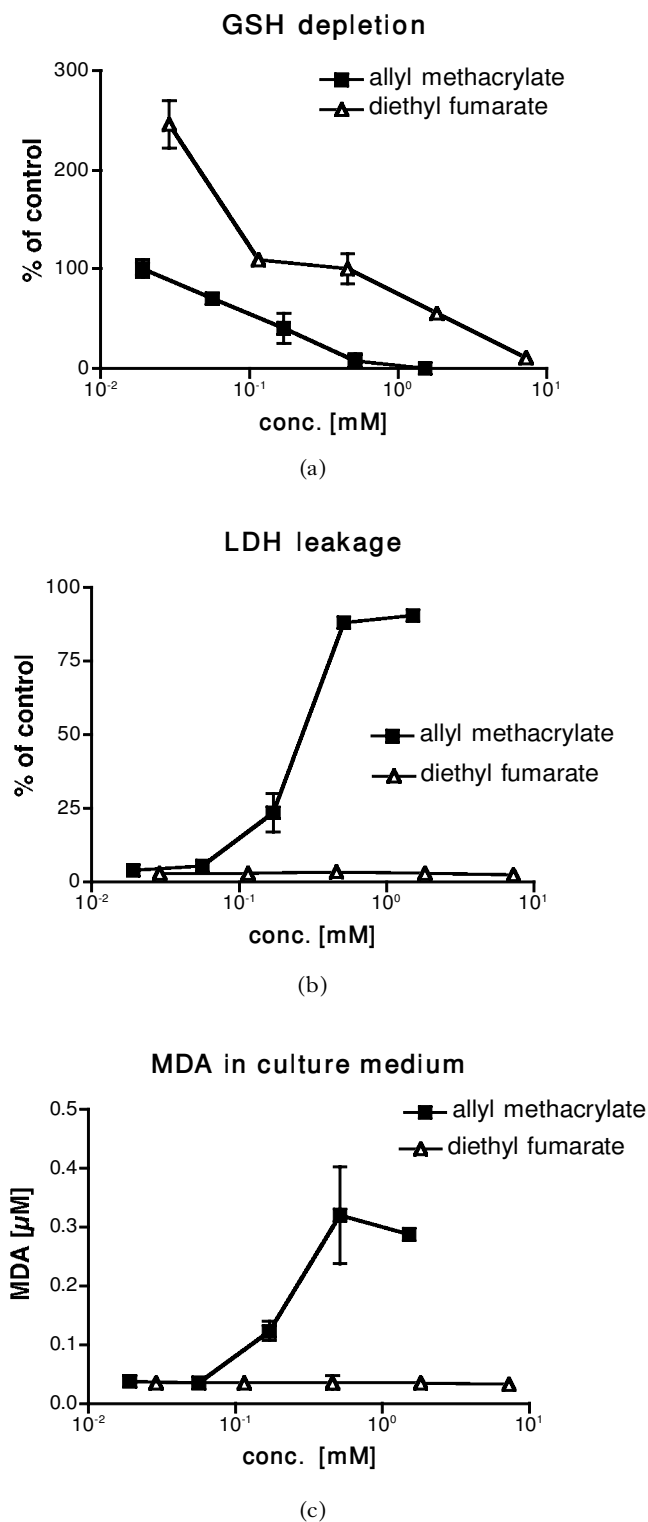


Figure 2. For caption see next page.

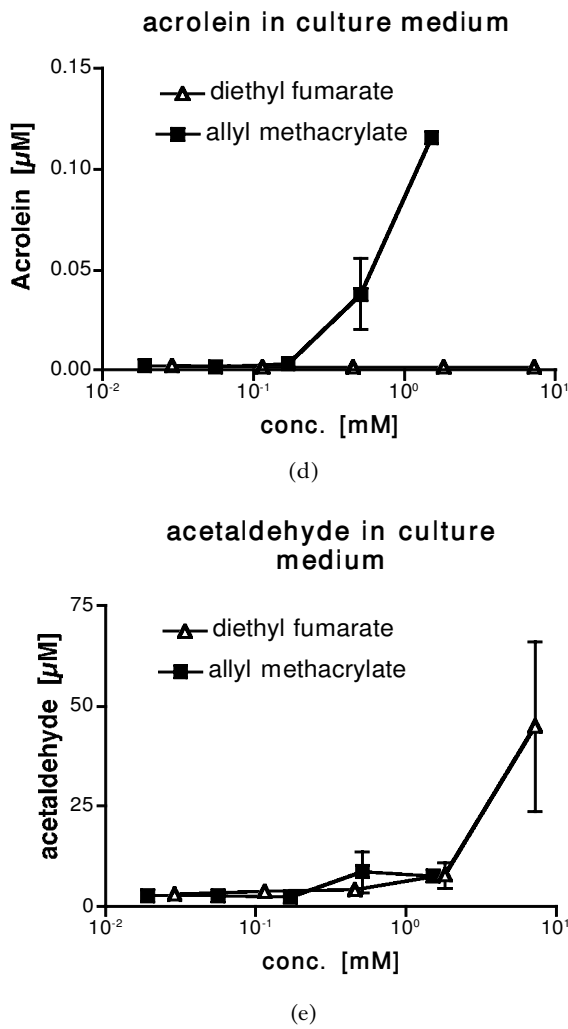


Figure 2 (continued). Dose-effect curves for allyl methacrylate and diethyl fumarate. Both compounds induce GSH depletion (a), but vary in LDH leakage (b), lipid peroxidation as measured by malondialdehyde production (c) and formation of the oxidized hydrolysis product (d, e).

because hepatocytes exposed to DF showed no sign of membrane damage or leakage.

Discussion

GSH depletion by α,β -unsaturated esters

Chemicals with α,β unsaturated carbonyl groups are known to cause GSH depletion in rat liver (Boyland and Chasseaud 1969). Accordingly, all esters tested in the present investigation depleted GSH in primary rat hepatocytes. Their potency to deplete GSH only weakly correlated with their chemical reactivity with GSH (table 1). Two compounds (allyl methacrylate and 2-hydroxyethyl acrylate) were substantially more potent than the other compounds. EC₅₀ for the other nine

esters differ only by a factor of 5 although their chemical reactivity, given in table 1, differs by >2 orders of magnitude. It can be concluded that chemical reactivity with GSH is not the predominant mechanism that leads to GSH depletion in isolated hepatocytes. Moreover, because most esters share a relatively high effect concentration (1–10 mM) and because they are readily hydrolyzed by hepatic carboxylases (Frederick *et al.* 1992), the metabolites can be expected to play an important role.

There are a number of processes apart from pure chemical reactivity that can govern the extent of depletion of GSH in the hepatocytes. First, rapid enzyme-catalysed conjugation of the less reactive methacrylates by glutathione transferases (GST) might be responsible for the small difference in observed EC_{50} . Enzymatic conjugation by GST has been described for ethyl acrylate in rat liver (Potter and Tran 1992). However, no information is available about the selectivity of GST isoenzymes towards acrylates and methacrylates. Second, rapid enzymatic hydrolysis of the esters can decrease the extent of GSH-depletion. *In vivo* inhibition of hydrolysis with tri-orthotolyl phosphate caused an increase of thioether production in rats exposed to methyl acrylate and methyl methacrylate (Delbressine *et al.* 1981) and increased the extent of depletion of non-protein thiols in various tissues of rats exposed to ethyl- and methyl acrylate (Silver and Murphy 1981). Third, metabolites resulting from hydrolysis that form or accumulate in cells can have an impact on the GSH level. This seems to be the case for allyl methacrylate. Allyl alcohol, a possible product of AMA metabolism was found to produce GSH depletion *in vitro* (Ohno *et al.* 1985, Penttilä *et al.* 1987, Penttilä 1988) and *in vivo* in rats (Badr *et al.* 1986, Belinsky *et al.* 1986). This was explained by oxidation of allyl alcohol to acrolein by alcohol dehydrogenase. Acrolein, the reactive metabolite, has been reported to be ~ 100 times more reactive with GSH than any of the esters tested in this work (Freidig *et al.* 1999b). In the present investigation, cells exposed to allyl methacrylate produced MDA, an indicator of lipid peroxidation and MDA was also found in allyl alcohol exposed cells (Dogterom *et al.* 1988, 1989a, b, Hormann *et al.* 1989). Furthermore, acrolein was detected in the culture medium. Neither acrolein nor MDA were found in DF exposed cells. We therefore suggest, that the low EC_{50} for GSH depletion of allyl methacrylate is caused by its metabolite acrolein which is formed through hydrolysis and subsequent oxidation. This is in agreement with reports on the toxicity of other esters of allyl alcohol (Droy *et al.* 1998, Silver and Murphy 1978). The induction of GSH, observed at the lowest exposure concentration of DF (figure 2a) may be caused by an 'electrophilic counterattack' as described by Talalay and co-workers (Prochaska and Santamaria 1988, Talalay *et al.* 1988, Talalay 1989, Prestera *et al.* 1993). The latter authors reported that many chemicals with unsaturated carboxyl and carbonyl groups were able to co-induce a battery of phase 2 enzymes in hepatoma cells at very low concentrations.

Formation of acid equivalents by ester-hydrolysis in the cell could be another mechanism by which GSH is depleted. Lowering of intracellular pH from 7.35 to 7.05 was found to cause GSH depletion in hepatocarcinoma cells (García-Cañero *et al.* 1999). Acrylic acid itself was also found to induce membrane permeability transitions in isolated mitochondria (Custodio *et al.* 1998). This, in turn could lead to an energy deprivation and subsequently to a loss of reduced GSH.

GSH depletion of mixtures

In the mixture experiment it was clearly shown that GSH depletion of α,β -unsaturated esters is an additive effect in hepatocytes. Mixtures of compounds, diluted to 10-16% of their individual effect concentration induced a comparable GSH depletion as one compound at 100% of its EC_{50} . Two additivity models, dose addition and response addition were used to predict the effect of mixtures of α,β -unsaturated esters. Both mixtures were well predicted with dose addition. Response addition, which should be able to predict the joint effect of compounds with independent modes of action (Plackett and Hewlett 1952) underestimated the potency of both mixtures. Maybe, the effects at low concentrations (P_i , equation 4) could not be predicted correctly with a dose-response curve based on a geometric dilution series with a factor of two. The dose addition model seems to be more stable under such circumstances.

Use of GSH depletion as effect equivalent in risk assessment

The advantage of an effect equivalent above a target concentration is that it allows to aggregate the effect of several compounds. This study clearly shows, that GSH depletion in hepatocytes can be used as additive effect-equivalent for the toxic effects of acrylic and methacrylic acid esters in primary rat hepatocytes. The use of GSH depletion data from individual esters made it possible to predict the potency of a complex mixture. For a translation to *in vivo* effects, information about the toxicokinetics of each compound in the mixture is needed to define their target tissue concentrations. Verhaar *et al.* (1997) reported, how an existing PBPK model can be adapted to predict target tissue concentrations of compounds of jet fuel mixture with varying physico-chemical properties. Using such a distribution model together with a toxicodynamic model for GSH depletion (D'Souza *et al.* 1988, Frederick *et al.* 1992), it may be possible to predict the GSH depletion due to a complex mixture of e.g. acrylic monomers.

References

- BADR, M. Z., BELINSKY, S. A., KAUFFMAN, F. C. and THURMAN, R. G., 1986, Mechanism of hepatotoxicity to periportal regions of liver lobule due to allyl alcohol: Role of oxygen and lipid peroxidation. *Journal of Pharmacology and Experimental Therapeutics*, **238**, 1138-1142.
- BELINSKY, S. A., BADR, M. Z., KAUFFMAN, F. C. and THURMAN, R. G., 1986, Mechanism of Hepatotoxicity in periportal regions of the liver lobule due to allyl alcohol: studies on thiols and energy status. *Journal of Pharmacology and Experimental Therapeutics*, **238**, 1132-1137.
- BERGMAYER, H. U., BENT, E. and HESS, B., 1965, Lactic dehydrogenase. In H. U. Bergmeyer (ed.), *Methods of Enzymatic Analysis* (Weinheim: Chemie), pp. 736-743.
- BOYLAND, E. and CHASSEAUD, L. F., 1969, The effect of some carbonyl compounds on rat liver glutathione levels. *Biochemical Pharmacology*, **19**, 1526-1528.
- BRADFORD, M. M., 1976, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Annals of Biochemistry*, **72**, 248-254.
- BROWN, V. M., 1968, The calculation of the acute toxicity of mixtures of poisons to rainbow trout. *Water Research*, **2**, 723-733.
- CARLOCK, L. L., CHEN, W. L., GORDON, E. B., KILLEEN, J. C., MANLEY, A., MEYER, L. S., MULLIN, L. S., PENDINO, K. J., PERCY, A., SARGENT, D. E., SEAMAN, L. R., SVANBORG, N. K., STANTON, R. H., TELLONE, C. I. and VAN GOETHEM, D. L., 1999, Regulating and assessing risks of cholinesterase-inhibiting pesticides: divergent approaches and interpretations. *Journal of Toxicology and Environmental Health B Critical Reviews*, **2**, 105-160.
- CUSTODIO, J. B. A., PALMEIRA, C. M., MORENO, A. J. and WALLACE, K. B., 1998, Acrylic acid induces the glutathione-independent mitochondrial permeability transition *in vitro*. *Toxicological Sciences*, **43**, 19-27.

- D'SOUZA, R. W., FRANCIS, W. R. and ANDERSEN, M. E., 1988, Physiological model for tissue glutathione depletion and increased resynthesis after ethylene dichloride exposure. *Journal of Pharmacology and Experimental Therapeutics*, **245**, 563-568.
- DELBRESSINE, L. P. C., SEUTTER-BERLAGE, F. and SEUTTER, E., 1981, Identification of urinary mercapturic acids formed from acrylate, methacrylate and crotonate in the rat. *Xenobiotica*, **11**, 241-247.
- DEZWART, L., VENHORST, J., GROOT, M., COMMANDEUR, J. N. M., HERMANN, R. C. A., MEERMAN, J. H. M., VANBAAR, B. L. M. and VERMEULEN, N. P. E., 1997, Simultaneous determination of eight lipid peroxidation degradation products in urine of rats treated with carbon tetrachloride using gas chromatography with electron-capture detection. *Journal of Chromatography B*, **694**, 277-287.
- DOGTEROM, P., KROESE, E. D., MULDER, G. J. and NAGELKERKE, J. F., 1989a, Extracellular calcium alleviates cell toxicity due to hepatotoxins that induce lipid peroxidation, but has no effect on toxins that do not cause lipid peroxidation. *Biochemical Pharmacology*, **38**, 4225-4230.
- DOGTEROM, P., MULDER, G. J. and NAGELKERKE, J. F., 1989b, Lipid peroxidation-dependent and -independent protein thiol modifications in isolated rat hepatocytes: differential effects of vitamin E and disulfiram. *Chemico-Biological Interactions*, **71**, 291-306.
- DOGTEROM, P., NAGELKERKE, J. F., VANSTEVENICK, J. and MULDER, G. J., 1988, Inhibition of lipid peroxidation by disulfiram and diethyldithiocarbamate does not prevent hepato-toxin-induced cell death in isolated rat hepatocyte. A study with allyl alcohol, tert-butyl hydroperoxide, diethyl maleate, bromoisovalerylurea and carbon tetrachloride. *Chemico-Biological Interactions*, **66**, 251-265.
- DROY, B. F., DAVIS, M. E. and HINTON, D. E., 1998, Mechanism of allyl formate-induced hepatotoxicity in rainbow trout. *Toxicology and Applied Pharmacology*, **98**, 313-324.
- ELOVAARA, E., KIVISTÖ, H. and VAINIO, H., 1983, Effects of methyl methacrylate on non-protein thiols and drug metabolizing enzymes in rat liver and kidneys. *Archives in Toxicology*, **52**, 109-121.
- FERON, V. J., CASSEE, F. R. and GROTEN, J. P., 1998, Toxicology of chemical mixtures: international perspective. *Environmental Health Perspectives*, **106** (suppl. 6), 1281-1281.
- FREDERICK, C. B., POTTER, D. W., MIDEY, I. C. and ANDERSEN, M. E., 1992, A physiologically based pharmacokinetic and pharmacodynamic model to describe the oral dosing of rats with ethyl acrylate and its implications for risk assessment. *Toxicology and Applied Pharmacology*, **114**, 246-260.
- FREDERICK, C. B., UDINSKY, J. R. and FINCH, L., 1994, The regional hydrolysis of ethyl acrylate to acrylic acid in the rat nasal cavity. *Toxicology Letters*, **70**, 49-56.
- FREIDIG, A. P., VERHAAR, H. J. M. and HERMENS, J. L. M., 1999b, Comparing the potency of chemicals with multiple modes of action in aquatic toxicology: acute toxicity due to narcosis versus reactive toxicity of acrylic compounds. *Environmental Sciences Technology*, **33**, 3038-3043.
- FREIDIG, A. P., VERHAAR, H. J. M. and HERMENS, J. L. M., 1999a, Quantitative structure property relationships for the chemical reactivity of acrylates and methacrylates. *Environmental Toxicology and Chemistry*, **18**, 1133-1139.
- FUJITA, M., SANO, M., TEDEKA, K. and TOMITA, I., 1993, Fluorescence detection of glutathione S conjugate with aldehyde by high-performance liquid chromatography with post-column derivatisation. *Analyst*, **118**, 1289-1292.
- GARCIA-CAÑERO, R., TRILLA, C., PEREZ DE DIEGO, J., DIAZ-GIL, J. J. and COBO, J. M., 1999, $\text{Na}^+:\text{H}^+$ exchange inhibition induces intracellular acidosis and differentially impairs cell growth and viability of human and rat hepatocarcinoma cells. *Toxicology Letters*, **106**, 215-228.
- GHANAYEM, B. I., MARONPOT, R. R. and MATTHEWS, H. B., 1985, Ethyl acrylate-induced gastric toxicity. II. Structure-toxicity relationships and mechanism. *Toxicology and Applied Pharmacology*, **80**, 336-344.
- GHANAYEM, B. I., MARONPOT, R. R. and MATTHEWS, H. B., 1986, Ethyl acrylate-induced gastric toxicity. III. Development and recovery of lesions. *Toxicology and Applied Pharmacology*, **83**, 576-583.
- GREIM, H., AHLERS, J., BIAS, R., BROECKER, B., HOLLANDER, H., GELKE, H. P., JACOBI, S., KLIMISCH, H. J., MANGELSDORF, I., MAYR, W., SCHÖN, N., STROPP, G., STAHNCKER, P., VOGEL, R., WEBER, C., ZIEGLER-SKYLAKAKIS, K. and BAYER, E., 1995, Assessment of structurally related chemicals: toxicity and ecotoxicity of acrylic acid and acrylic acid alkyl esters (acrylates), methacrylic acid and methacrylic acid alkyl esters (methacrylates). *Chemosphere*, **31**, 2637-2659.
- HASHIMOTO, K. and ALDRIDGE, W. N., 1970, Biochemical studies on acrylamide, a neurotoxic agent. *Biochemical Pharmacology*, **19**, 2591-2604.
- HENDRIKS, A. J., MAAS-DIEPEVEEN, J. L., NOORDSIJ, A. and VANDERGRAAG, M. A., 1994, Monitoring response of XAD-concentrated water in the Rhine delta: a major part of the toxic compounds remains unidentified. *Water Research*, **28**, 581-598.

- HORMANN, V. A., MOORE, D. R. and RIKANS, L. E., 1989, Relative contributions of protein sulphydryl loss and lipid peroxidation to allyl alcohol-induced cytotoxicity in isolated rat hepatocytes. *Toxicology and Applied Pharmacology*, **98**, 375-384.
- KÖNEMANN, W. H., 1981b, Fish toxicity tests with mixtures of more than two chemicals: a proposal for a quantitative approach and experimental results. *Toxicology*, **19**, 229-238.
- KÖNEMANN, W. H., 1981a, Quantitative structure-activity relationships in fish toxicity studies. Part 1. Relationship for 50 industrial pollutants. *Toxicology*, **19**, 209-221.
- KRETZSCHMAR, M., 1996, Regulation of hepatic glutathione metabolism and its role in hepatotoxicity. *Experimental and Toxicological Pathology*, **48**, 439-446.
- LOMAX, L. G., KRIVANEK, N. D. and FRAME, S. R., 1997, Chronic inhalation toxicity and oncogenicity of methyl methacrylate in rat and hamsters. *Food Chemistry and Toxicology*, **35**, 393-407.
- MCCARTHY, T. J., HAYES, E. P., SCHWARTZ, C. S. and WITZ, G., 1994, The reactivity of selected acrylate esters toward glutathione and deoxyribonucleosides *in vitro*: structure-activity relationships. *Fundamentals of Applied Toxicology*, **22**, 543-548.
- MCCARTHY, T. J. and WITZ, G., 1997, Structure-activity relationships in the hydrolysis of acrylate and methacrylate esters by carboxylesterase *in vitro*. *Toxicology*, **116**, 153-158.
- MCCARTHY, L. S., MACKAY, D. and SMITH, A. D., 1992, Residue-based interpretation of toxicity and bioconcentration QSARs from aquatic bioassays: neutral narcotic organics. *Environmental Toxicology and Chemistry*, **11**, 917-930.
- MUSCH, A., 1996, Toxicity of mixtures. In R. J. M. Niesink, J. de Vries and M. A. Hollinger (eds), *Toxicology: Principles and Applications* (Boca Raton: CRC Press), pp. 270-287.
- OHNO, Y., ORNSTAD, K., ROSS, D. and ORRENIUS, S., 1985, Mechanism of allyl alcohol toxicity and protective effects of low-molecular-weight thiols studied with isolated rat hepatocytes. *Toxicology and Applied Pharmacology*, **78**, 169-179.
- PENTTILÄ, K. E., 1988, Allyl alcohol cytotoxicity and glutathione depletion in isolated periportal and previous rat hepatocytes. *Chemico-Biological Interactions*, **65**, 107-121.
- PENTTILÄ, K. E., MAKINEN, J. and LINDROS, K. O., 1987, Allyl alcohol liver injury: suppression by ethanol and relation to transient glutathione depletion. *Pharmacology and Toxicology*, **60**, 340-344.
- PLACKETT, R. L. and HEWLETT, P. S., 1952, Quantal response to mixtures of poisons. *Journal of the Royal Statistical Society Series B*, **14**, 141-163.
- POTTER, D. W. and TRAN, T. B., 1992, Rates of ethyl acrylate binding to glutathione and protein. *Toxicology Letters*, **62**, 275-285.
- PRESTERA, T., ZHANG, Y., SPENCER, S. R., WILCZAK, C. A. and TALALAY, P., 1993, The electrophile counterattack response: protection against neoplasia and toxicity. *Advances in Enzyme Regulation*, **33**, 281-296.
- PROCHASKA, H. J. and SANTAMARIA, A. B., 1988, Direct measurement of NAD(P)H:quinone reductase from cells cultured in microtiter wells: a screening assay for anticarcinogenic enzyme inducers. *Annals of Biochemistry*, **169**, 328-336.
- RUSSOM, C. L., DRUMMOND, R. A. and HOFFMAN, A. D., 1988, Acute toxicity and behavioral effects of acrylates and methacrylates to juvenile fathead minnows. *Bulletin of Environmental Contamination and Toxicology*, **41**, 589-596.
- SAFE, S., 1987, Determination of 2,3,7,8-TCDD toxic equivalent factors (TEFs): support for the use of the *in vitro* AHH induction assay. *Chemosphere*, **16**, 791-803.
- SAFE, S., MASON, G., FARELL, K., KEYS, B., J., P.-P., MADGE, J. A. and CHITTIM, B., 1987, Validation of *in vitro* bioassays for the 2,3,7,8-TCDD equivalents. *Chemosphere*, **16**, 1723-1728.
- SEGLEN, P. O., 1976, Preparation of isolated rat liver cells. In D. M. Prescott (ed.), *Methods in Cell Biology* (New York: Academic Press), pp. 29-78.
- SILVER, E. H. and MURPHY, S. D., 1978, Effect of carboxylesterase inhibitors on the acute hepatotoxicity of esters of allyl alcohol. *Toxicology and Applied Pharmacology*, **45**, 377-389.
- SILVER, E. H. and MURPHY, S. D., 1981, Potentiation of acrylate ester toxicity by prior treatment with the carboxylesterase inhibitor triorthotolyl phosphate (TOTP). *Toxicology and Applied Pharmacology*, **57**, 208-219.
- TALALAY, P., 1989, Mechanisms of induction of enzymes that protect against chemical carcinogenesis. *Advances in Enzyme Regulation*, **28**, 237-250.
- TALALAY, P., DELONG, M. J. and PROCHASKA, H. J., 1988, Identification of a common chemical signal regulating the induction of enzymes that protect against chemical carcinogenesis. *Proceedings of the National Academy of Sciences, USA*, **85**, 8261-8265.
- TANI, H. and HASHIMOTO, K., 1982, Structure-toxicity relationship of acrylates and methacrylates. *Toxicology Letters*, **11**, 125-129.
- VERHAAR, H. J. M., MORRONI, J. S., REARDON, K. F., HAYS, S. M., GAVER, D. P., JR., CARPENTER, R. L. and YANG, R. S. H., 1997, A proposed approach to study the toxicology of complex mixtures of petroleum products: the integrated use of QSAR, lumping analysis and PBPK/PD modeling. *Environmental Health Perspectives*, **105** (suppl. 1), 179-195.

- VODICKA, P., GUT, I. and FRANTIŠEK, E., 1990, Effects of inhaled acrylic acid derivatives in rats. *Toxicology*, **65**, 209-221.
- YANG, R. S., THOMAS, R. S., GUSTAFSON, D. L., CAMPAIN, J., BENJAMIN, S. A., VERHAAR, H. J. and MUMTAZ, M. M., 1998, Approaches to developing alternative and predictive toxicology based on PBPK/PD and QSAR modeling. *Environmental Health Perspectives*, **106** (suppl. 6), 1385-1393.

Disposition of naproxen, naproxen acyl glucuronide and its rearrangement isomers in the isolated perfused rat liver

A. LO, R. S. ADDISON*, W. D. HOOPER and R. G. DICKINSON

Centre for Studies in Drug Disposition, The University of Queensland, Clinical Sciences Building, Royal Brisbane Hospital, Qld 4029 Australia

Received 16 November 2000

1. An isolated perfused rat liver (IPRL) preparation was used to investigate separately the disposition of the non-steroidal anti-inflammatory drug (NSAID) naproxen (NAP), its reactive acyl glucuronide metabolite (NAG) and a mixture of NAG rearrangement isomers (isoNAG), each at 30 µg NAP equivalents ml⁻¹ perfusate ($n = 4$ each group).

2. Following administration to the IPRL, NAP was eliminated slowly in a log-linear manner with an apparent elimination half-life ($t_{1/2}$) of 13.4 ± 4.4 h. No metabolites were detected in perfusate, while NAG was the only metabolite present in bile in measurable amounts ($3.9 \pm 0.8\%$ of the dose). Following their administration to the IPRL, both NAG and isoNAG were rapidly hydrolysed ($t_{1/2}$ in perfusate = 57 ± 3 and 75 ± 14 min respectively). NAG also rearranged to isoNAG in the perfusate. Both NAG and isoNAG were excreted intact in bile (24.6 and 14.8% of the NAG and isoNAG doses, respectively).

3. Covalent NAP-protein adducts in the liver increased as the dose changed from NAP to NAG to isoNAG (0.20 to 0.34 to 0.48% of the doses, respectively). Similarly, formation of covalent NAP-protein adducts in perfusate were greater in isoNAG-dosed perfusions. The comparative results suggest that isoNAG is a better substrate for adduct formation with liver proteins than NAG.

Introduction

Conjugation with glucuronic acid is a major metabolic pathway for most drugs containing a carboxylic acid functional group. Acyl glucuronides are intrinsically reactive, able to undergo a number of reactions including hydrolysis, rearrangement and formation of adducts with biological macromolecules (Faed 1984, Dickinson 1993, Fenselau 1994). Hydrolysis, catalysed by hydroxide ion, β -glucuronidases, esterases or serum albumin, leads to the liberation of the parent aglycone. Rearrangement, via hydroxide ion-catalysed acyl migration, yields the 2-, 3- and 4-*O* positional isomers, which are resistant to hydrolysis by β -glucuronidases (but not by hydroxide ion). In contrast to the 1-*O*-acyl glucuronide, the positional isomers can undergo transient ring opening, with the production of a reactive aldehyde intermediate which can react with amino groups on proteins. In this rearrangement/glycation mechanism of drug-protein adduct formation, both the aglycone and glucuronic acid elements of the conjugated xenobiotic are incorporated into the adduct (Smith *et al.* 1986, Ding *et al.* 1993, Williams and Dickinson 1994). If rearrangement does not occur, the 1-*O*-acyl glucuronide can react directly with nucleophilic —SH, —OH and —NH₂

* Author for correspondence: e-mail: R.Addison@medicine.uq.edu.au

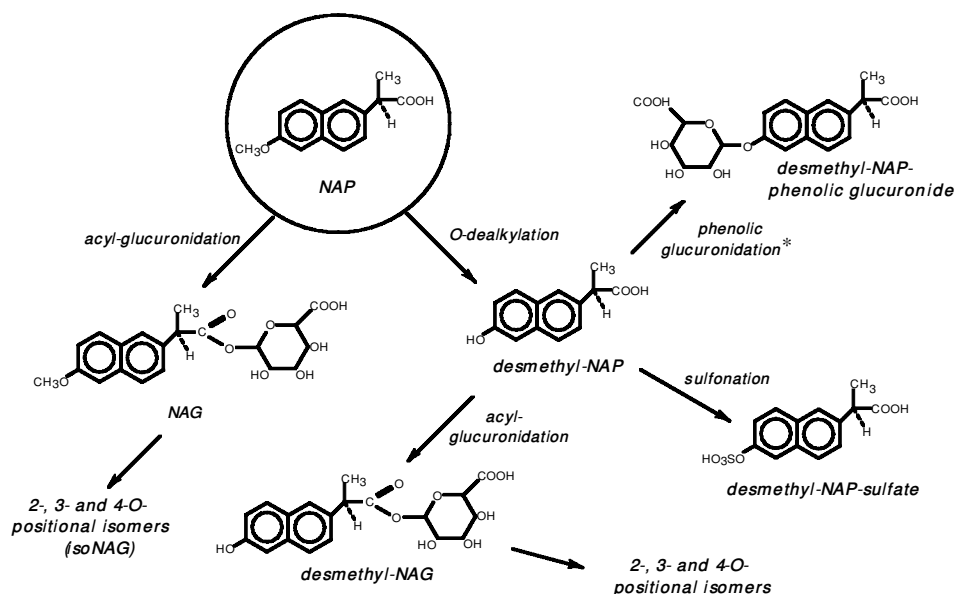


Figure 1. Metabolism of NAP via dealkylation and conjugation. *Not yet demonstrated in human or animal studies.

groups on proteins, resulting in loss of the glucuronic acid moiety (i.e. transacylation mechanism) (van Breeman and Fenselau 1985, Williams and Dickinson 1994, Ding *et al.* 1995). Both mechanisms of adduct formation have been shown to occur for carboxylate drugs, with many examples coming from the non-steroidal anti-inflammatory drugs (NSAIDs).

Naproxen (NAP, 6-methoxy- α -methyl-2-naphthaleneacetic acid) is an NSAID primarily metabolized in humans by acyl glucuronidation to form naproxen acyl glucuronide (NAG) and by *O*-dealkylation to form 6-*O*-desmethyl-naproxen (DMN), which in turn can be further metabolized to glucuronide and sulphate conjugates (Vanggard-Anderson and Hansen 1992, Vree *et al.* 1993, Kiang *et al.* 1989) (figure 1). Limited data are available on irreversible binding of NAG with proteins. Bischer *et al.* (1995) described the covalent binding of the diastereomers of NAG (as the 1-*O*-acyl glucuronide) to human serum albumin, and human and rat plasma proteins *in vitro*, and reported a higher extent of adduct formation with the *R*-enantiomer in all cases. Additionally, Iwaki *et al.* (1998) reported that, *in vitro*, human serum albumin forms adducts with NAG (via the 1-*O*- and 2-*O*-isomers) through both the transacylation and glycation mechanisms, with the latter forming more slowly but showing greater stability than the former. No data are available with respect to the formation of covalent adducts of DMN-glucuronides with proteins.

Drug or xenobiotic molecules that possess both carboxy and phenolic functional groups can be subject to glucuronidation at either or, perhaps, both groups. The latter reactions, little described for drug molecules, would form novel 'di-glucuronide' metabolites. This novel metabolism has been previously described in the rat for the NSAID diflunisal, which forms a 'di-glucuronide mixture' comprised of the phenolic glucuronides of the 2-, 3- and possibly 4-*O*-positional isomers of diflunisal acyl glucuronide (King and Dickinson 1991). However, we

found no evidence to support appreciable phenolic glucuronidation of the 1-*O*-acyl glucuronide. Furthermore, acyl glucuronidation of diflunisal phenolic glucuronide did not occur. Interestingly, the formation of the phenolic glucuronide of DMN has not been described in human or animal studies.

Our interest in NAP was ultimately directed to the possibility of the formation of novel di-glucuronide metabolites of DMN, due to the presence of both carboxy and phenolic groups in this Phase I metabolite of NAP. In order to gain a better overall understanding of the metabolism of NAP in the rat, we have investigated its metabolic disposition following administration to the isolated perfused rat liver (IPRL) in the form of parent drug, its acyl glucuronide conjugate (NAG), and a mixture of acyl migration rearrangement isomers (isoNAG), with specific aims of (1) probing preferential mechanisms of covalent adduct formation and (2) exploring hepatic disposition of DMN.

Materials and methods

Chemicals and reagents

NAP, as the *S*-enantiomer, β -glucuronidase (*Helix pomatia* crude extract, 134800 units ml⁻¹, containing 4000 units sulphatase ml⁻¹), D-glucose and bovine serum albumin (fraction V) were from Sigma Chemical Co. (St Louis, MO, USA). Methanol, acetonitrile (HPLC grade), 1-chlorobutane, ethyl acetate, hexane and diethyl ether (AR grade) were from Mallinckrodt (Melbourne, Australia). Naphthyl acetic acid (NAA) was from BDH chemicals (Sydney, Australia), and isoflurane was supplied by Abbott Australasia Pty Ltd (Sydney, Australia). All other reagents used were of AR grade purity. DMN was prepared by refluxing a solution of NAP in 4 M HCl (1:5 w/v) for 5 h. The solution was cooled over ice water, the precipitate recrystallized in boiling water, and filtered and dried. DMN was formed with a yield of 84% and was shown to be of high purity by HPLC.

IPRL experiments

Animal experiments were approved by the Animal Experimentation Ethics Committee of the University of Queensland. Male Sprague-Dawley rats (300-350 g) were obtained from the Herston Medical Research Centre (Brisbane, Australia).

The IPRL experiments were conducted in recirculating mode as described by Wang and Dickinson (1998). After an initial equilibration of 40 min, perfused livers meeting viability criteria (bile flow $\geq 5 \mu\text{l min}^{-1}$; portal vein pressure ≤ 40 mmHg; perfusate pH maintained at 7.4 ± 0.1 ; adequate oxygen consumption as indicated by the difference in colour between perfusate entering (bright red) and leaving (dark brownish-red) the liver; consistency of colour of perfused tissue) were dosed with either NAP, NAG or isoNAG (3 mg NAP equivalents/100 ml perfusate), administered as a bolus dose to the reservoir. Serial perfusate samples (500 μl) were collected predose and at 5, 10, 20, 30, 60, 90, 120, 150 and 180 min after dosing, and immediately centrifuged. The supernatant was immediately frozen on dry ice and stored at -20°C until analysis. Bile samples were collected predose and half-hourly over the perfusion period. The bile was collected on ice in pretared vessels and the acyl glucuronide was stabilized by titrating the pH to 5.0, using small volumes of 0.1 M phosphoric acid. These samples were also frozen on dry ice and stored at -20°C until analysis. At the completion of perfusion, the remaining perfusate was centrifuged and the supernatant frozen. The liver was rinsed with saline, dried, weighed, homogenized with 2 vols (w/v) 0.1 M phosphate buffer, pH 4.5, and frozen at -20°C until analysis.

Biosynthesis of glucuronides

NAG was isolated from the bile of male Sprague-Dawley rats dosed with NAP, as described for diflunisal (King and Dickinson 1991). Following recovery from surgery, the rats were injected via the jugular vein with three doses of NAP (50 mg kg⁻¹ in 0.1 M NaHCO₃) at 1-h intervals. Bile was collected in tared vessels over ice and was titrated to pH 5.0 with 1 M phosphoric acid to stabilize the NAG. The bile samples were adjusted to pH 1.0-1.5 with 2 M HCl, and extracted three times with 3 vols 1-chlorobutane to remove any NAP present. The NAG was then extracted three times with 3 vols ethyl acetate, the extracts combined and evaporated at 37°C under a gentle stream of air. NAG was obtained as a viscous green syrup and was reconstituted in water (10 ml). The pH was adjusted to 5.0 with small additions of 0.1 M NaOH. The purity of the bile extract was checked by HPLC and found to contain only small amounts (<5%) of NAP and isoNAG.

IsoNAG was prepared as an unspecified mixture of acyl migration isomers by rearrangement of NAG under mild alkaline conditions. Briefly, the purified NAG solution was adjusted to pH 8.5 with 2 M NaOH and incubated at 37°C for 5 h. Under these conditions, acyl migration is the dominant reaction, although some hydrolysis of the NAG and isoNAG being generated can also occur. The reaction was monitored by taking serial hourly samples for analysis by HPLC. After 5 h, β -glucuronidase was added to the solution of isoNAG, to a final concentration of 1000 units ml⁻¹, and incubated overnight at 37°C to hydrolyse any residual NAG. The final solution of isoNAG was readjusted to pH 1.0–1.5 with 2 M HCl and any residual NAP removed by extraction three times with 3 vols 1-chlorobutane. The isoNAG solution was then extracted three times with 3 vols ethyl acetate, the extracts combined and evaporated at 37°C under a gentle stream of air. IsoNAG was obtained as a thick green syrup and was reconstituted in water (10 ml), and the pH adjusted to 5.0 with small additions of 0.1 M NaOH. The purity of the isoNAG solution was checked by HPLC and found to be >96%.

HPLC analyses

The analytical system consisted of a model 510 HPLC pump, a model 481 UV detector and a WISP 712 autosampler (Waters Associates, Milford, MA, USA). The analytical column was a 4- μ m Novapak C₁₈ cartridge column (100 $\times$ 8 mm i.d.) housed in an RCM 8 $\times$ 10 compression unit, and was preceded by a μ Bondapak C₁₈ Corasil guard column (Waters). The mobile phase consisted of water (1000 ml), acetonitrile (670 ml) and glacial acetic acid (2 ml), and the flow rate was 2 ml min⁻¹. Using these conditions, NAP eluted at 13.3 min, NAG at 2.8 min (as a single peak well resolved from positional isomers), the 4-*O*-positional isomer of NAG as a single peak at 2.0 min and the 2-*O*- and 3-*O*-positional isomers as non-baseline-resolved doublets with peaks between 3.3 and 3.8 min. UV detection at 225 nm was used in all cases. All quantitative analyses were performed in conjunction with standard curves constructed from blank samples of an appropriate matrix, spiked with increasing concentrations of NAP. Standard curves had a coefficient of determination of ≥ 0.99 in all cases.

Analysis of non-covalently bound drug and metabolites

Perfusate samples obtained from the NAP-, NAG- and isoNAG-dosed IPRL experiments were analysed for NAP and its metabolites as follows. Aliquots of perfusate samples (100 μ l) were added to 150 μ l 20 μ g ml⁻¹ NAA in acetonitrile containing 4% (v/v) acetic acid. After vortexing and centrifugation, 20 μ l samples were injected into the HPLC system. For analysis of the bile samples, aliquots (20 μ l) were diluted with 150 μ l 200 μ g ml⁻¹ NAA in acetonitrile containing 4% (v/v) acetic acid and 230 μ l 0.1 M phosphate buffer, pH 4.5. Aliquots (10 μ l) of the prepared bile samples were injected into the HPLC system.

Analysis of non-covalently bound drug species in tissue was conducted as follows. Liver homogenate (1 g) was added to 4 vols acetonitrile containing 4% acetic acid (v/v), and the samples vortexed and centrifuged (3000g). The supernatant was removed and evaporated at 37°C under a gentle stream of air. To the residues, 1 ml NaOH (1 M) was added and the samples were then incubated at 80°C to allow hydrolysis of glucuronides. Internal standard (50 μ l 20 μ g ml⁻¹ NAA in water) was then added and the samples extracted twice with 4 ml hexane/diethyl ether (1:1 v/v). After gentle mixing and centrifugation, the organic layer was removed and evaporated under a gentle stream of air at 37°C. The residue was reconstituted in 100 μ l mobile phase, and 30 μ l was injected into the HPLC system.

Analysis of covalently bound drug metabolites

NAP-protein adducts were measured in final perfusate and liver homogenate samples as follows. Samples of perfusate (1 ml) or liver homogenate (1 g) were added to 4 vols acetonitrile containing 4% (v/v) acetic acid to precipitate proteins. After mixing (30 s) and centrifugation (3000g), the supernatant was discarded, and the pellet resuspended and washed with methanol/diethyl ether (3:1 v/v, 4 ml). This washing procedure was repeated seven times to remove non-covalently bound drug and metabolites from the protein pellets. After vortexing and centrifugation, the last wash was analysed to confirm that the protein pellet was free of non-covalently bound drug. The pellet was dried under a gentle stream of air and digested overnight in 1 ml 1 M NaOH at 80°C. The digest was acidified with 200 μ l 10 M HCl, and 50 μ l 20 μ g ml⁻¹ NAA in water was added. The sample was then extracted twice with 4 ml hexane/diethyl ether (1:1 v/v), and the combined organic layers dried down under a gentle stream of air at 37°C. The residue was reconstituted in 100 μ l mobile phase, and 30 μ l was injected into the HPLC system.

Data analysis

The apparent perfusate half-lives of NAP, NAG and isoNAG were determined from the slope of the log concentration-time profile using linear regression analysis. Statistical comparisons between perfusion experiments were performed using unpaired Student's *t*-tests.

Results

The concentration-time profile of NAP following its administration to the isolated perfused rat liver preparation is shown in figure 2. NAP concentrations in serial perfusate samples showed a slow linear decrease over the perfusion period, with an elimination half-life ($t_{1/2}$), calculated between 30 and 180 min of perfusion, of 13.4 ± 4.4 h. At 3 h, the recovery of unchanged NAP from perfusate was $75.8 \pm 12.0\%$ of the dose (table 1). No metabolites of NAP were measurable in serial or 3-h perfusate samples. Analysis of bile samples revealed NAG to be the only metabolite present in measurable amounts ($3.9 \pm 0.8\%$ of the dose), with the majority excreted between 30 and 60 min after dosing. Covalent NAP-protein adducts were found in the liver, accounting for $0.20 \pm 0.06\%$ of the dose, while liver homogenate contained $1.5 \pm 0.3\%$ of the dose as non-bound NAP derived species. No NAP-protein adducts were found in the perfusate above the detection limit of the assay.

Rapid hydrolysis of NAG occurred after its administration to perfused livers (figure 2), with a $t_{1/2}$, calculated over the essentially linear 5-30 min period, of 11.3 ± 1.4 min. Its elimination thereafter (60-180 min) was slower, although essentially linear, with a $t_{1/2} = 57.2 \pm 2.7$ min. NAP appeared rapidly in perfusate during the first hour of perfusion, followed by a more gradual rise from 60 to 180 min. NAG also rearranged rapidly to form isoNAG, with the concentration in perfusate at 5 min after dosing being the same as that of NAP. However, isoNAG was also rapidly eliminated under the perfusion conditions with a $t_{1/2} = 23.6 \pm 5.1$ min, and fell below quantifiable limits 60 min after dosing. At the end of perfusion, only 1.6% of administered NAG was recovered in perfusate,

Table 1. Recovery of NAP-derived species after administration of NAP, NAG and isoNAG at 3 mg NAP equivalents to the isolated perfused rat liver preparation.

	% Recovery of dose ^a					
	NAP	NAG	isoNAG	NAP-protein adducts	Non-bound NAP-derived species	Total
NAP dosed						
Perfusate	75.8 ± 12.0	<	<	-	-	75.8 ± 12.0
Bile	< ^b	3.9 ± 0.8	<	<	-	3.92 ± 0.80
Liver	- ^c	-	-	0.20 ± 0.06	1.52 ± 0.27	1.71 ± 0.33
Total	75.8 ± 12.0	3.9 ± 0.8		0.20 ± 0.06	1.52 ± 0.27	81.4 ± 12.1
NAG dosed						
Perfusate	60.9 ± 8.3	1.6 ± 0.6	<	0.65 ± 0.10	<	63.4 ± 9.0
Bile	<	24.6 ± 7.3^d	3.0 ± 0.86	-	-	27.8 ± 8.2^d
Liver	-	-	-	0.34 ± 0.03^d	2.33 ± 0.86	2.7 ± 0.8^d
Total	60.9 ± 8.3	25.8 ± 6.5^d	3.0 ± 0.86	0.99 ± 0.12^d	2.33 ± 0.86	92.9 ± 2.2
IsoNAG dosed						
Perfusate	55.8 ± 5.0^d	<	5.8 ± 1.7	1.53 ± 0.14^e	-	63.1 ± 6.7
Bile	<	<	14.8 ± 3.4^e	-	-	14.8 ± 3.4^d
Liver	-	-	-	0.48 ± 0.12^d	$7.65 \pm 1.91^{d,e}$	$8.1 \pm 2.0^{d,e}$
Total	55.8 ± 5.0^d		20.6 ± 1.8^e	$2.02 \pm 0.25^{d,e}$	$7.65 \pm 1.91^{d,e}$	86.1 ± 5.2

^a Values are means $\pm$ SD ($n = 4$).

^b Below the limit of the assay.

^c No measurement made.

^d Significantly different ($p < 0.05$) from the corresponding value from NAP-dosed perfusions.

^e Significantly different ($p < 0.05$) from the corresponding value from NAG-dosed perfusions.

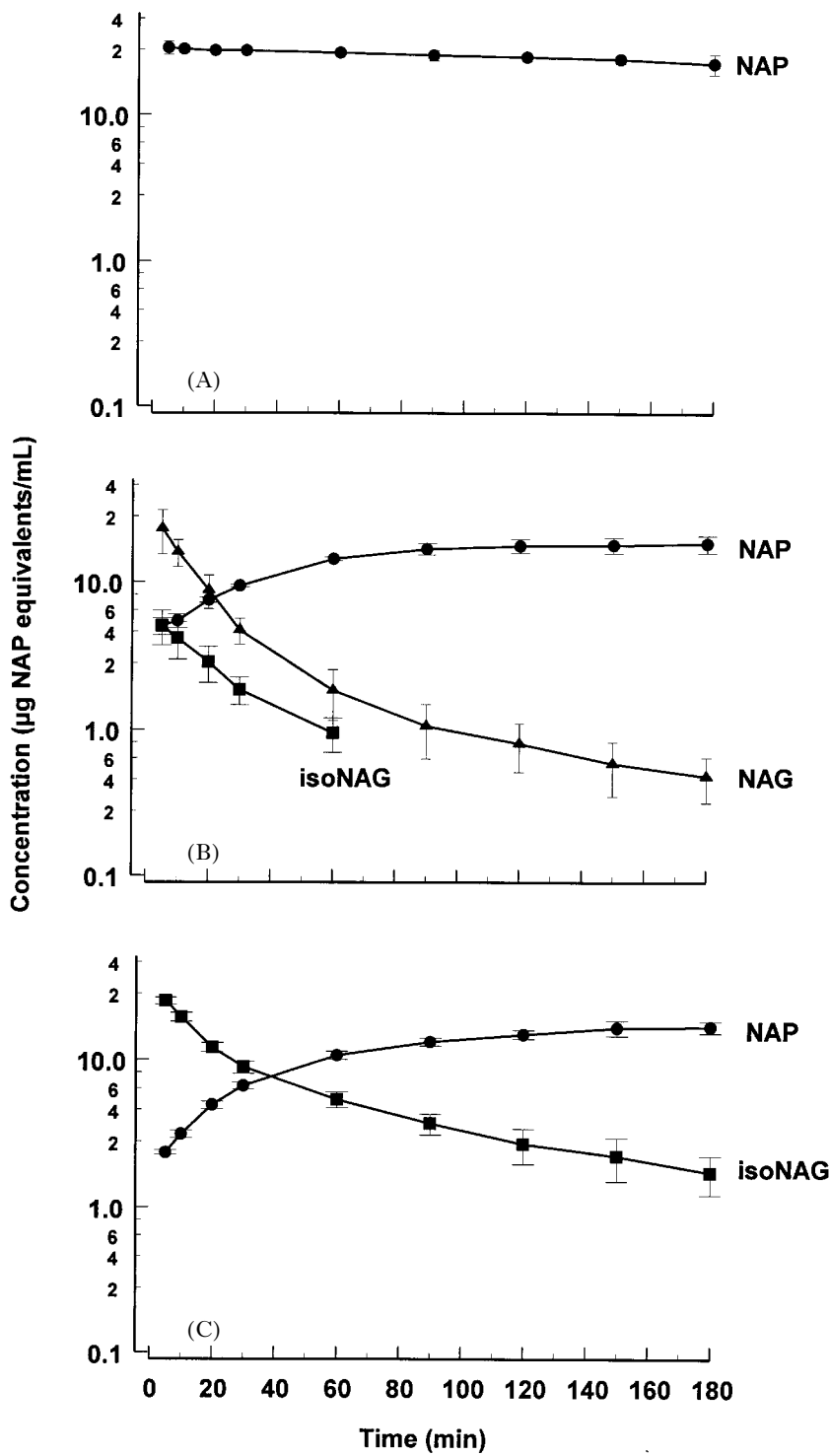


Figure 2. Concentration-time profiles of NAP, NAG and isoNAG after dosing the recirculating isolated perfused rat liver preparation with (A) NAP, (B) NAG and (C) isoNAG at 3 mg NAP equivalents. Results are mean $\pm$ SD ($n = 4$).

while NAP in perfusate accounted for $60.9 \pm 8.3\%$ of the dose (table 1). NAG was rapidly excreted in bile, with $24.6 \pm 7.3\%$ of the dose being recovered, significantly higher than the recovery observed for this species in NAP-dosed IPRL. The majority of this excretion occurred in the first 90 min after dosing. Covalent NAP-protein adducts were higher in the liver of NAG-dosed IPRL compared with NAP-dosed preparations, accounting for $0.34 \pm 0.03\%$ of the dose, while recovery of non-bound drug was also found in higher levels ($2.3 \pm 0.9\%$ of the dose). NAP-protein adducts were also detected in low levels in the perfusate at the end of the perfusion period (0.65% of the dose).

Following its administration to IPRL preparations, isoNAG decreased in serial perfusates (figure 2) with an initial (0-30 min) $t_{1/2} = 16.7 \pm 1.0$ min, followed by a slower elimination phase (60-180 min) with $t_{1/2} = 75.0 \pm 14.5$ min. Hydrolysis of the isoNAG dose to the parent drug was evident, with the NAP concentration in final perfusate being similar to that obtained in NAG-dosed perfusions. Recoveries of isoNAG and NAP from perfusate were $5.8 \pm 1.7\%$ and $55.8 \pm 5.0\%$ of the dose respectively (table 1). Serial bile samples predominantly contained isoNAG ($14.8 \pm 3.4\%$ of the dose), with no other metabolites present above the detection limit of the assay.

The formation of NAP-protein adducts in liver was significantly higher ($p < 0.05$) in both NAG-dosed (recovery = $0.34 \pm 0.03\%$ of the dose) and isoNAG-dosed ($0.48 \pm 0.12\%$ of the dose) IPRL than that observed in those dosed with the parent drug (table 1). Non-bound NAP-derived species were higher ($p < 0.05$) in isoNAG dosed IPRL ($7.7 \pm 1.9\%$ of the dose) than in NAP- and NAG-dosed IPRL experiments. The perfusate at the end of the 3-h isoNAG-dosed preparations also showed the highest concentration of covalent NAP-protein adducts, with a mean recovery of $1.53 \pm 0.14\%$ of the dose.

Discussion

The carboxylate NSAID NAP is metabolized in humans and animals to its acyl glucuronide conjugate NAG (Vree *et al.* 1993). Also documented is demethylation to DMN, which bears both carboxy and phenolic functions, and which can therefore be conjugated variously by acyl glucuronidation and sulphonation (Kiang *et al.* 1989, Vanggard-Anderson and Hansen 1992, Vree *et al.* 1993), and perhaps even by novel diconjugation (as shown earlier for the carboxy/phenolic NSAID diflunisal; King and Dickinson 1991). One of our longer term goals was to explore hypotheses regarding diconjugation mechanisms. The present study was concerned not only with establishing the detailed *in vivo* disposition of NAP (as a prelude to establishing that of DMN), but also to probe intrahepatic transport of and protein adduct formation from NAG and, more specifically, its acyl migration rearrangement isomers (i.e. isoNAG). The role of NAG and isoNAG as reactive substrates for covalent modification of plasma protein *in vitro* has already been established (Iwaki *et al.* 1998). However, *in vivo* studies are lacking.

The present study administered NAP, its preformed acyl glucuronide (NAG), and a prepared mixture of NAG rearrangement isomers (isoNAG), to the IPRL. After administration of NAP, its elimination from perfusate was very slow (apparent $t_{1/2} = \sim 13$ h, almost three times slower than that observed in the rat *in vivo*; Runkel *et al.* 1972). NAG in bile was the only metabolite quantifiable, and accounted for $\sim 4\%$ of the dose. By comparison, when preformed NAG was

administered at the same dose, systemic hydrolysis to parent NAP was the major, and systemic rearrangement to isoNAG the minor, disposition pathway observed in perfusate (figure 2). The perfusate profiles of the isoNAG generated *in situ* appeared to be biphasic and essentially parallel to those of its precursor NAG. Following dosing of preformed isoNAG itself to perfused livers, hydrolysis to NAP was the dominant pathway discerned from perfusate analyses. As is known generally, an acyl glucuronide is not reformed during rearrangement amongst its isomers (figure 1) (Spahn-Langguth and Benet 1992, Fenselau 1994). Therefore, after dosing to perfusions, NAG will not be reformed spontaneously from isoNAG by rearrangement; any 'new' NAG must originate from glucuronidation of NAP released by hydrolysis of isoNAG. However, this must be minimal given the results obtained after administration of NAP itself to perfused livers. In this context it is noteworthy that <2 and 6% of the administered NAG or isoNAG doses respectively were recovered as such in perfusate at 3 h, in contrast to the recovery of NAP at 3 h (table 1).

The perfusate profiles obtained were not dissimilar to those reported earlier following dosage of the salicylate NSAID diflunisal, its acyl glucuronide, and rearrangement isomers to the IPRL (Wang and Dickinson 1998). Thus, while hydroxide ion-catalysed rearrangement seems to be the predominant mechanism of degradation of acyl glucuronides in physiological buffers *in vitro*, hydrolysis seems to predominate *in vivo*, presumably due to the combined effects of hydroxide ion, esterases and β -glucuronidases (Smith *et al.* 1990, King and Dickinson 1991, Williams and Dickinson 1994). Interestingly, the 'terminal' elimination half-life of NAG in the IPRL system, where the perfusate contains 1% BSA, was 57.2 ± 2.7 min, slightly longer than those reported by Bischer *et al.* (1995) who found that NAG degraded with half-lives of 47 and 27 min in phosphate buffer containing 0.3 and 3.0% human serum albumin respectively. The β -glucuronidase-resistant rearrangement isomers, isoNAG, were, like NAG, eliminated from perfusate in a facile manner, though less rapidly than NAG. This was not unexpected, as acyl glucuronide isomers have previously been shown to be more stable in the IPRL (Andrews and Dickinson 1998, Wang and Dickinson 1998) and in the rat *in vivo* (King and Dickinson 1991).

Perfused livers dosed with NAP yielded only ~4% of the dose as the acyl glucuronide NAG in bile in 3 h (table 1), indicating the relatively poor capacity of the IPRL to metabolize NAP. However, after dosing with preformed NAG, ~25% of the dose was recovered (as NAG) in bile in 3 h, the great majority (>80%) of this being recovered in the first 1.5 h of perfusion. For perfused livers dosed with isoNAG, ~15% of the dose was recovered (as isoNAG) in bile in 3 h, with the major part again being in the first 1.5 h. The only explanation for these results is that considerable portions of intact NAG and isoNAG in perfusate are taken up by hepatocytes at the sinusoidal membrane, transported intrahepatically to the canalicular membrane, and excreted into bile. Such a transport phenomenon will be involved in the recovery of 3% of the NAG dose as isoNAG in bile (most likely subsequent to rearrangement of NAG in perfusate, though rearrangement of NAG subsequent to its biliary excretion will also play some role). A small portion of the NAG dose recovered in bile as NAG will be produced by intrahepatic glucuronidation and biliary excretion of NAP, generated by hydrolysis of NAG in perfusate.

Small choleretic effects were observed (data not shown) in this study during the first 1.5 h following dosing with NAG and isoNAG, but not after dosing with NAP. This is consistent with the relative recovery data for NAG or isoNAG in bile after the various doses, as choleretic effects are known to accompany biliary excretion of the acyl glucuronides of valproate (Dickinson *et al.* 1982, Andrews and Dickinson 1998) and diflunisal (Wang and Dickinson 1998). The observed choleretic effects coincided with the period of major excretion of NAG and isoNAG in bile (i.e. in the first 1.5 h after dosing).

The perfusate AUC_{0-3h} for NAP, NAG and isoNAG following their administration to the IPRL were 4370 ± 436 , 480 ± 130 and $953 \pm 113 \mu\text{g min ml}^{-1}$, respectively. Covalent NAP-protein adducts in perfusate (notionally with BSA), measured at 3 h, comprised 0, 0.65 and 1.53% of the doses, respectively. If exposure of perfusate to NAP-derived species is measured by AUC_{0-3h} , then NAP itself cannot be a candidate substrate for covalent adduct formation, though clearly both NAG and isoNAG can act in this capacity. For comparison here, it is instructive to remember that NAG can spontaneously form isoNAG, though the reverse does not happen. Thus, an exposure of perfusate proteins to $953 \pm 113 \mu\text{g min ml}^{-1}$ yielded $1.53 \pm 0.14\%$ of the isoNAG dose as covalent adducts (i.e. a 'conversion rate' of $1.605 \times 10^{-3} \text{ min}^{-1}$). After dosage with NAG, perfusate proteins were exposed to spontaneously generated isoNAG ($AUC_{0-3h} = 123 \pm 28 \mu\text{g min ml}^{-1}$). If the same adduct yield determined above applied, this exposure would account for 0.20% of the 0.65% yield of adducts following dosage with NAG. On this basis, it could be suggested that isoNAG is a slightly better substrate (via the glycation mechanism) than NAG (presumably *via* the transacylation mechanism, 'conversion rate' = $9.409 \times 10^{-4} \text{ min}^{-1}$) for covalent NAP-protein adduct formation in perfusate of the IPRL.

Covalent NAP-protein adducts were also found in liver, with the extent of formation increasing as the dose changed from NAP to NAG to isoNAG (table 1). This trend is consistent with our earlier observations for diflunisal, its acyl glucuronide and rearrangement isomers following dosing to the IPRL (Wang and Dickinson 1998). It is difficult to estimate the intrahepatic exposure to NAG and/or isoNAG after each of the three dose types. However, some comparative measure might be taken from the biliary excretion data. Thus, the values for the sum of NAG and isoNAG excreted in bile after dosing with NAP, NAG and isoNAG were 3.9 ± 0.8 , 27.6 ± 8.2 and $14.8 \pm 3.4\%$ of the doses, respectively (i.e. ratio 1:7:4). This corresponds with a ratio of 1:1.7:2.4 for the respective recoveries of the doses as adducts in liver. This is a somewhat coarse analysis, but it may serve to illustrate the point that isoNAG appears to be a somewhat better substrate for adduct formation in liver, in agreement with the observations discussed above for adduct formation in perfusate.

The relative levels of non-covalently bound NAP-derived species in liver tissue at the end of the 3-h perfusion period (table 1) were quite intriguing. Thus, 1.5, 2.3 and 7.7% of the doses of NAP, NAG and isoNAG respectively were recovered in liver as non-covalently bound species (measured as NAP after alkaline hydrolysis of liver extracts). It is presumed that the liver at 3 h would be in equilibrium with the drug species circulating in perfusate, and it would further be expected that the more lipophilic species (in this case NAP, as compared with NAG and isoNAG) would achieve relatively higher equilibrium concentrations. However, the concentrations of NAP in perfusate at 3 h were 22.7, 18.3 and $16.7 \mu\text{g ml}^{-1}$ following

dosing with NAP, NAG and isoNAG respectively, i.e. the opposite trend to that found for non-covalently bound NAP-derived species in liver. There are, of course, residual concentrations of NAG and isoNAG at 3 h after dosing with these species (0.5 and 1.7 μg NAP equivalent ml^{-1} respectively), though these concentrations are considerably lower than the concurrent concentrations of NAP in these perfusions. The data appear to point to an unexplained avid but non-covalent association of isoNAG with liver tissue.

Conclusion

This study has shown that NAP is only very slowly glucuronidated in the IPRL. Its acyl glucuronide, NAG, undergoes both hydrolysis and rearrangement, is taken up intact by the liver and excreted in bile, and serves as a substrate for covalent adduct formation with both perfusate and liver proteins. The rearrangement isomers, isoNAG, also undergo hydrolysis, uptake and biliary excretion, and seem to be even better substrates for adduct formation.

References

- ANDREWS, J. A. and DICKINSON, R. G., 1998, Metabolic discrimination between valproic acid glucuronide and its rearrangement isomers in the perfused rat liver. *Proceedings of the Australasian Society of Clinical and Experimental Pharmacologists and Toxicologists*, **5**, P2-38.
- BISCHER, A., ZIA-AMIRHOSSEINI, P., IWAKI, M., McDONAGH, A. F. and BENET, L. Z., 1995, Stereoselective binding properties of naproxen glucuronide diastereomers to proteins. *Journal of Pharmacokinetics and Biopharmaceutics*, **23**, 379-395.
- DICKINSON, R. G., 1993, Acyl glucuronide conjugates: reactive metabolites of nonsteroidal anti-inflammatory drugs. *Proceedings of the Western Pharmacological Society*, **36**, 157-162.
- DICKINSON, R. G., HARLAND, R. C., KAUFMAN, S. N., LYNN, R. K. and GERBER, N., 1982, An osmotic explanation for valproic acid induced choleresis in the rat. *Arzneimittelforschung*, **32**, 241-247.
- DING, A., OJINGWA, J. C., McDONAGH, A. F., BURLINGAME, A. L. and BENET, L. Z., 1993, Evidence of covalent binding of acyl glucuronides to serum albumin via an imine mechanism as revealed by tandem mass spectrometry. *Proceedings of the National Academy of Sciences, USA*, **90**, 3797-3801.
- DING, A., ZIA-AMIRHOSSEINI, P., McDONAGH, A. F., BURLINGAME, A. L. and BENET, L. Z., 1995, Reactivity of tolmetin glucuronide with human serum albumin: Identification of binding sites and mechanisms of reaction by tandem mass spectrometry. *Drug Metabolism and Disposition*, **23**, 369-376.
- FAED, E. M., 1984, Properties of acyl glucuronides: Implications for studies of the pharmacokinetics and metabolism of acidic drugs. *Drug Metabolism and Disposition*, **15**, 1213-1249.
- FENSELAU, C., 1994, Acyl glucuronides as chemically reactive intermediates. *Handbook of Experimental Pharmacology*, **112**, 367-389.
- IWAKI, M., OGISO, T., INAGAWA, S. and KAKEHI, K., 1998, *In vitro* regioselective stability of β -1-O- and 2-O-acyl glucuronides of naproxen and their covalent binding to human serum albumin. *Journal of Pharmaceutical Sciences*, **88**, 52-57.
- KIANG, C.-H., LEE, C. and KUSHINSKY, S., 1989, Isolation and identification of 6-desmethyl-naproxen sulfate as a new metabolite of naproxen in human plasma. *Drug Metabolism and Disposition*, **17**, 43-48.
- KING, A. R. and DICKINSON, R. G., 1991, Studies on the reactivity of acyl glucuronides: I. Phenolic glucuronidation of isomers of diflunisal acyl glucuronide in the rat. *Biochemical Pharmacology*, **42**, 2289-2299.
- RUNKEL, R., CHAPLIN, M., BOOST, G., SEGRE, E. and FORCHIELLI, E., 1972, Absorption, distribution, metabolism and excretion of naproxen in various laboratory animals and human subjects. *Journal of Pharmaceutical Sciences*, **61**, 703-708.
- SMITH, P. C., McDONAGH, A. F. and BENET, L. Z., 1986, Irreversible binding of zomepirac to plasma protein *in vitro* and *in vivo*. *Journal of Clinical Investigation*, **77**, 934-939.
- SMITH, P. C., McDONAGH, A. F. and BENET, L. Z., 1990, Effect of esterase inhibition on the disposition of zomepirac glucuronide and its covalent binding to plasma proteins in the guinea pig. *Journal of Pharmacology and Experimental Therapeutics*, **252**, 218-224.

- SPAHN-LANGGUTH, H. and BENET, L. Z., 1992, Acyl glucuronides revisited: is the glucuronidation process a toxification as well as a detoxification mechanism? *Drug Metabolism Reviews*, **24**, 5-47.
- VAN BREEMAN, R. B. and FENSELAU, C., 1985, Acylation of albumin by 1-*O*-acyl glucuronides. *Drug Metabolism and Disposition*, **13**, 318-320.
- VANGGARD-ANDERSON, J. and HANSEN, S. H., 1992, Simultaneous quantitative determination of naproxen, metabolite 6-*O*-desmethylnaproxen and their five conjugates in plasma and urine samples by high performance liquid chromatography on dynamically modified silica. *Journal of Chromatography*, **577**, 325-333.
- VREE, T. B., VAN DEN BIGGELAAR-MARTEA, M., VERWEY-VAN WISSEN, C. P. W. G. M., VREE, M. L. and GUELEN, P. J. M., 1993, The pharmacokinetics of naproxen, its metabolite *O*-desmethylnaproxen, and their acyl glucuronides in humans. Effect of cimetidine. *British Journal of Clinical Pharmacology*, **35**, 467-472.
- WANG, M. and DICKINSON, R. G., 1998, Disposition and covalent binding of diflunisal and diflunisal acyl glucuronide in the isolated perfused rat liver. *Drug Metabolism and Disposition*, **26**, 98-104.
- WILLIAMS, A. M. and DICKINSON, R. G., 1994, Studies on the reactivity of acyl glucuronides - IV. Modulation of reversible and covalent interaction of diflunisal acyl glucuronide and its isomers with human plasma protein *in vitro*. *Biochemical Pharmacology*, **47**, 457-467.

Carbamazepine: a 'blind' assessment of CYP-associated metabolism and interactions in human liver-derived *in vitro* systems

O. PELKONEN^{†*}, P. MYLLYNEN[†], P. TAAVITSAINEN[†],
A. R. BOOBIS[‡], P. WATTS[‡], B. G. LAKE[§], R. J. PRICE[§],
A. B. RENWICK[§], M.-J. GÓMEZ-LECHÓN[¶], J. V. CASTELL[¶],
M. INGELMAN-SUNDBERG^{||}, M. HIDESTRAND^{||},
A. GUILLOUZO^{††}, L. CORCOS^{††}, P. S. GOLDFARB^{‡‡} and
D. F. V. LEWIS^{‡‡}

[†] University of Oulu, Department of Pharmacology and Toxicology, PO Box 5000, FIN-90014 Oulu, Finland

[‡] Imperial College School of Medicine, Department of Medicine, Section of Clinical Pharmacology, London, UK

[§] TNO BIBRA International Ltd, Carshalton, UK

[¶] Unidad Hepatología Experimental, Centro de Investigación, Hospital Universitario La Fe. Valencia, Spain

^{||} Karolinska Institutet, Institute of Environmental Medicine, Division of Molecular Toxicology, Stockholm, Sweden

^{††} University of Rennes, Faculty of Pharmacy, INSERM U456, Rennes, France

^{‡‡} University of Surrey School of Biological Sciences, Guildford, UK

Received 11 December 2000

1. The ability of various *in vitro* systems for CYP enzymes (computer modelling, human liver microsomes, precision-cut liver slices, hepatocytes in culture, recombinant enzymes) to predict various aspects of *in vivo* metabolism and kinetics of carbamazepine (CBZ) was investigated.

2. The study was part of the EUROCYP project that aimed to evaluate relevant human *in vitro* systems to study drug metabolism.

3. CBZ was given to the participating laboratories without disclosing its chemical nature.

4. The most important enzyme (CYP3A4) and metabolic route (10,11-epoxidation) were predicted by all the systems studied.

5. Minor enzymes and routes were predicted to a different extent by various systems.

6. Prediction of a clearance class, i.e. slow clearance, was correctly predicted by microsomes, slices, hepatocytes and recombinant enzymes (CYP3A4).

7. The 10,11-epoxidation of CBZ by the recombinant CYP3A4 was enhanced by the addition of exogenous cytochrome-b₅, leading to a considerable over-prediction.

8. Induction potency of CBZ was predicted in cultured hepatocytes in which 7-ethoxycoumarin O-deethylase was used as an index activity.

9. It seems that for a principally CYP-metabolized substance such as CBZ, all liver-derived systems provide useful information for prediction of metabolic routes, rates and interactions.

* Author for correspondence; e-mail: olavi.pelkonen@oulu.fi

Introduction

Unsatisfactory pharmacokinetic properties have been pointed out as a major reason for the failure of new chemical entities (NCEs) in drug development in man (Prentis *et al.* 1988, Kennedy 1997). Prediction of human pharmacokinetic properties of NCEs at an early phase of drug discovery and development is therefore of outmost importance for the success rate later on. The pharmaceutical industry has therefore directed great efforts to develop fast and reliable *in vitro* methods for this purpose (Rodrigues 1994).

Prediction of hepatic clearance is especially important for drugs eliminated by hepatic metabolism. The enzymes responsible for drug metabolism in humans have been intensively studied during recent years and the knowledge has increased considerably (e.g. Pelkonen *et al.* 1998). It is now possible to determine which cytochrome P450 (CYP) enzyme is involved in the metabolism of a certain drug by using cDNA-expressed enzymes (Rodrigues 1999). The metabolism in liver preparations like microsomes and S9 fractions can reflect the metabolic profile and be the basis for clearance and bioavailability predictions. More complex systems like isolated hepatocytes and tissue slices give a more complete picture of the metabolism. Isolated human hepatocytes have been proposed to be the best model for *in vivo* metabolic clearance predictions (Houston and Carlile 1997).

This study was a part of the EURO CYP project of the EU Biomed 2 program that aimed to evaluate relevant human *in vitro* systems to study drug metabolism and compare the results with the situation *in vivo*. A substance, carbamazepine, was given to participants without disclosing its chemical nature. The *in vitro* assay systems used were cDNA-expressed CYP enzymes, liver microsomes, isolated hepatocytes and precision-cut liver slices. Furthermore, a panel of human hepatocytes and CYP-selective chemical inhibitors was used to elucidate the metabolism of carbamazepine. All assays were run at participating laboratories specialized in the specific *in vitro* systems and the samples were sent to University of Oulu for analysis.

Carbamazepine (CBZ; 5-carbamoyl-5*H*-dibenz-[*b,f*]azepine) is a widely used anti-epileptic drug, which is nowadays the first-line drug of choice in most types of epilepsy. CBZ was selected for this blind assessment of *in vitro* liver-derived systems because there is a lot of knowledge about its kinetic and metabolic characteristics in humans. Furthermore, a reliable assay for measuring the metabolic profile of CBZ was available in the University of Oulu.

CBZ is metabolized to over 30 metabolites both in rat and humans (Lertratanakoon and Horning 1982). In humans it is mainly oxidized to its major metabolite carbamazepine-10,11-epoxide (CBZ-E; figure 1). The epoxide is mainly hydrolyzed to the 10,11-*trans*-dihydrodiol metabolite (10,11-D) prior to excretion in urine (Faigle and Feldman 1995). Several minor metabolites are also formed including metabolites analysed in this study: 9-hydroxymethyl-10-carbamoyl acridan (9-AC), 3-hydroxy-carbamazepine (3-OH-CBZ) and 2-hydroxy-carbamazepine (2-OH-CBZ; figure 1). A summary of *in vivo* kinetics of CBZ is presented in table 1. CBZ is a known inducer of CYP systems (Faigle and Feldman 1995). It induces its own metabolism and its metabolism is also inducible by many other drugs (Levy and Wurden 1995). Clinically significant interactions are due to induction by CBZ (also autoinduction, Levy and Wurden 1995).

In order to perform predictions of *in vivo* characteristics of CBZ in a 'blind' fashion, specific goals of the study were: (1) determination of enzymes responsible

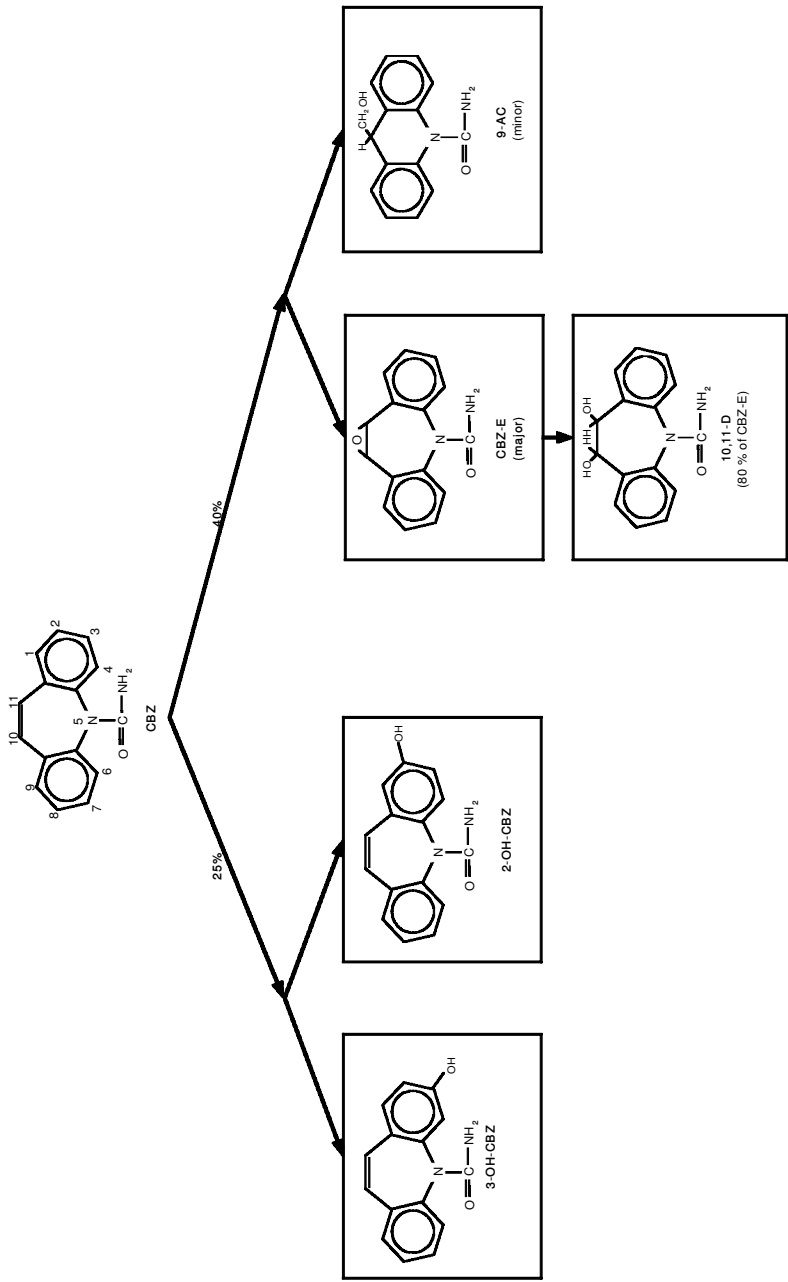


Figure 1. Principal metabolic pathways of CBZ in humans. Carbamazepine is metabolized to over 30 metabolites (Lertranagoon and Horning 1982) and only the metabolites detected in this study are presented. In humans, metabolism of CBZ proceeds down four major pathways: oxidation of 10,11 double bond (40%), hydroxylation of the six-membered aromatic rings (25%), direct *N*-glucuronidation (15%) and substitution of the six-membered rings with sulphur-containing groups (5%; Faigle and Feldman 1995). 10-OH-CBZ represents < 0.1% of CBZ dose and has only been detected in the haemofiltrate of one uraemic patient *in vivo* (Beyer and Spittler 1992) and hence it is not presented. About 15% of the dose is directly *N*-glucuronidated (Faigle and Feldman 1995). About 1% is excreted unchanged in urine (e.g. Morselli 1995).

Table 1. Summary of the *in vivo* kinetics of carbamazepine (Tomson 1983, Dollery 1991, Morselli 1995).

Oral bioavailability	75-85%
Clearance	25-35 ml min ⁻¹
Half-life of CBZ	18-55 h
Half-life of CBZ (after induction)	5-26 h
Half-life of CBZ-E	6 h
Volume of distribution	0.8-2.0 Lkg ⁻¹

for metabolism (molecular modelling, microsomes, recombinant enzymes), (2) elucidation of metabolism and kinetics (microsomes, recombinant enzymes, liver slices, hepatocytes), (3) determination of induction potential (hepatocytes), and (4) comparison of findings and prediction with *in vivo* literature data on CBZ in order to find a system(s) that best predicts the *in vivo* situation.

Material and methods

Materials

Carbamazepine (CBZ), carbamazepine-10,11-epoxide (CBZ-E), 10,11-*trans*-dihydroxy-10,11-dihydro-carbamazepine (10,11-D), 2-hydroxy-carbamazepine (2-OH-CBZ), 3-hydroxy-carbamazepine (3-OH-CBZ), 9-hydroxymethyl-10-carbamoyl acridan (9-AC) and 10-hydroxy-10,11-dihydro-carbamazepine (10-OH-CBZ) were gifts from Novartis (Basel, Switzerland). According to Novartis, the chemical purity of CBZ and OCBZ was > 99%. HPLC grade acetonitrile and methanol were purchased from Merck (Darmstadt, Germany). Triethylamine was purchased from Fluka AG Chemische Fabrik (Buchs, Switzerland).

Determination of CBZ and its metabolites

HPLC analysis was done as described by Pienimäki *et al.* (1995). The HPLC system consisted of a Merck-Hitachi L-6200 gradient pump and L-4250 UV-VIS Detector set at 212 nm. The data were processed with a personal computer using Merck Hitachi D-6000 HPLC software. Chromatographic separation was achieved at room temperature using a mobile phase consisting of acetonitrile (20%) and buffer (80%, 20 mM KH₂PO₄, pH 6.3, with 0.05% triethylamine) at a flow rate 1.0 ml min⁻¹ in conjunction with a LiChrospher 100 RP-18 (4 × 4 mm i.d., 5 µm) precolumn and Superspher 60 RP-select B (125 × 4 mm i.d., 4 µm) column from Merck.

Samples were extracted twice with 5 ml diethyl ether. Cells were dissolved in 500 µl 0.1 M Na-K phosphate buffer, pH 5, and sonicated prior to extractions. The evaporated samples were dissolved in 80 µl methanol:H₂O (5:2 v/v). A 20-µl aliquot was injected into HPLC. If necessary (high CBZ concentration) samples were further diluted. In these cases, both samples were analysed to maximize the accuracy in the detection of different concentrations of substrates and metabolites.

The extraction ratios were determined for hepatocytes (medium and cells) and liver slices (medium only). Generally, extraction ratios were very close to 100% except for 10,11-*trans*-dihydroxy-10,11-dihydro-carbamazepine (10,11-D). The extraction ratio for it was quite inefficient varying from 29 to 61%. This may have caused false-negative results in the analysis of this metabolite in the experiments where the amount of 10,11-D produced was low.

Candidate in vitro assay systems

Human liver microsomes

Origin of livers and preparation of microsomes. Human liver samples used were obtained from kidney transplantation donors. Appropriate Ethics Committees approved the collection of surplus tissue in the University of Oulu and in the Imperial College School of Medicine in London. For studies with CYP specific inhibitors microsomes from three livers were used. All patients died of intracerebral haemathoma (two male, one female) and they had no known liver pathology. Patients' ages ranged from 40 to 54. For other studies pool of microsomes from 10 livers were used. Microsomes were

prepared according to standard procedures. The final microsomal pellet was suspended in 0.1 M phosphate buffer, pH 7.4, to achieve a concentration of ~ 20 mg protein ml^{-1} . Protein content was measured by the method of Bradford (1976). The livers were thoroughly characterized for their CYP-specific model activities and showed to contain all expected activities, which could be inhibited by CYP-selective chemical inhibitors (data not shown). These were considered a proof of existence of specified CYP enzymes.

Inhibition by CBZ of CYP-specific enzyme activities. The following enzyme assays, which display at least some CYP-isoform specificity, were employed: ethoxyresorufin *O*-deethylation (CYP1A1/2) (Burke *et al.* 1977), coumarin 7-hydroxylation (CYP2A6) (Aitio 1978), with the slight modifications of Raunio *et al.* (1988, 1990), tolbutamide methylhydroxylation (CYP2C9) (modified from Knodell *et al.* 1987, Sullivan-Klose *et al.* 1996), mephenytoin 4'-hydroxylation (CYP2C19) (Wrighton *et al.* 1993), dextromethorphan *O*-demethylation (CYP2D6) (modified from Park *et al.* 1984, Kronbach *et al.* 1987), chlorzoxazone 6-hydroxylation (CYP2E1) (Peter *et al.* 1990), and testosterone 6 β -hydroxylation (CYP3A4/5) (Waxman *et al.* 1983). The incubation conditions were basically similar in different reactions. If not otherwise stated below, the analytical method was applied according to the stated references.

In the primary determination of IC_{50} , CBZ was added in different concentrations (final concentrations in the incubation mixture: 0.1, 1, 10, 100 and 1000 μM) into the incubation mixture in a small volume of DMSO and the activity was compared with the control incubates into which only solvent was added. IC_{50} (concentration of inhibitor to cause 50% inhibition of the original enzyme activity) was determined graphically from the plot of the logarithm of inhibitor concentration versus per cent of activity remaining after inhibition using MicrocalTM OriginTM, v.4.10 (Microcal Software, Inc., MA, USA). The reference inhibitors used were furafylline for CYP1A2, methoxsalen for CYP2A6, sulphaphenazole for CYP2C9, omeprazole for CYP2C19, quinidine for CYP2D6, pyridine for CYP2E1 and ketoconazole for CYP3A4. Methoxsalen and omeprazole are known to inhibit also other CYPs than their targets, but there are no sufficiently selective reference inhibitors for CYP2A6 and CYP2C19 (Pelkonen *et al.* 1998).

In vitro incubation system for CBZ metabolism. CBZ was incubated up to 2 h at 37°C with human liver microsomes in an incubation system, which included human liver microsomes (pooled sample from 10 livers; 1 mg), NADPH (1.2 mM), NADPH-regenerating system (glucose 6-phosphate dehydrogenase; 4 mM NADP), phosphate buffer, pH 7.4, and 1 mM CBZ.

Inhibition of microsomal metabolism of CBZ by CYP-selective inhibitors. Inhibition of CBZ metabolism in human liver microsomes by various CYP-specific inhibitors was studied in the incubation system described above (CBZ concentration 1 mM, incubation time 60 min). The following inhibitors were used: CYP1A2: furafylline (concentrations used: 1, 2, 5 μM); CYP2A6: coumarin (10, 20, 50 μM); CYP2C8: quercetin (3, 10, 30 μM); CYP2C9: sulphaphenazole (3, 10, 30 μM); CYP2C19: S-mephenytoin (100, 200, 500 μM); CYP2D6: quinidine (1, 5, 20 μM); CYP2E1: pyridine (1, 5, 20 μM); CYP3A4: ketoconazole (1, 5, 20 μM). All inhibitors were added in methanol (1%), except pyridine, which was added in water. The formation of 10,11-epoxide, 3-hydroxy-CBZ and 10,11-diol was estimated as described below.

Correlation study. CBZ was incubated in an above-described incubation system and metabolism was determined in nine different human liver samples, which had previously been characterized for their content and activity of all of the major drug metabolizing forms of P450 (CYP1A2, CYP1B1, CYP2A6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4, CYP3A5, CYP4A11). The formation of 10,11-epoxide, 3-hydroxy-CBZ and 10,11-diol were correlated with CYP-specific activities and the amounts of CYP proteins quantitated by antipeptide antibodies in Western immunoblots as described by Edwards *et al.* (1998).

Precision-cut human liver slices

Preparation and culture of liver slices. The sources of the tissue culture materials were as described by Beamand *et al.* (1993) and Lake *et al.* (1998). Samples of human liver (surplus to transplant requirements) were collected and transported to TNO BIBRA on ice. Samples from two different livers were used. Tissue cylinders from liver samples were prepared using a 10-mm diameter motor-driven tissue-coring tool. From the cylinders, liver slices (200–300 μm) were prepared in oxygenated (95% O_2 /5% CO_2) Earle's balanced salt solution containing 25 mM D-glucose, 50 $\mu\text{g ml}^{-1}$ gentamicin and 2.5 $\mu\text{g ml}^{-1}$ fungizone using a Krumdieck tissue slicer (Alabama Research and Development Corporation, Munford, AL, USA). The liver slices were floated onto Vitron, Inc. (Tucson, AZ, USA) type C titanium roller inserts (two slices per insert) and cultured in glass vials containing 1.7 ml culture medium in a Vitron dynamic organ incubator. The culture medium consisted of Williams' medium E

containing 2 mM L-glutamine, 0.1 μM insulin, 0.1 μM dexamethasone, 50 $\mu\text{g ml}^{-1}$ gentamicin and 2.5 $\mu\text{g ml}^{-1}$ fungizone. Liver slice cultures were maintained at 37°C in an atmosphere of 95% O₂/5% CO₂. After 60 min, the medium was changed to fresh medium containing 2 mM L-glutamine, 0.1 μM insulin, 0.1 μM dexamethasone and 0–2500 μM CBZ (added in 10 μl dimethyl sulphoxide ml^{-1} medium). Liver slices were incubated for 40–160 min and the incubations terminated by removing the vials from the incubator and plunging them into ice. Appropriate blank incubations (i.e. liver slices in medium without any CBZ and CBZ in medium without any liver slices) were also performed. In order to achieve a good recovery of parent compound and metabolites (Worboys *et al.* 1995), the liver slices were removed from the mesh of the roller inserts and homogenized in the culture medium by sonication (Beamand *et al.* 1993). Liver slice/medium homogenates were assayed for total protein content by the method of Lowry *et al.* (1951) employing bovine serum albumin as standard. Total protein content was determined in order to allow for any differences in liver slice thickness between vials and to provide a scaling factor for intrinsic clearance calculations. The liver slice/medium homogenates stored at –80°C prior to despatch to the laboratory undertaking the analysis of CBZ metabolites.

Studies on CBZ metabolism and kinetics. Slices were incubated for various periods ranging from 0.5 to 6 h with CBZ concentrations ranging from 0.5 to 5000 μM . CBZ was prepared as stock solutions in dimethyl sulphoxide (DMSO) and then added to the culture media at a final DMSO concentration of 10 $\mu\text{l ml}^{-1}$ medium. For all incubations, serum free culture media were employed. The media comprised either RPMI 1640 medium with hormones (see above), Williams' medium E with L-glutamine and hormones (see above) or phenol red free RPMI 1640 medium containing 0.1 μM insulin and 0.1 μM dexamethasone. Because of the short-term nature of these incubations, it was not considered necessary to supplement the media with either gentamicin or fungizone. The CBZ concentrations employed were based on solubility and toxicity considerations, together with other data (e.g. concentrations employed in incubations with liver microsomal fractions). The CBZ concentrations and incubation periods were modified as a result of initial experiments. In all experiments, the incubations were terminated by removing the vials from the incubator and placing them in ice. The liver slices were then removed from the mesh of the roller inserts and homogenized in the culture medium by sonication. It was considered necessary to prepare liver slice/medium homogenates, rather than just harvest the media, as other studies have demonstrated that xenobiotic metabolites may be retained inside the liver slices (Worboys *et al.* 1995). Appropriate blank incubations (e.g. liver slices in medium without any CBZ and CBZ in medium without any liver slices) were also performed. Samples of the CBZ dilutions in tissue culture medium and culture medium alone were also provided for analysis. Liver slice/medium homogenates were assayed for total protein content to allow for any differences in liver slice thickness between vials and to provide a scaling factor for kinetic calculations (see below). The liver slice/medium homogenates were stored at –80°C prior to dispatch to the appropriate laboratory undertaking the analysis of CBZ and its metabolites.

Human cultured hepatocytes

Procedures in Laboratory A

Isolation and culture of human hepatocytes. Surgical liver biopsies (weighting 1–3 g) were obtained from patients undergoing cholecystectomy after informed consent was obtained. Patients had no known liver pathology nor did they receive medication during the weeks prior to surgery. None of the patients were habitual consumers of alcohol or other drugs. A total of 6 liver biopsies (two males, four females) were used. Patients' ages ranged from 35 to 77 years. Two liver biopsies were used for metabolic studies because the concentration of CBZ was too low in four of the incubations and only incubations from two biopsies could be used for metabolite analysis and calculations for hepatic clearances. Hepatocytes were isolated using a two-step tissue microperfusion technique as described elsewhere (Gómez-Lechón *et al.* 1997). Cellular viability, estimated after isolation by the dye exclusion test with 0.4% trypan blue in saline, was > 90%. Hepatocytes were seeded on fibronectin-coated plastic dishes (3.5 $\mu\text{g cm}^{-2}$) at a density of 8×10^4 viable cells cm^{-2} and cultured in Ham's F-12/Lebovitz L-15 (1:1) medium supplemented with 2% new-born calf serum, 10 mM glucose, 50 mU ml^{-1} penicillin, 50 $\mu\text{g ml}^{-1}$ streptomycin, 0.2% bovine serum albumin and 10 nM insulin. One hour later the medium was changed, and after 24 h, cells were shifted to serum-free, hormone-supplemented medium (10 nM insulin, 10 nM dexamethasone). The medium was changed daily. Under these culture conditions, cells are metabolically competent (Donato *et al.* 1995, Gómez-Lechón *et al.* 1997).

Preparation of CBZ for incubations. A 50 mM stock solution of the compound was prepared in DMSO and then diluted in culture medium. The final concentration of the solvent in culture medium did not exceed 0.5% (v/v). Control cultures were treated with the same amount of solvent.

Cytotoxicity assays. Hepatocytes were seeded on 96-well microtitre plates, and treatment with increasing concentrations of the CBZ started after 24 h of culture. Cells were exposed to the compound during the following 48 h, and the compound was added daily at the time of medium renewal. Cellular

viability was assessed at the end of the treatments by the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) test as described by Borenfreund *et al.* (1988).

IC calculations for cytotoxicity. IC₅₀ and IC₁₀ represent the concentration of the compound that reduces viability by 50 and 10% with respect to the controls and were determined from the dose-response curves obtained. In five individual hepatocyte preparations, IC₁₀ was usually > 250 µM except in one culture, in which it was 50 mM. The IC₅₀ value was 1 mM > IC₅₀ > 0.75 mM.

7-Ethoxycoumarin O-deethylation activity assay. This reaction was catalysed by several P450 isozymes including CYP1A2, CYP2A6, CYP2B6, CYP2C8-9, CYP2E1, CYP3A4/5 (Waxman *et al.* 1991). Activity was measured in intact cells (Gómez-Lechón *et al.* 1997). Monolayers were washed twice with warm PBS and the assay was initiated by adding 800 µM 7-ethoxycoumarin to the culture medium. Cells were incubated for ~30 min at 37°C, and the reaction was stopped by aspirating the incubation medium from plates. To hydrolyze any 3-hydroxycoumarin conjugates formed, 1 ml of the medium sample was incubated with β-glucuronidase/arylsulphatase (200 Fishman units/1200 Roy units) for 2 h at 37°C. Hydrolysis was stopped by adding 125 µl TCA (15%) and 2 ml chloroform and then shaking the mixture for 5-10 min at 37°C. After centrifugation (2000g for 10 min), the organic phase was extracted with 1 N NaOH and the fluorescence of the aqueous phase was measured (368 nm excitation, 456 nm emission) in a microplate fluorimeter. Activity is expressed as pmol 3-hydroxycoumarin formed min⁻¹ and mg⁻¹ cellular protein assessed by the Lowry method (Lowry *et al.* 1951).

Incubation of human hepatocytes with CBZ. For metabolic studies hepatocytes were incubated with various concentration of the compound (75, 125, 250 µM). Treatment was started at 24 h of culture and incubation medium and cells were subsequently frozen after 10, 24 and 48 h of continuous incubation with the compound, to be further analysed for the metabolic profile. Parallel plates without cells, incubated with medium containing the compound, were collected and also frozen after the same incubation periods.

Procedures in Laboratory B

The tissue sample was obtained from a liver that could not be used for transplantation. The French National Ethics Committee has approved collection of human liver tissue. Hepatocytes were isolated by the two-step collagenase perfusion method as previously described (Guguen-Guillouzo *et al.* 1982) then seeded at a density of 10⁶ cells per dish in 1 ml Williams' medium supplemented with 0.2% bovine insulin, 3.2% bovine serum albumin, 1% glutamine, 0.1% penicillin/streptomycin, 0.2% gentamycin and 10% foetal calf serum. This medium supplemented with 7 × 10⁻⁵ hydrocortisone hemisuccinate but lacking foetal calf serum was renewed daily.

Cell viability estimated by trypan blue exclusion was 85%. CBZ was dissolved in DMSO before addition to the culture medium at the concentrations of 0.02, 0.1, 0.5, 1 and 2.5 mM (in 0.25% DMSO v/v). CBZ was first added after 24 h and then with each medium renewal. Media and cells were harvested after 16, 24, 48 and 72 h of exposure to CBZ and stored at -80°C before analysis. EROD activity in hepatocytes was quite low: 0.23 pmol min⁻¹ mg⁻¹ cellular proteins (min-max in our hands: 0.2-8.0; mean 3.0 with 19 cell populations). Nifedipine oxidation for CYP3A4 was 6.24 nmol h⁻¹ mg⁻¹ cellular protein.

cDNA-expressed enzymes

Yeasts were produced that expressed one of the following human cytochrome P450 enzymes: CYP1A1, CYP1A2, CYP2A6, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4, and the expression level was relatively high (30-300 pmol mg⁻¹ microsomal protein). All microsomes produce good difference spectra showing a homogenous peak at ~450 nm in the reduced carbon monoxide bound state of the haemoprotein with no cytochrome P420 present. In order to achieve good expression of the CYP2C19 and CYP2D6 enzymes, the plasmid cDNA:s were modified to include a triplet of adenine bases just prior to the initiating ATG codon (Krynetski *et al.* 1995).

The expression was carried out with the *Saccharomyces cerevisiae* strain W(R), which had been genetically modified also to over express the yeast reductase (Truan *et al.* 1993). A galactose-inducible promoter in the plasmid and in the yeast genome, respectively, was used to control expression. After transformation with a lithium acetate method of the plasmid into the W(R) yeast strain, selection of clones was achieved by growing the yeast in adenine and uracil deficient media. To achieve higher expression levels, the yeasts were first grown to a high density with glucose as main energy source, thereafter the expression of the plasmid was initiated by the addition of galactose. Preparation of microsomes was done as described by Oscarson *et al.* (1997) with the exception that the microsomes were collected in the last step by ultracentrifugation. Cytochrome P450 and NADPH-cytochrome P450 reductase were measured as described (Oscarson *et al.* 1997). Control microsomes were prepared from yeast transformed with the V60 plasmid without insert. The CYP2C19 and CYP2B6 pV60 plasmids were generous gifts from Charlotta Otter (Astra Zeneca, Mölndal, Sweden). The catalytic properties of

the yeast microsomes were evaluated using probe substrates for different P450 isoforms and found to appropriate (Anderson *et al.* 2001).

Incubations with yeast microsomes, corresponding to 400 µg protein, were carried out for 30 min with 1 mM CBZ in 0.5 ml phosphate buffer, pH 7.4, and 1 mM NADPH. For further determination of K_m and V_{max} , 250 µg CYP3A4 containing microsomes was used and the time was shortened to 8–10 min to ensure linearity. All incubations were done at 37°C and the samples were pre-incubated for 2 min prior to the addition of NADPH. In order to investigate the influence of NADPH cytochrome P450 reductase and cytochrome-b5 on CYP3A4-mediated CBZ metabolism, human or rat NADPH cytochrome P450 reductase and rat cytochrome-b5 were mixed with the CYP3A4 containing microsomes and left on ice for 10 min prior to the incubation procedure. Human reductase was produced recombinantly in *Escherichia coli* and purified on an 2',5'-ADP Sepharose column essentially as described by Shen *et al.* (1989). Rat NADPH cytochrome P450 reductase and rat cytochrome-b5 were purified as described by Ingelman-Sundberg *et al.* (1980) to a specific content of 13 nmol flavin mg⁻¹ and 30 nmol mg⁻¹, respectively.

Modelling

Molecular modelling of mammalian microsomal P450s from the CYP102 bacterial crystal structure template has been carried out for all of the major P450 families associated with the Phase 1 metabolism of drugs and other foreign compounds (Lewis 1999). Three-dimensional models of human P450s have been constructed from the CYP102 haemoprotein domain, for which the X-ray coordinates are known, both in the substrate-bound (Li and Poulos 1997) and substrate-free states (Ravichandran *et al.* 1993). The methodologies employed in homology modelling of P450 isoforms from CYP102 are described in detail elsewhere, including a general reference to the rationale for using the CYP102 structure as a preferred modelling template (Lewis 1996). The model of CYP2C8 was constructed as part of the EURO CYP collaboration and details have not been published elsewhere, although the related enzymes CYP2C9 and CYP2C19 have been modelled previously and the current CYP2C8 model is based on these former CYP2C subfamily models, being closer to CYP2C9 in both sequence and active site structure. A template of known CYP2C8 substrates fits the enzyme's active site closely, and this shows how hydrogen bonds and π - π stacking of aromatic rings cooperately assist in orientating substrates for metabolism in the experimentally observed positions.

A number of potential probe substrates (25 in total) for various human P450 isozymes have been identified and tested in models for CYP1A1, CYP1A2, CYP2A6, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4 (Lewis 1999). After this preliminary work, CBZ was tested in these models.

Calculations

Scaling from *in vitro* candidate systems to the whole liver. Intrinsic clearance (CL_{int}) is defined as the proportional constant between initial rate (v_0) and the drug concentration (C). According to the Michaelis-Menten equation:

$$V_0/C = V_{max}/K_m + C = CL_{int}.$$

At low substrate concentrations $CL_{int} = V_{max}/K_m$. At low substrate concentrations CL_{int} could be calculated from initial rates and from V_{max}/K_m (Ito *et al.* 1998).

In all *in vitro* systems, either the disappearance of the parent compound or the appearance of metabolites was used to determine K_m and V_{max} using either Michaelis-Menten or Eadie-Hofstee plots. It was not always possible strictly to use the initial linear conditions, but the deviations were probably not too large. Furthermore, not all data/time points were necessarily used for all calculations and also no allowance was made for either loss or recovery of CBZ metabolites in the incubations. Consequently, one should bear in mind that calculated K_m and V_{max} are approximate because of experimental imprecisions due to the inbuilt nature of experiments (very little advanced knowledge about the compound and optimal experimental set-up, i.e. a situation typical at the screening phase of NCEs) and because of interindividual biological variability (liver samples are from a limited number of individuals). In all systems, appropriate scaling factors are required to convert the primary kinetic data (V_{max}/K_m or intrinsic clearance) to whole human liver data (hepatic intrinsic clearance). The scaling of the results from system intrinsic clearance was somewhat different in each *in vitro* system.

In the recombinant system, velocity was calculated as pmol metabolite formed pmol⁻¹ spectrally determined P450 min⁻¹. Subsequent scaling to human liver microsomes was achieved using V_{max}/K_m . In order to extrapolate the data from the recombinant system into the situation in human liver microsomes, we used the concentrations of the various cytochromes P450 as provided by Gentest Co. (http://www.gentest.com/prod_inserts/H161_2.htm#immunoquantitation). Further scaling was carried out as described below.

In liver microsomes, the microsomal intrinsic clearance has been converted to the whole liver clearance by assuming that 1 g liver contains 50 mg microsomal protein and that the liver mass is 1500 g.

In liver slices scaling was performed on the basis of liver slice whole homogenate protein content compared to human liver total protein content (B. Houston, University of Manchester, personal

communication). Human liver whole homogenate protein has been determined by homogenizing samples of liver used to prepare the liver slices and determining the protein content by the method of Lowry *et al.* (1951). For seven separate human liver samples total protein content was determined to be $128.4 \pm 6.5 \text{ mg g}^{-1}$ wet weight liver (mean $\pm$ SEM, $n = 7$, range 102–153 mg g^{-1} liver). Also higher values, e.g. 200 mg protein g^{-1} liver wet weight, have been used in the literature.

In hepatocytes, the value for calculating whole liver clearance was estimated according to the following assumptions: average hepatocyte volume $11\,000 \mu\text{m}^3$, density of liver tissue (1.1), percentage of volume that hepatocytes represent within the liver tissue (80%). Based on these assumptions, 60–70 million hepatocytes g^{-1} liver tissue were obtained. These values are much closer to the yields obtained after gentle digestion of liver tissue with collagenase ($30\text{--}40 \times 10^6$ cells g^{-1}). Consequently, the average of 65×10^6 cells g^{-1} liver was used for calculations of hepatic intrinsic clearance. Also much higher values, e.g. 120×10^6 cells g^{-1} liver, have been used; consequently the activity values presented here should be doubled.

Scaling from liver to in vivo

To calculate the hepatic organ clearance the well-stirred model including or excluding protein binding was applied with the following equation:

$$\text{CL}_H = Q_H * \text{CL}_{\text{int}} * F_u / Q_H + \text{CL}_{\text{int}} * F_u,$$

where $Q_H = 1450 \text{ ml min}^{-1}$ and F_u is the unbound fraction; or

$$\text{Organ clearance (CL}_H) = Q_H * \text{CL}_{\text{int}} / (Q_H + \text{CL}_{\text{int}}).$$

The prediction of presystemic elimination was calculated on the basis of equation:

$$F_H = Q_H / (Q_H + \text{CL}_{\text{int}}),$$

where F_H is bioavailability. For the calculation of total body clearance and half-life, renal clearance was assumed to be negligible, human body weight was assumed as 70 kg and distribution volume V_D was taken from literature ($= 1 \text{ kg}^{-1}$ body weight). The equations used were as follows:

$$K_{\text{el}} = \text{CL}_{\text{total}} / V_D \text{ and } t_{1/2} = 0.693 / K_{\text{el}}.$$

Results

Metabolite patterns and metabolic rates of CBZ in various in vitro systems

Metabolites found in the different in vitro systems. The formation of CBZ metabolites was studied in various *in vitro* systems and the results are summarized in table 2. CBZ 10,11-epoxide was the major metabolite in all *in vitro* systems. The corresponding diol metabolite was detected in

Table 2. Carbamazepine metabolites detected in the different *in vitro* systems. Percentages are only approximate and one must also take into consideration the interindividual variability in activities.

System	Carbamazepine metabolites (% of total)				
	CBZ-E	3-OH-CBZ	10,11-DIOL	9-AC	2-OH-CBZ
Liver	major	minor	trace	n.d.	n.d.
microsomes	(> 90%)	(< 10%)			
Liver slices	major	minor	minor	medium	minor
	(70–80%)	(1–3%)	(2%)	(15–25%)	(1–2%)
Hepatocytes	major	minor	minor	medium	trace
	(> 80%)	(1%)	(1–2%)	(5–15%)	
Recombinant enzymes	major	n.d.	n.d.	n.d.	n.d.
	(100%)				
<i>In vivo</i> *	major	minor	about 80% of epoxide	minor	minor

n.d., Not detected.

* Data from Faigle and Feldman (1995).

microsomes, slices and hepatocytes. In the recombinant enzyme system, only the epoxide metabolite was detectable. The lack of diol metabolite in recombinant enzyme system was presumably due to a lack of epoxide hydrolase, which is responsible of the formation of 10,11-diol (Kerr and Levy 1995) in this system. It is also possible that the formation rate of the minor metabolites may be too slow to produce detectable amounts in some *in vitro* systems. Hepatocytes produced one unidentified metabolite. More detailed results as well as some quantitative data are given below for each *in vitro* system (table 3).

Human liver microsomes. Metabolites were detected at a substrate concentration of $\geq 50 \mu\text{M}$. 10,11-Epoxy was the major metabolite, 3-hydroxycarbamazepine was quantifiable and 10,11-dihydrodihydroxycarbamazepine was present in trace amounts at $\geq 500 \mu\text{M}$. Average formation rates were for CBZ 10,11-epoxide: $460 \text{ pmol mg}^{-1} \text{ min}^{-1}$; for 3-hydroxy-carbamazepine: $10 \text{ pmol mg}^{-1} \text{ min}^{-1}$, and for 10,11-dihydrodihydroxycarbamazepine: $5 \text{ pmol mg}^{-1} \text{ min}^{-1}$. Assuming that the average formation of all metabolites is $500 \text{ pmol mg}^{-1} \text{ min}^{-1}$ and $K_m = 400 \mu\text{M}$, Cl_{int} for microsomes is $1.2 \mu\text{l min}^{-1} \text{ mg}$, $\text{Cl}_{\text{int}} \text{ g}^{-1} \text{ liver}$ (assuming 50 mg microsomal protein $\text{g}^{-1} \text{ liver}$) $60 \mu\text{l g}^{-1} \text{ min}$, and for the whole liver (assuming 1500 g) 90 ml min^{-1} .

Precision-cut human liver slices. CBZ metabolism was time-dependent and metabolite formation was linear with respect to incubation time up to 160 min. It was assumed that metabolite production was linear with respect to liver slice protein concentration. The following metabolites were detected (table 2 and figure 2): the 10,11-epoxide was the major metabolite at all time points measured ($\sim 70\text{--}80\%$ of all the detectable metabolites at $1000 \mu\text{M}$ concentration and after 2.5 h of incubation). The amount of the 9-acridane metabolite was about a one-sixth to one-quarter (15–25%) and other metabolites, 10,11-diol, 3-hydroxy and 2-hydroxy (between 1 and 3%), were barely detectable at only longer incubation times and at higher concentrations of CBZ.

The kinetics of CBZ metabolism were estimated by first calculating the total (i.e. sum of all detected) metabolites and then performing kinetic calculations by use of Michaelis-Menten and Eadie-Hofstee plots. The estimated $K_m = \sim 360 \mu\text{M}$ and $V_{\text{max}} = \sim 17 \text{ pmol min}^{-1} \text{ mg}^{-1} \text{ liver slice whole homogenate protein}$. In the two individual studies, the values were similar. Assuming that 1 g liver contains 200 mg homogenate protein, CL_{int} was calculated to be $9.4 \mu\text{l min}^{-1} \text{ g}^{-1} \text{ liver}$ and $\sim 14 \text{ ml min}^{-1}$ per whole liver.

Human hepatocytes

Laboratory A. The 10,11-epoxide was the major metabolite in all cultures studied (table 2), representing $> 75\%$ of all the detectable metabolites. Other metabolites, including 3-hydroxy, 10,11-diol and 9-acridane, were formed in much lower quantities. Traces of 2-hydroxycarbamazepine were found in one experiment. Also an unidentified metabolite was present. CBZ has several metabolites

Table 3. Hepatic intrinsic clearances (based on disappearance of carbamazepine and/or appearance of metabolites) in various *in vitro* systems. K_m of S in parentheses are estimates. Carbamazepine has a relatively low plasma clearance *in vivo* in man (~ 27.5 ml min⁻¹) (Dollery 1991).

Candidate system	K_m or S^+ (μM)	V_{max}^+ (averages)	Assumptions in extrapolations	Cl ⁺ (ml min ⁻¹)
Liver microsomes: disappearance	(500)	224 pmol min ⁻¹ mg ⁻¹ microsomal protein	50 mg microsomal protein g ⁻¹ liver	42
Metabolite formation	(400)	500 pmol min ⁻¹ mg ⁻¹ microsomal protein	50 mg microsomal protein g ⁻¹ liver	90
Liver slices: metabolite formation	300	17 pmol min ⁻¹ mg ⁻¹ slice whole homogenate	200 mg protein g ⁻¹ liver	15.8
Hepatocytes (laboratory A): disappearance	(170)	2.2 pmol min ⁻¹ mg ⁻¹ protein	67 million hepatocytes g ⁻¹ liver	18
Metabolite formation	(170)	0.4 pmol min ⁻¹ mg ⁻¹ cell protein	67 million hepatocytes g ⁻¹ liver	4
Hepatocytes (laboratory B): metabolite formation	362	cell protein 457 pmol min ⁻¹ 10 ⁶ cells	65 million hepatocytes g ⁻¹ liver	0.5
Recombinant CYP3A4: metabolite formation	250	1.3 pmol min ⁻¹ pmol ⁻¹ p450	127 pmol CYP3A4 mg ⁻¹ microsomes	40
Recombinant CYP3A4+ b ₃ : metabolite formation	250	4.7 pmol min ⁻¹ pmol ⁻¹ p450		160

⁺ Values are calculated on the basis of the following number of individual livers: microsomes, a pool of 10 livers; slices, two livers; hepatocytes, A: two livers; B, one liver; recombinants enzymes, one set of experiments.

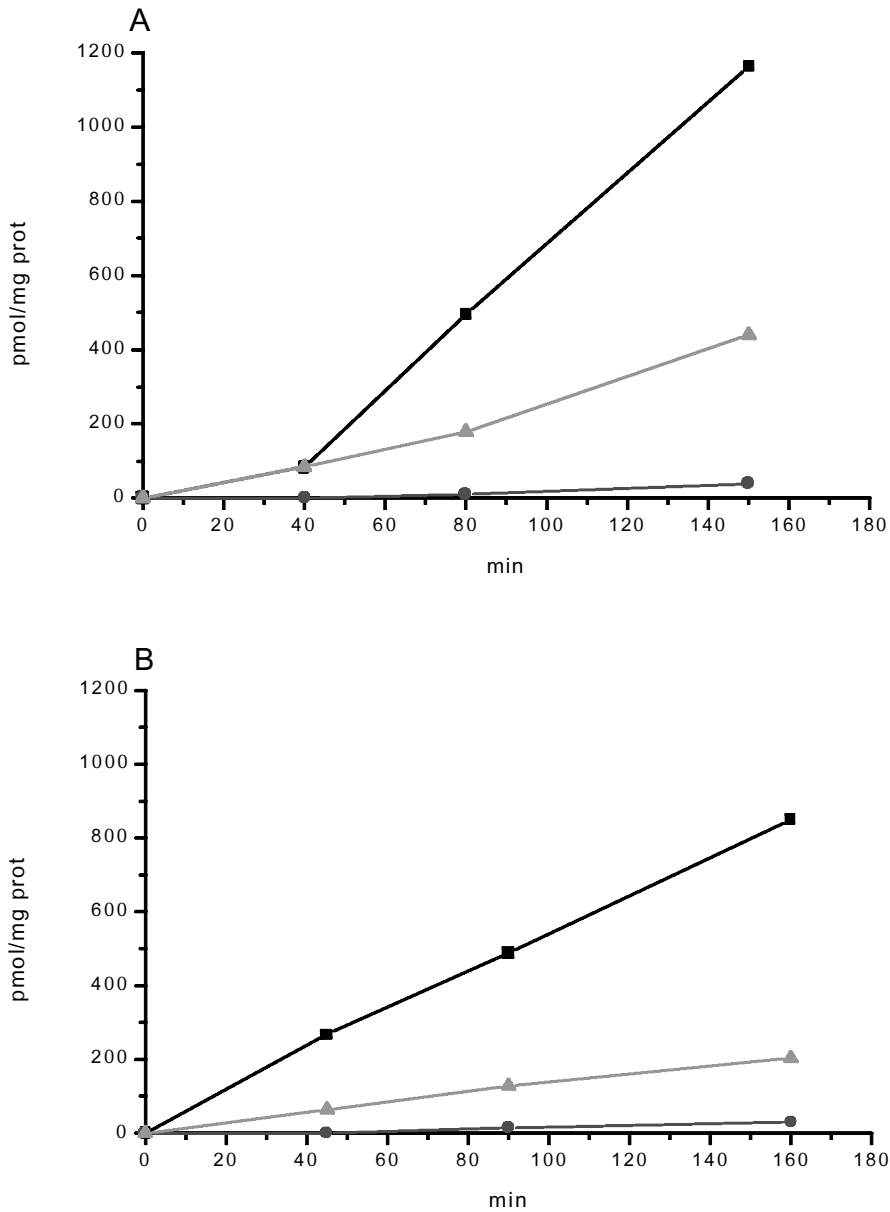


Figure 2. Formation of CBZ-E (■), 9-AC (▲) and 10,11-D (●) by human liver slices incubated with 500 μM CBZ. Slices from two livers were used and incubations are shown separately.

that were not analysed in this study and this unidentified peak most likely presents one of those metabolites.

Studies of control incubations indicated that CBZ does not extensively bind to plastic (data not shown). The amount of drug metabolized by human hepatocytes was fairly linear for 48 h. The appearance of metabolites was also linear (figure 3), but for unknown reasons the amount of metabolites produced was only ~ 20-25% of CBZ disappearance from the incubation. Therefore, the data for both drug

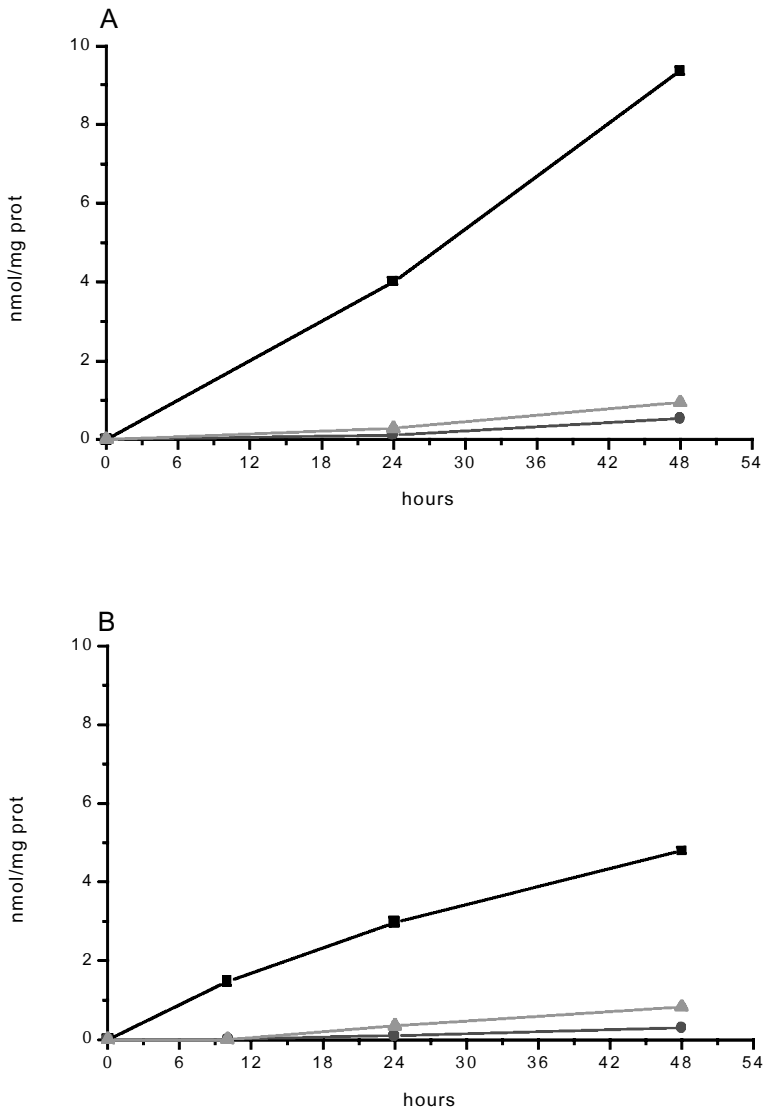


Figure 3. Formation of CBZ-E (■), 9-AC (▲) and 10,11-D (●) by hepatocytes incubated with 100 μM CBZ. Hepatocytes originating from two livers were used and they are presented separately.

disappearance and metabolite appearance were used for the determination of intrinsic clearances (table 3). Assuming that 1 g liver contains 65 million hepatocytes, hepatic intrinsic clearance was $\sim 18 \text{ ml min}^{-1}$ for disappearance and 4 ml min^{-1} for metabolite production.

Laboratory B. The 10,11-epoxide was the major metabolite in all cultures studied (table 2), representing $> 80\%$ of all the detectable metabolites. Other metabolites, 3-hydroxy, 10,11-diol and 9-acridane, were formed in much lower quantities. Traces of 2-hydroxycarbamazepine were found in a few of the experiments.

Incubations without cells were not done and intrinsic clearances were calculated only using the data for metabolite formation. Assuming that 1 g liver contains 65 million hepatocytes, hepatic intrinsic clearance was $\sim 0.5 \text{ ml min}^{-1}$ for metabolite production.

Recombinant expressed human CYP enzymes. Yeast microsomes were incubated with CBZ initially in the blind study. The conversion of the compound under the standard conditions used was, however, very low. No significant conversion was seen using standard conditions and the different recombinant microsomes. Addition of cytochrome-b₅ to the different microsomes revealed a significant, but low, conversion to the 10,11 epoxide in CYP3A4 containing microsomes where the reaction exhibited an apparent $K_m > 1000 \mu\text{M}$ and $V_{\max} = 0.6\text{--}0.9 \text{ pmol 10,11-oxide min}^{-1}$ and pmol P450 yielding a clearance = 8 ml min^{-1} .

At this point the identity of the substance was revealed and we tried to get conditions suitable for obtaining significant metabolism. It was hypothesized that the use of NADPH-cytochrome yeast reductase was not optimal for the reaction and yeast reductase was therefore replaced with recombinant human reductase. This had a substantial effect on catalysis and a velocity of $1.2 \text{ pmol pmol min}^{-1}$ was obtained in the absence of cytochrome-b₅ and upon addition of cytochrome-b₅ $V_{\max}$ was calculated to be $4.7 \text{ pmol pmol min}^{-1}$ and the apparent K_m was lower, namely $250 \mu\text{M}$.

Comparison of clearance estimates in various in vitro systems. Intrinsic clearances, based on the disappearance of CBZ and/or appearance of metabolites, in various *in vitro* systems, as detailed above, were compared (table 3). Although values for microsomes, liver slices and hepatocytes differ quite considerably from each other, they nevertheless place CBZ pharmacokinetically into the class of low-clearance drugs. An interesting finding was the dependence of the metabolic activity of the recombinant CYP3A4 on the presence of cytochrome-b₅ and human versus yeast reductase: the activity was about four times higher in the presence of cytochrome-b₅ and a 20-fold increase in activity was seen when using human reductase instead of yeast reductase.

Identification of CYP enzymes with affinity and/or metabolic capacity in various in vitro systems

Molecular modelling: computer prediction of affinity of CBZ for CYP enzymes. CBZ was tested in models for CYP1A1, CYP1A2, CYP2A6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4. On the basis of the homology model calculations, CBZ is probably a substrate (or at least has affinity to) for CYP3A4 (figure 4B), CYP2C8 (figure 4C), CYP2C9 and, possibly, CYP1A2, in this order of preference with positions of metabolism as indicated in figure 4A. In each case, the substrate molecule is orientated for metabolism via a combination of hydrogen bond and π -stacking interactions.

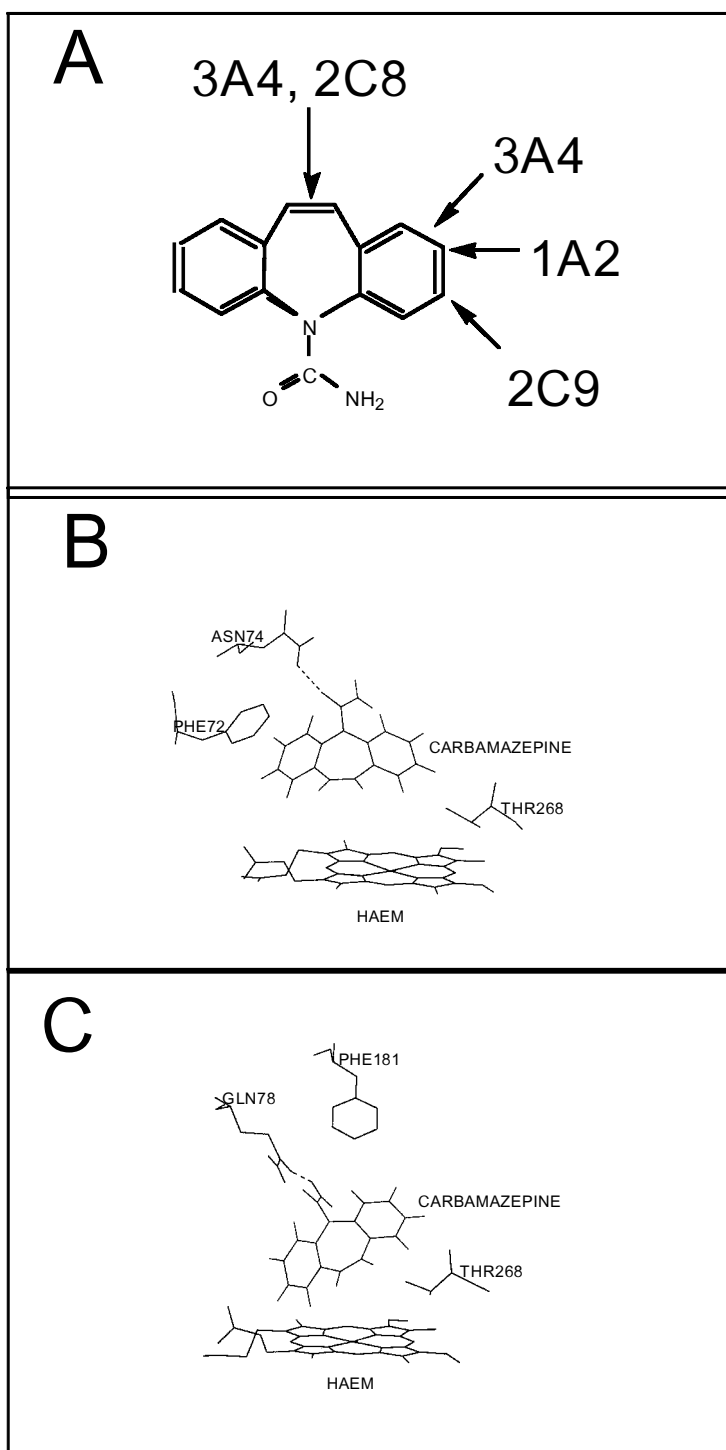


Figure 4. Computer modelling. (A) Positions of metabolisms for CBZ indicated by computer modelling. (B) Active site region of CYP3A4 containing the bound substrate, CBZ. (C) Active site region of CYP2C8 containing the bound substrate, CBZ.

Inhibition of CYP-selective model activities in human liver microsomes

The inhibitory effect of CBZ on CYP-specific model activities was screened by determining approximate IC₅₀ (table 4). CBZ inhibited CYP2C19 most potently, with IC₅₀ = 37 µM, and less potently with CYP2C9 and CYP3A4. CBZ had little or no effect on CYP1A2, CYP2A6, CYP2D6, and CYP2E1 activities. CYP2C8 was not studied because of the unavailability of a suitable marker enzyme assay.

Inhibition of microsomal metabolism of CBZ by CYP-specific inhibitors. The effect of various CYP-specific inhibitors on CBZ metabolism in human liver microsomes was studied (table 5). It has to be noted that the inhibitors might not be absolutely specific. First, a wide range of concentrations were employed for the determination of approximate IC₅₀ and thereafter concentrations near known or slightly higher than the K_i for CYP-specific model activities were selected to measure the percentage of inhibition. The main enzyme involved in the formation of CBZ-10,11-epoxide appears to be CYP3A4, with possible minor contributions from CYP1A2, CYP2A6 and

Table 4. Effect of carbamazepine on CYP-model activities in human liver microsomes.

CYP	Reference inhibitor	IC ₅₀ /K _i (µM)		Reference inhibitor IC ₅₀
		IC ₅₀ (n = 2)	K _i	
1A2	furafylline	> 1000		0.045
2A6	methoxsalen	> 1000		0.7
2C9	sulphaphenazole	226	90	4.1
2C19	omeprazole	37	8	67
2D6	quinidine	> 1000		0.41
2E1	pyridine	> 1000		4.7
3A4/5	ketoconazole	327	109	0.34

Table 5. Inhibition of microsomal metabolism of carbamazepine by CYP-specific inhibitors.

Target P450	Inhibitor	Highest concentrations tested (µM)	CBZ-E formation (% inhibition)	3-OH-CBZ formation (% inhibition)	10,11-D formation (% inhibition)
1A2	furafylline	5	26	41	5-30
2A6	coumarin	50	33	69	*
2C8	quercetin	30	42	36	n.q.
2C19	S-mephenytoin	500	negligible	negligible	n.q.
2D6	quinidine	20	35	44	n.q.
2E1	pyridine	20	negligible	negligible	n.q.
3A4	ketoconazole	20	94	27	n.q.

All inhibitors were added in methanol (1%), except pyridine, which was added in water.

n.q., quantification not possible.

* Stimulation.

Data are the average of six determinations.

CYP2C8. The main enzyme involved in the formation of 3-hydroxycarbamazepine appears to be CYP2A6, with smaller contributions from CYP1A2, CYP2C8, CYP2D6 and CYP3A4. Levels of 10,11-dihydrodiol were such that quantification was difficult, and no conclusions could be reached regarding specificity of inhibition.

Correlation study. The following correlations between CBZ metabolite formation and amount/activity of various CYPs in nine human liver samples are presented in table 6. It seems evident that only CYP3A4 is involved in the formation of CBZ-E. There is evidence for the involvement of CYP2A6 and to a lesser extent CYP2D6 in the formation of 3-OH-CBZ. The formation of 10,11-D, a secondary metabolite, was detected with only three samples and there was some evidence for CYP2C8 and CYP2C19 in its formation.

The overall conclusion with respect to specificity from the microsomal studies is that CBZ 10,11-epoxide is produced by CYP3A4, with possible minor contributions from CYP1A2, CYP2A6 and CYP2C8. 3-Hydroxycarbamazepine appears to be produced by CYP2A6 and CYP2D6, with smaller contributions from CYP1A2, CYP2C8 and CYP3A4. 10,11-Dihydrodiol appears to be produced by CYP2C8 and CYP2C19.

Recombinant expressed human CYP enzymes. All recombinantly expressed isoforms were incubated with 1 mM CBZ. Only CYP3A4 formed appreciable amounts of any metabolite as described above (table 2).

Induction potential of CBZ in human hepatocytes

The potential effects of CBZ on CYP induction were examined after 48-72 h incubation, by measuring 7-ethoxycoumarin *O*-deethylase activity, after incubating hepatocytes for 48-72 h with increasing concentrations of CBZ up to 250 μ M. CBZ produced a concentration-dependent induction (table 7). The concentration causing maximal induction, $\sim$ 2-fold, in these conditions appeared to be between 25 and 75 μ M.

Table 6. Significant correlations between carbamazepine metabolite formation and amount/activity of various CYPs in nine human liver samples.

Metabolite	Correlation with enzyme	Corresponding enzyme (CYP)	Correlation with immunohistochemically determined CYP content
CBZ-E	midazolam 1'-hydroxylase activity ($r = 0.915, p < 0.001$)	3A4	3A4 ($r = 0.736, p < 0.01$)
10,11-D	<i>S</i> -mephenytoin 4-hydroxylase activity ($r = 0.843, p < 0.02$)	2C19	total P450 ($r = 0.807, p < 0.01$) 2C8 ($r = 0.755, p < 0.05$)
3-OH-CBZ	coumarin 17-hydroxylase activity ($r = 0.765, p < 0.02$)	2A6	2A6 ($r = 0.839, p < 0.01$) 2D6 ($r = 0.695, p < 0.05$)

Table 7. Effect of carbamazepine on 7-ethoxycoumarin *O*-deethylase activity in human cultured hepatocytes. Control activities in four individual cultures studied ranged from 2.4 ± 0.05 to 4.41 ± 0.8 pmol min⁻¹ mg⁻¹.

Carbamazepine concentration (μM)	<i>n</i>	7-Ethoxycoumarin <i>O</i> -deethylase activity (% control culture)
5	1	159
10	2	135
25	3	167
50	1	207
75	3	205
125	2	193
250	3	207

Discussion

Elucidation of metabolism and kinetics (microsomes, liver slices, hepatocytes, recombinant enzymes)

Detection and formation rates of metabolites. In plasma and urine of patients, CBZ-10,11-epoxide is the major metabolite. All the systems studied produced the major *in vivo* metabolite, and in this respect well predicted the *in vivo* situation. Some other minor metabolites were detectable in liver microsomes, liver slices and hepatocytes, roughly in the proportions found *in vivo*. In recombinant enzymes, only the epoxide metabolite could be produced (by CYP3A4). In hindsight, it seems probable the inability of the recombinant system to detect other minor metabolites might be due to a slow metabolic turnover of CBZ.

Metabolic rate in various in vitro systems. CBZ is typically a slow-clearance drug with a half-life of 18-55 hours. Hepatic intrinsic clearances calculated were in most systems clearly < 100 ml min⁻¹, predicting low extraction and slow clearance. The only exception was the recombinant CYP3A4 enzyme fortified with cytochrome-b₅, which produced a clearance = ~160 ml min⁻¹. However, the omission of cytochrome-b₅ led to a value of one-quarter of the above value, i.e. 40 ml min⁻¹.

Substrate disappearance corresponded to the formation of metabolites in various systems, but not in every case. In hepatocytes, the disappearance of CBZ itself was much larger than the appearance of the metabolites. The reason for this discrepancy is not known, but it could have been due to the binding of CBZ to the incubation vessels, but this was not apparent in the liver slice system (or in microsomes).

Regarding, prediction ability, microsomes, slices and hepatocytes were reasonably accurate in the prediction of the *in vivo* situation. The recombinant system, although predicting the major metabolite, gave predictions which were higher than with the other systems and clearly needs further refinement.

Determination of enzymes responsible for substrate affinity and metabolism (molecular modelling, microsomes, recombinant enzymes)

On the basis of extensive *in vivo* studies in association of various *in vitro* investigations, the major metabolizing CYP enzyme is CYP3A4, which catalyses the major 10,11-epoxidation of CBZ (Kerr *et al.* 1994). This enzyme and reaction were correctly identified by all the systems studied. It is of interest that while microsomes and computer modelling predicted possible minor participants for this and other (less important) metabolic routes, the recombinant system identified only CYP3A4. A possible explanation for this 'insensitivity' (if it is indeed so, because it is difficult to prove at the present moment, whether the predictions of other systems as to minor participants and metabolic routes are actually true or only apparent or artificial) is that CBZ is a slowly metabolized substance and would need some refinement of the system. From the *in vivo* point of view, it is assuring that all the *in vitro* systems predicted the major enzyme catalysing the most important pathway.

CYP2C8 as a minor contributor to the 10,11-epoxidation was implicated by computer modelling and by microsomal inhibitor and correlation studies, but not by the use of recombinant enzymes. The study of Kerr *et al.* (1994) has convincingly demonstrated the minor role of CYP2C8. The inability of recombinant CYP2C8 to catalyse 10,11-epoxidation might be due to the low formation rate, but may also be related to the artificial nature of the recombinant system.

Inhibitor and correlation studies in liver microsomes indicated CYP2A6 as a main enzyme for the formation of 3-hydroxycarbamazepine, with a minor contribution from the other four CYPs. However, CBZ at the maximum concentration of 1 mM did not inhibit microsomal coumarin 7-hydroxylation and the recombinant CYP2A6 did not catalyse the formation of this metabolite. Again, it is probable that the slow metabolic rate is responsible for this discrepancy between different systems.

Prediction of induction potential

CBZ is one of the 'classical' *in vivo* inducers of several CYP-related activities. In this study, isolated hepatocytes was the only system in which induction potential was studied. A 2-day exposure of cells to CBZ led to a 2-3-fold induction of the marker activity 7-ethoxycoumarin *O*-deethylase, thus correctly predicting the *in vivo* induction potential of this substance (Levy and Wurden 1995). It has recently been demonstrated that long-term culturing of liver slices in the presence of potential inducers would lead to an increase of CYP-associated activities (Lake *et al.* 1996, 1997, 1998). However, in this survey we did not employ the liver slice system in this manner.

The detection of induction potential of CBZ by cultured hepatocytes would certainly have led to a more detailed scrutiny of this property of the compound (if it were an NCE) and revelation of the CYP3A4 induction. Induction is involved in many important *in vivo* interactions caused by CBZ (Levy and Wurden 1995), and consequently, cultured hepatocytes correctly predicted this phenomenon. It is also probable that liver slices would have been predicted the induction potential of CBZ (see above).

Prediction of potential inhibitory interactions

All systems demonstrated that CYP3A4 is the principal CBZ metabolizing enzyme in human liver systems. From the drug interaction point of view, it seems unlikely that CBZ itself would cause problems because its K_m for CYP3A4, $\geq 400 \mu\text{M}$, is much higher than the highest total plasma concentrations achieved, $\sim 40\text{--}80 \mu\text{M}$ (Morselli 1995). A high degree of accumulation in the liver should be assumed if significant interactions caused by CBZ would be implicated. On the other hand, the interference by potent CYP3A4 inhibitors of CBZ metabolism is much more likely and has actually been demonstrated *in vivo* in a number of instances for known CYP3A4 substrates such as calcium-channel blocking agents and macrolide antibiotics (Levy and Wurden 1995).

It is of interest that CBZ inhibited CYP2C19 and CYP2C9 activities at lower concentrations than CYP3A4. These enzyme were not implicated, at least prominently, by any other candidate system. However, a high affinity for CYP2C19, if indeed true, would possibly lead to CBZ associated interactions with compounds metabolized by CYP2C19 such as omeprazole and imipramine (Pelkonen *et al.* 1998). The effect of CBZ on omeprazole metabolism has also been studied *in vivo*. CBZ induced CYP3A4 catalysed sulfoxidation of omeprazole but had no or less effect on CYP2C19 catalysed hydroxylation (Bertilsson *et al.* 1997).

As stated above, specific aspects of *in vivo* pharmacokinetics are often reasons for drug failure and there is a need for *in vitro* systems to be better able to predict the *in vivo* data. With respect to CBZ, hepatocytes predicted the induction potential of CBZ. All systems predicted the slow clearance of the compound, although there was quite a large variability (which, incidentally, is also true for the *in vivo* situation). All systems predicted the major enzyme, CYP3A4, was responsible for catalysing the principal metabolic pathway, the 10,11-epoxidation of CBZ. This finding would certainly have led to studies on the reactivity of this epoxide metabolite, if CBZ had really been an NCE. Minor metabolites and less important enzymes catalysing both the principal and less important pathways were variously predicted by different systems, which may related to the relatively low formation of these metabolites resulting in sensitivity problems. In summary, whatever the system, important qualitative and quantitative aspects of CBZ pharmacokinetics were predicted, and the next task should be the refinement of various systems as to their predictive power.

Acknowledgements

The authors acknowledge the contributions of the other members of the EUROCYP project: Dieter Paul, Rainer Klocke (Fraunhofer Institute, Hannover, Germany), Tommy B. Andersson (AstraZeneca, Mölndal, Sweden), Jarmo S. Salonen (Orion Pharma, Turku, Finland), and Gisela Backfisch (Boehringer-Mannheim, Germany). They also thank Novartis for providing carbamazepine, oxcarbazepine and their metabolites.

References

- ARTIO, A., 1978, A simple and sensitive assay of 7-hydroxycoumarin deethylation. *Annals of Biochemistry*, **85**, 488-491.

- ANDERSSON, T. B., SJÖBERG, H., HOFFMAN, K.-J., BOOBIS, A. R., WATTS, P., EDWARDS, R. J., LAKE, B. G., PRICE, R. J., RENWIC, A. B., GÓMEZ-LECHÓN, M. J., CASTELL, J. V., INGELMAN-SUNDBERG, M., HIDESTRAND, M., GOLDFARB, P., LEWIS, D., CORCOS, L., GUILLOUZO, A., TAAVITSAINEN, P. and PELKONEN, O., 2001, An assessment of human liver-derived *in vitro* systems to predict the *in vivo* metabolism and clearance of Almqalant, *Drug Metabolism and Disposition*, **29**, 712-720.
- BEAMAND, J. A., PRICE, R. J., CUNNINGHAM, M. E. and LAKE, B. G., 1993, Culture of precision-cut liver slices: effect of some peroxisome proliferators. *Food and Chemical Toxicology*, **31**, 137-147.
- BERTILSSON, L., TYBRING, G., WIDEN, J., CHANG, M. and TOMSON, T., 1997, Carbamazepine treatment induces the CYP3A4 catalysed sulphoxidation of omeprazole, but has no or less effect on hydroxylation via CYP2C19. *British Journal of Clinical Pharmacology*, **44**, 186-189.
- BEYER, C. and SPITTELLER, G., 1992, A new carbamazepine metabolite in uraemic filtrate. *Xenobiotica*, **22**, 1029-1035.
- BORENFREUND, H., BABICH, H. and MARTIN-ALGUACIL, N., 1988, Comparisons of two *in vitro* cytotoxicity assays — the neutral red (NR) and tetrazolium MTT tests. *Toxicology In Vitro*, **2**, 1-6.
- BRADFORD, M. M., 1976, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Annals of Biochemistry*, **72**, 248-254.
- BURKE, M. D., PROUGH, R. A. and MAYER, R. T., 1977, Characteristics of a microsomal cytochrome P-448-mediated reaction ethoxymresorufin O-deethylation. *Drug Metabolism and Disposition*, **5**, 1-8.
- DOLLERY, C., 1991, Carbamazepine. In C. Dollery (ed.), *Therapeutic Drugs* (Edinburgh: Churchill Livingstone), pp. C49-53.
- DONATO, M. T., CASTELL, J. V. and GÓMEZ-LECHÓN, M. J., 1995, Effect of model inducers on cytochrome P450 activities of human hepatocytes in primary cultures. *Drug Metabolism and Disposition*, **23**, 553-558.
- EDWARDS, R. J., ADAMS, D. A., WATTS, P. S., DAVIES, D. S. and BOOBIS, A. R., 1998, Development of a comprehensive panel of antibodies against the major xenobiotic metabolising forms of cytochrome P450 in humans. *Biochemical Pharmacology*, **56**, 377-387.
- FAIGLE, J. W. and FELDMAN, K. F., 1995, Carbamazepine. Chemistry and biotransformation. In R. H. Levy, R. H. Mattson and B. S. Meldrum (eds), *Antiepileptic Drugs*, 4th edn (New York: Raven), pp. 499-513.
- GÓMEZ-LECHÓN, M. J., DONATO, M. T., PONSODA, X., FABRA, R., TRULLENQUE, R. and CASTELL, J. V., 1997, Isolation, culture and use of human hepatocytes in drug research. In J. V. Castell and M. J. Gómez-Lechón (eds), *In Vitro Methods in Pharmaceutical Research* (London: Academic Press), pp. 129-154.
- GUGUEN-GUILLOUZO, C., CAMPION, J. P., BRISSOT, P., GLAISE, D., LAUNOIS, B., BOUREL, M. and GUILLOUZO, A., 1982, High yield preparation of isolated human adult hepatocytes by enzymatic perfusion of the liver. *Cell Biology and International Reports*, **6**, 625-628.
- HOUSTON, J. B. and CARLILE, D. J., 1997, Prediction of hepatic clearance from microsomes, hepatocytes, and liver slices. *Drug Metabolism Reviews*, **129**, 891-922.
- INGELMAN-SUNDBERG, M. and JOHANSSON, I., 1980, Cytochrome b₅ as electron donor to rabbit liver cytochrome P-450 in reconstituted phospholipid vesicles. *Biochemistry and Biophysical Research Communications*, **97**, 582-589.
- ITO, K., IWATSUBO, T., KANAMITSU, S., NAKAJIMA, Y. and SUGIYAMA, Y., 1998, Quantitative prediction of *in vivo* drug clearance and drug interactions from *in vitro* data on metabolism, together with binding and transport. *Annual Reviews in Pharmacology and Toxicology*, **38**, 461-499.
- KENNEDY, T., 1997, Managing the drug discovery/development interface. *Drug Discovery Today*, **2**, 436-444.
- KERR, B. M. and LEVY, R. H., 1995, Carbamazepine. Carbamazepine epoxide. In R. H. Levy, R. H. Mattson and B. S. Meldrum (eds), *Antiepileptic Drugs*, 4th edn (New York: Raven), pp. 529-541.
- KERR, B. M., THUMMEL, K. E., WURDEN, C. J., KLEIN, S. M., KROETZ, D. L., GONZALEZ, F. J. and LEVY, R. H., 1994, Human liver carbamazepine metabolism. Role of CYP3A4 and CYP2C8 in 10,11-epoxide formation. *Biochemical Pharmacology*, **47**, 1969-1070.
- KNOELL, R. G., HALL, S. D., WILKINSON, G. R. and GUENGERICH, F. P., 1987, Hepatic metabolism of tolbutamide: Characterization of the form of cytochrome P-450 involved in methyl hydroxylation and relationship to *in vivo* disposition. *Journal of Pharmacology and Experimental Therapeutics*, **241**, 1112-1119.
- KRONBACH, T., MATHYS, D., GUT, J., CATIN, T. and MEYER, U. A., 1987, High-performance liquid chromatographic assays for bufuralol 1'-hydroxylase, debrisoquine 4-hydroxylase, and dextromethorphan O-demethylase in microsomes and purified cytochrome P-450 isozymes of human liver. *Annals of Biochemistry*, **162**, 24-32.
- KRUMDIECK, C. L., DOS SANTOS, J. E. and HO, K. J., 1980, A new instrument for the rapid preparation of tissue slices. *Annals of Biochemistry*, **104**, 118-123.
- KRYNETSKI, E. Y., DRUTSA, V. L., KOVALEVA, I. E. and LUZIKOV, V. N., 1995, High yield expression of functionally active human liver CYP2D6 in yeast cells. *Pharmacogenetics*, **5**, 103-109.

- LAKE, B. G., BALL, S. E., RENWICK, A. B., TREDGER, J. M., KAO, J., BEAMAND, J. A. and PRICE, R. J., 1997, Induction of CYP 3A isoforms in cultured precision-cut human liver slices. *Xenobiotica*, **27**, 1165-1173.
- LAKE, B. G., TREDGER, J. M., RENWICK, A. B., BARTON, P. B. and PRICE, R. J., 1998, 3,3'-Diindolylmethane induces CYP1A2 in cultured precision-cut human liver slices. *Xenobiotica*, **28**, 803-811.
- LERTRATANANGKON, K. and HORNING, M. G., 1982, Metabolism of carbamazepine. *Drug Metabolism and Disposition*, **10**, 1-10.
- LEVY, R. H. and WURDEN, C. J., 1995, Carbamazepine. Interactions with other drugs. In R. H. Levy, R. H. Mattson and B. S. Meldrum (eds), *Antiepileptic Drugs*, 4th edn (New York: Raven), pp. 543-554.
- LEWIS, D. F. V., 1996, *Cytochromes P450: Structure, Function and Mechanism* (London: Taylor & Francis).
- LEWIS, D. F. V., DICKINS, M., EDDERSHAW, P. J., TARBIT, M. H. and GOLDFARB, P. G., 1999, Cytochrome P450 substrate specificities, substrate structural templates and enzyme active site geometries. *Drug Metabolism and Drug Interactions*, **15**, 1-49.
- LI, H. and POULOS, T. L., 1997, The structure of the cytochrome p450BM-3 haem domain complexed with the fatty acid substrate, palmitoleic acid. *Nature Structural Biology*, **4**, 140-146.
- LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. and RANDALL, R. J., 1951, Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry*, **193**, 265-275.
- MORSELLI, P. L., 1995, Carbamazepine. Absorption, distribution and excretion. In R. H. Levy, R. H. Mattson and B. S. Meldrum (eds), *Antiepileptic Drugs*, 4th edn (New York: Raven), pp. 515-528.
- OSCARSON, M., HIDESTRAND, M., JOHANSSON, I. and INGELMAN-SUNDBERG, M., 1997, A combination of mutations in the CYP2D6*17 (CYP2D6Z) allele causes alterations in enzyme function. *Molecular Pharmacology*, **52**, 1034-1040.
- PARK, Y. H., KULLBERG, M. P. and HINSVARK, O. N., 1984, Quantitative determination of dextromethorphan and three metabolites in urine by reverse-phase high-performance liquid chromatography. *Journal of Pharmacological Science*, **73**, 24-29.
- PELKONEN, O., MÄENPÄÄ, J., TAAVITSAINEN, P., RAUTIO, A. and RAUNIO, H., 1998, Inhibition and induction of human cytochrome P450 (CYP) enzymes. *Xenobiotica*, **28**, 1203-1253.
- PETER, R., BOCKER, R., BEAUNE, P. H., IWASAKI, M., GUENGERICH, F. P. and YANG, C. S., 1990, Hydroxylation of chlorzoxazone as a specific probe for human liver cytochrome P-450IIE1. *Chemical Research in Toxicology*, **3**, 566-573.
- PIENIMÄKI, P., FUCHS, S., ISOJÄRVI, J. and VÄHÄKANGAS, K., 1995, Improved detection and quantitation of carbamazepine and oxcarbazepine and their metabolites by high-performance liquid chromatography. *Journal of Chromatography B*, **673**, 97-105.
- PRENTIS, R. A., LIS, Y. and WALKER, S. R., 1988, Pharmaceutical innovation by the seven UK-owned pharmaceutical companies 1964-1985. *British Journal of Clinical Pharmacology*, **25**, 387-396.
- RAUNIO, H., SYNGELMÄ, T., PASANEN, M., JUONEN, R., HONKAKOSKI, P., KAIRALUOMA, M. A., SOTANIEMI, E., LANG, M. A. and PELKONEN, O., 1988, Immunochemical and catalytical studies on hepatic coumarin 7-hydroxylase in man, rat and mouse. *Biochemical Pharmacology*, **37**, 3889-3895.
- RAUNIO, H., VALTONEN, J., HONKAKOSKI, P., LANG, M. A., STAHLBERG, M., KAIRALUOMA, M. A., RAUTIO, A., PASANEN, M. and PELKONEN, O., 1990, Immunochemical detection of human liver cytochrome P450 forms related to phenobarbital-inducible forms in the mouse. *Biochemical Pharmacology*, **40**, 2503-2509.
- RAVICHANDRAN, K. G., BODDUPALLI, S. S., HASERMANN, C. A., PETERSON, J. A. and DEISENHOFER, J., 1993, Crystal structure of hemoprotein domain of P450BM-3, a prototype for microsomal P450's. *Science*, **261**, 731-736.
- RODRIGUES, A. D., 1994, Use of *in vitro* human metabolism studies in drug development. An industrial perspective. *Biochemical Pharmacology*, **48**, 2147-2156.
- RODRIGUES, A. D., 1999, Integrated cytochrome P450 reaction phenotyping. Attempting to bridge the gap between cDNA-expressed cytochromes p450 and native human liver microsomes. *Biochemical Pharmacology*, **57**, 465-480.
- SHEN, A. L., PORTER, T. D., WILSON, T. E. and KASPER, C. B., 1989 Structural analysis of the FMN binding domain of NADPH-cytochrome P-450 oxidoreductase by site-directed mutagenesis. *Journal of Biological Chemistry*, **264**, 7584-7589.
- SULLIVANKLOSE, T. H., GHANAYEM, B. I., BELL, D. A., ZHANG, Z. Y., KAMINSKY, L. S., SHENFIELD, G. M., MINERS, J. O., BIRKETT, D. J. and GOLDSTEIN, J. S., 1996, The role of the CYP2C9-Leu359 allelic variant in the tolbutamide polymorphism. *Pharmacogenetics*, **6**, 341-349.
- TOMSON, T., TYBRING, G. and BERTILSSON, L., 1983, Single-dose kinetics and metabolism of carbamazepine-10,11-epoxide. *Clinical Pharmacology and Therapy*, **33**, 58-65.
- TRUAN, G., CULLIN, C., REISDORF, P., URBAN, P. and POMPON, D., 1993, Enhanced *in vivo* monooxygenase activities of mammalian P450s in engineered yeast cells producing high levels of NADPH-P450 reductase and human cytochrome b₅. *Gene*, **125**, 49-55.

- WAXMAN, D. J., LAPENSON, D. P., AOYAMA, T., GELBOIN, H. V., GONZALES, F. J. and KORZEKWA, K., 1991, Steroid hormone hydroxylase specificities of eleven cDNA-expressed human cytochrome P450s. *Archives in Biochemistry and Biophysics*, **290**, 160-166.
- WORBOYS, P. D., BRADBURY, A. and HOUSTON, J. B., 1995, Kinetics of drug metabolism in rat liver slices. Rates of oxidation of ethoxycoumarin and tolbutamide, examples of high- and low-clearance compounds. *Drug Metabolism and Disposition*, **23**, 393-397.
- WRIGHTON, S. A., STEVENS, J. C., BECKER, G. W. and VANDENBRANDEN, M., 1993, Isolation and characterization of human liver cytochrome P450 2C19: correlation between 2C19 and S-mephenytoin 4'-hydroxylation. *Archives of Biochemistry and Biophysics*, **306**, 240-245.

Co-expression of human cytochrome P4501A1 (CYP1A1) variants and human NADPH-cytochrome P450 reductase in the baculovirus/insect cell system

D. SCHWARZ^{†*}, P. KISSELEV[‡], H. HONECK[§], I. CASCORBI[†], W.-H. SCHUNCK[§] and I. ROOTS[†]

[†] Institute of Clinical Pharmacology, University Medical Centre Charité, Humboldt University of Berlin, 10098 Berlin, Germany,

[‡] Institute of Bioorganic Chemistry, Academy of Sciences Belarus, 220141 Minsk, Belarus

[§] Max Delbrueck Centre for Molecular Medicine, 13125 Berlin-Buch, Germany

Received 15 January 2001

1. Three human cytochrome P4501A1 (CYP1A1) variants, wild-type (CYP1A1.1), CYP1A1.2 (I462V) and CYP1A1.4 (T461N), were co-expressed with human NADPH-P450 reductase (OR) in *Spodoptera frugiperda* (Sf9) insect cells by baculovirus co-infection to elaborate a suitable system for studying the role of CYP1A1 polymorphism in the metabolism of exogenous and endogenous substrates.

2. A wide range of conditions was examined to optimize co-expression with regard to such parameters as relative multiplicity of infection (MOI), time of harvest, haem precursor supplementation and post-translational stabilization. Under optimized conditions, almost identical expression levels and molar OR/CYP1A1 ratios (20:1) were attained for all CYP1A1 variants.

3. Microsomes isolated from co-infected cells demonstrated ethoxyresorufin deethylase activities (nmol/min⁻¹ nmol⁻¹ CYP1A1) of 16.0 (CYP1A1.1), 20.5 (CYP1A1.2) and 22.5 (CYP1A1.4). Pentoxyresorufin was dealkylated ~10-20 times slower with all enzyme variants.

4. All three CYP1A1 variants were active in metabolizing the precarcinogen benzo[a]pyrene (B[a]P), with wild-type enzyme showing the highest activity, followed by CYP1A1.4 (60%) and CYP1A1.2 (40%). Each variant produced all major metabolites including B[a]P-7,8-dihydrodiol, the precursor of the ultimate carcinogenic species.

5. These studies demonstrate that the baculovirus-mediated co-expression-by-co-infection approach all CYP1A1 variants yields functionally active enzyme systems with similar molar OR/CYP1A1 ratios, thus providing suitable preconditions to examine the metabolism of and environmental chemicals by the different CYP1A1 variants.

Introduction

The human *CYP1A1* gene is polymorphic (the names of alleles recommended by the Human Cytochrome P450 (CYP) Allele Nomenclature Committee are used throughout; Ingelman-Sundberg *et al.* 2000, also URL: <http://www.imm.ki.se/CYPalleles/cyp1a1.htm>). In addition to the wild-type *CYP1A1**1A (protein: CYP1A1.1) (Jaiswal *et al.* 1985), seven *CYP1A1* alleles have been characterized to date. Two show single nucleotide exchanges leading to amino acid exchanges: *CYP1A1**2B (protein: CYP1A1.2, I462V variant) (Hayashi *et al.* 1991) and

* Author for correspondence at: Institute of Clinical Pharmacology, Charité, Humboldt University of Berlin, c/o Max Delbrueck Centre for Molecular Medicine, Robert Roessle Str. 10, D-13125 Berlin-Buch, Germany; e-mail: schwarz@mdc-berlin.de

*CYP1A1*4* (protein: CYP1A1.4, T461N variant) (Cascorbi *et al.* 1996). Certain *CYP1A1* genotypes are associated with increased incidence of cancer by epidemiological investigations (for a review about lung cancer, see Houlston 2000, *passim*, Guengerich and Shimada 1998, Huang *et al.* 1999, Nebert *et al.* 1999, Sato *et al.* 1999).

Those CYP1A1 polymorphisms with amino acid changes may affect catalytic activities towards precarcinogens as substrate because these substitutions have been hypothesized to be located near the substrate-binding site of the enzyme (Hayashi *et al.* 1991). Previous studies have compared wild-type and the CYP1A1.2 variant that were expressed in *E. coli* and yeast, and different reconstituted systems were used to measure catalytic activity towards benzo[a]pyrene (B[a]P) as substrate (Zhang *et al.* 1996, Persson *et al.* 1997). Moreover, it was demonstrated that high levels of unmodified, native CYP1A1 can be produced using the baculovirus/insect cell system (Buters *et al.* 1995, Gonzalez and Korzekwa 1995) and catalytically active systems can be reconstituted with purified NADPH-cytochrome P450 reductase. Using baculovirus (bv) expression, we recently expressed the three CYP1A1 variants in insect cells and reconstituted functionality with cumene hydroperoxide as oxygen donor, without OR, to characterize specificities of the three CYP1A1 variants in steroid hydroxylations (Schwarz *et al.* 2000).

While each approach for the production of catalytically active CYP1A1 enzymes has unique advantages and disadvantages, the current study utilizes the co-expression-by-co-infection approach introduced to co-express cytochrome P450 (CYP) enzymes with its associated reductase (Tamura *et al.* 1992, Chen *et al.* 1997). This approach enables the production of high levels of enzymatically active CYP enzymes in the natural microsomal membrane and has the potential to provide the ability to control the CYP:OR ratio (by changing the multiplicity of infection (MOI) for each bv). Further advantages are the use of either whole cells or microsomal preparations for catalytic studies and the potential to incorporate several CYP enzymes with OR (and/or cytochrome-b₅) to study electron transfer and protein-protein interaction of the monooxygenase enzymes in their natural membrane environment (Wang *et al.* 1996, Tan *et al.* 1997, Ong *et al.* 1998). We report a systematic study of the co-infection method to co-express the three human variants CYP1A1.1, CYP1A1.2 or CYP1A1.4 with human NADPH-cytochrome P450 reductase. Many conditions were examined to optimize co-expression with regard to such parameters as relative multiplicity of infection (MOI) for bvCYP1A1 and bvOR, time of harvest, and haem precursor supplementation and post-translational stabilization. The functionality of the system was characterized by ethoxyresorufin *O*-deethylation (EROD), pentoxyresorufin deethylation (PROD), and B[a]P oxidation activity of microsomal preparations.

Materials and methods

Materials

Sf9 insect cells, the initial bv transfer vector pBlueBac4.5 and linear bv DNA Bac-N-Blue DNA were purchased from Invitrogen (Groningen, The Netherlands), insect cell medium Excell 401 from Biozol (Eching, Germany), foetal bovine serum and penicillin/streptomycin from Life Technologies (Karlsruhe, Germany), and the transfection kit was bought from Pharmingen (San Diego, CA, USA).

Goat anti-rat CYP1A1 (for the detection of human CYP1A1 and/or CYP1A2) and anti-rat OR (for the detection of NADPH-cytochrome P450 reductase in rat liver) antibodies were purchased from

Daiichi Pure Chemicals (Tokyo, Japan), and the BM chemiluminescence blotting system with anti-goat secondary antibodies (peroxidase-labelled) were from Boehringer Mannheim (Mannheim, Germany). In Western blot analysis, as negative and positive control for the expression of CYP1A1 and OR, we used microsomes prepared from uninfected Sf9 cells, CYP1A1-supersomes purchased from Gentest (Woburn, MA, USA), and microsomes prepared from Sf9 cells expressing only human OR, respectively. For supplementation of the B[a]P assay with epoxide hydrolase, lymphoblastoid cell microsomes containing microsomal epoxide hydrolase were purchased from Gentest. Monoclonal antibodies to human CYP1A1 (MAb 1-7-1) used in inhibition studies were kindly provided by Dr H. V. Gelboin (National Cancer Institute, NIH, Bethesda, MD). [7,10-¹⁴C]B[a]P (specific activity 2 GBq mmol⁻¹; radiochemical purity (by HPLC) 97.2%) was purchased from Amersham Pharmacia Biotech (Freiburg, Germany). 7-ER, 7-pentoxoresorufin, resorufin, δ -amino levulinic acid (δ -ALA), and α -naphthoflavone were purchased from Sigma (Deisenhofen, Germany).

Construction and preparation of recombinant baculoviruses

Wild-type full-length cDNA for human CYP1A1 was kindly provided by Dr F. J. Gonzalez (National Cancer Institute, NIH, Bethesda). Construction of the CYP1A1.2 and CYP1A1.4 variants, cloning of the cDNAs carrying the CYP1A1.2 and CYP1A1.4 mutation, respectively, into bv transfer vectors pBlueBac4.5 (Invitrogen, Groningen, The Netherlands) under control of the polyhedrin promoter, cotransfection of Sf9 insect cells, and preparation of large-scale high-titre stocks of recombinant bv for the expression of the variants in insect cells were done as described by Schwarz *et al.* (2000). The titres of all virus preparations were determined by plaque assays. Recombinant bv for co-expression of human OR was kindly provided by Dr F. J. Gonzalez.

Expression and preparation of microsomes

Standard expression was done with 200–400 ml Sf9 cells seeded at 1.8×10^6 cells ml⁻¹ in spinner flasks and simultaneous infection with the two bv at a MOI for bvCYP1A1 and bvOR of 3.2 and 1.05 plaque-forming units (pfu)/cell, respectively. Culture media were supplemented 20–24 h *post-infection* (p.i.) with δ -ALA (0.5 mM final) and cells were harvested ~ 72 h p.i. Analyses were undertaken using whole cells taken as aliquots from the culture as described below.

To prepare microsomes, the harvested cell pellet was washed twice with phosphate-buffered saline and resuspended in standard phosphate buffer (0.1 M potassium phosphate, pH 7.4, 20% glycerol (v/v), 0.1 mM EDTA, 0.1 mM DTT). Microsomes were prepared according to Buters *et al.* (1995), except that an additional centrifugation step at 12 000g (15 min at 4°C) was done before the final pelleting of microsomes by centrifugation at 100 000g. Microsomal pellets were resuspended in standard phosphate buffer, aliquoted and stored until use at -80°C .

Analytical and other methods

CYP content (content of cytochrome P450 as the holoform) was measured by standard reduced CO-difference spectroscopy and protein concentration was determined by Coomassie Plus protein assay (Pierce, Rockford, IL, USA). OR activity was determined as NADPH-cytochrome c reductase activity. One unit is defined as amount of OR reducing 1 μmol cytochrome c min⁻¹ at 25°C (Yasukochi and Masters 1976). Data were statistically analysed using ANOVA (GraphPad Software, San Diego, CA, USA; <http://www.graphpad.com>). Quantitative analysis of immunoblots was done using a luminescent image analyser (Fuji Photo Film, Japan).

Whole-cell assays

For assays using whole cells, 14 ml cultured cells was harvested, washed in phosphate-buffered saline, sedimented and resuspended in 2 ml standard phosphate buffer. Cell density was 1.75×10^7 cells ml⁻¹. Reduced-CO difference spectra were recorded using 1 ml of this cell suspension, lysed by freeze-thawing. Cytochrome c reduction was measured as described above using 5–10 μl . Cell viability was checked by the trypan blue test. Cells that take up trypan blue and stain are considered non-viable. EROD activity was determined at 25°C according to the fluorometric assay of (Burke and Mayer 1983) using a spectrofluorometer RF5000-PC (Shimadzu, Japan). Of whole cells, 100 μl (1.75×10^7 cells ml⁻¹) was diluted with 1880 μl EROD buffer (50 mM Tris-HCl, pH 7.5, 3 mM MgCl₂). 7-Ethoxyresorufin (7-ER) dissolved in 10 μl DMSO was added (final concentration 4.3 μM) and the reaction was started by addition of 10 μl NADPH to a final concentration of 0.5 mM. The formation of resorufin was followed kinetically by monitoring its fluorescence (λ_{ex} at 535 nm, λ_{em} at 585 nm). Product was calibrated using authentic resorufin.

EROD assay with microsomes

EROD activity was determined at 37°C according to the same assay as for whole cells, but 2.5 pmol microsomal CYP1A1 was diluted with EROD buffer. Substrate dissolved in 10 μl DMSO was added to

give a final concentration of 4.3 μM and the solution was pre-incubated for 3 min at 37°C. After that the reaction was started by addition of 10 μl NADPH to a final concentration of 0.5 mM. After the 5-min reaction at 37°C, the reaction was stopped by addition of 2 ml ice-cold acetone. After centrifugation the fluorescence as above was recorded. In inhibition studies, 10 pmol CYP1A1 was pre-incubated with antibodies MAbs 1-7-1 (10–500 $\mu\text{g ml}^{-1}$) in $\sim 50 \mu\text{l}$ for 5 min at room temperature, the volume was adjusted by addition of buffer to 100 μl and further incubated for 5 min. The mixture was diluted with EROD buffer at 37°C to the final volume of 2 ml. Substrate dissolved in 1 μl DMSO was added (0.43 μM final concentration) and the reaction initiated as described above.

B[a]P metabolism and HPLC analysis

Microsomal CYP1A1 (20 pmol), 250 μg epoxide hydrolase and 10 μl DMSO solution containing a mixture of B[a]P and [^{14}C]B[a]P were diluted in EROD buffer (total volume 990 μl) and pre-incubated for 3 min at 37°C. The reaction was started by addition of 10 μl NADPH for 30 min at 37°C (final volume 1 ml; final concentrations 25 μM B[a]P including 3.3 kBq [^{14}C]B[a]P, 0.5 mM NADPH). The reaction was stopped by addition of 1 ml ice-cold acetone. Substrate and products were extracted twice with ethylacetate. Ethylacetate (2.5 ml) was added to the reaction suspension, vortexed for 30 s and centrifuged for 20 min. The upper phases were collected and combined followed by evaporation of the solvent. Residual substrate and products were redissolved with $\sim 50 \mu\text{l}$ methanol, sonicated in an ultrasonication bath and injected into the HPLC apparatus.

B[a]P metabolites were analysed following HPLC methods reported by Selkirk *et al.* (1974) and Holder *et al.* (1974). Two solvent delivery systems, LC-10 AD VP, a UV detector SPD-10 A VP, and CLASS VP software package, were used (Shimadzu, Japan). Separation was achieved with a reversed-phase column (Nucleosil 100-5 C18, 5 μm , 250 $\times$ 4 mm; Macherey-Nagel, Düren, Germany) at 25°C with a flow rate of 0.8 ml min $^{-1}$ using a linear methanol-water gradient starting with 60% methanol to 100% in 80 min. ^{14}C radioactivity was measured on-line using a radioactive monitor LB 507 A (Bertholdt, Wildbad, Germany). Simultaneously, absorbance was monitored at 254 nm. Metabolites were identified using authentic standards purchased from NCI Chemical Carcinogen Repository at Midwest Research Institute (Kansas City, MO, USA).

Results and discussion

Optimization of co-expression of human CYP1A1 variants and human OR in Sf9 cells

CYP1A1 and OR were co-expressed by simultaneous infection of Sf9 cells by two recombinant viruses, bvCYP1A1 and bvOR. For the CYP1A1 system this is, to our knowledge, the first co-expression by co-infection as a functionally active enzyme system. To compare activities of the various CYP1A1 variants - a major objective also of future studies - it was particularly important that co-infection had the advantage to control the CYP:OR ratio by changing the relative MOI by the two viruses and to use microsomal preparations. To achieve this goal and to obtain a relative high level of expression, experiments were undertaken to find optimal expression conditions.

It is well known that addition of a haem precursor is necessary to compensate for deficiency in haem synthesis by the insect cells and in this way to maximize expression of the CYP holoenzyme (Buters *et al.* 1995, Gonzalez and Korzekwa 1995). To establish optimal conditions for supplementation with δ -ALA, we studied CYP1A1 expression at various concentrations (0–0.85 mM) and different times of addition (0–24 h p.i.). A δ -ALA concentration of 0.35–0.67 mM added 20–24 h p.i. gave optimal CYP1A1 expression, being ~ 3 -fold higher than without addition. At concentrations $> 67 \text{ mM}$ the expression level decreased (data not shown).

MOI optimization experiments were done with cells infected at a MOI of 3 pfu/cell for bvCYP1A1.1. The MOI for bvOR was varied from 0.1 to 2 pfu/cell, therefore the ratio of MOIs of the both bv was varied between 1.5 and 30. Figure 1 shows that OR expression increased with increasing MOI of bvOR, reaching a

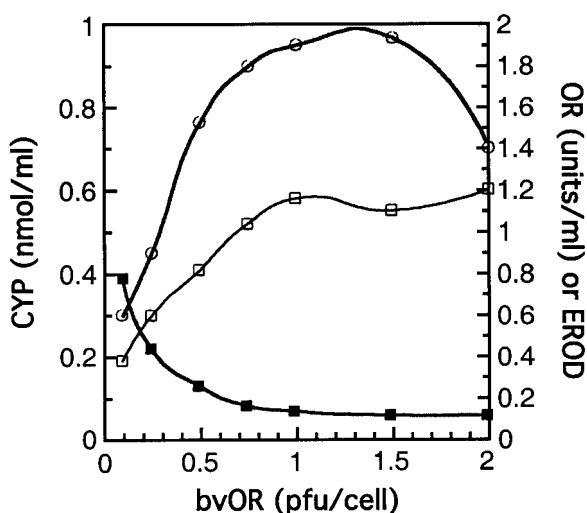


Figure 1. Reconstitution of CYP1A1.1 enzyme activity. CYP1A1.1 holoenzyme (■), OR activity (□) and EROD activity, given in $\text{nmol min}^{-1} \text{nmol}^{-1}$ CYP1A1 (○) in Sf9 cells co-infected with bvCYP1A1.1 and bvOR. Spinner cultures were infected with 3 pfu/cell bvCYP1A1.1 and the indicated pfu of bvOR and cultured for 72 h at 27°C as described in the Materials and methods. CYP contents, OR and EROD activity of whole cells were determined as described in the Materials and methods. OR content was determined by cytochrome c reduction using a specific activity of purified OR of $1.87 \text{ units nmol}^{-1}$ OR (Tamura *et al.* 1992). Data are the average of two determinations.

plateau at about a MOI(bvOR) of 1. However, as the OR level increased, CYP1A1 expression decreased. Levels of EROD activity were also measured in co-infected cells expressing different levels of OR and CYP1A1. The maximal catalytic rate was obtained at a MOI(bvOR) of ~ 1 , corresponding to an OR/CYP ratio of $\sim 10 \text{ (mol mol}^{-1}\text{)}$. Based on these data used, in standard expressions a $\text{MOI}(\text{bvCYP1A1}) = 3.2 \text{ pfu/cell}$ and a $\text{MOI}(\text{bvOR}) = 1.05 \text{ pfu/cell}$ for maximal activity and optimal expression of CYP1A1 and OR.

The time-course of co-expression of CYP1A1 and OR was studied using a fixed MOI. The level of CYP1A1 and OR expression was analysed by reduced-CO difference spectra and cytochrome c reduction, respectively, and the EROD activities were measured as described (figure 2). The optimal time to harvest, based on CYP content and enzymatic activity, was $\sim 72 \text{ h p.i.}$ At this time, $\sim 15\text{--}20\%$ of cells were non-viable according to the trypan blue test. The CYP holoenzyme content dropped significantly at later times whereas OR expression level was fairly constant up to 120 h.

Using the optimized conditions, the three CYP1A1 variants could be co-expressed with OR in Sf9 insect cells as native, spectrally and enzymatically active enzymes with expression levels for CYP1A1 and OR not significantly different. From whole cell extracts, expression levels were calculated of $\sim 10\text{--}20 \text{ nmol CYP1A1/l culture}$ and $160\text{--}220 \text{ units OR/l culture}$. Formation of cytochrome P420 was negligible (data not shown). Co-expression of CYP1A1 and OR resulted in greatly reduced CYP1A1 levels compared with expression without OR which is $\sim 50\text{--}100 \text{ nmol l}^{-1} \text{ culture}$ (Schwarz *et al.* 2000) (see below).

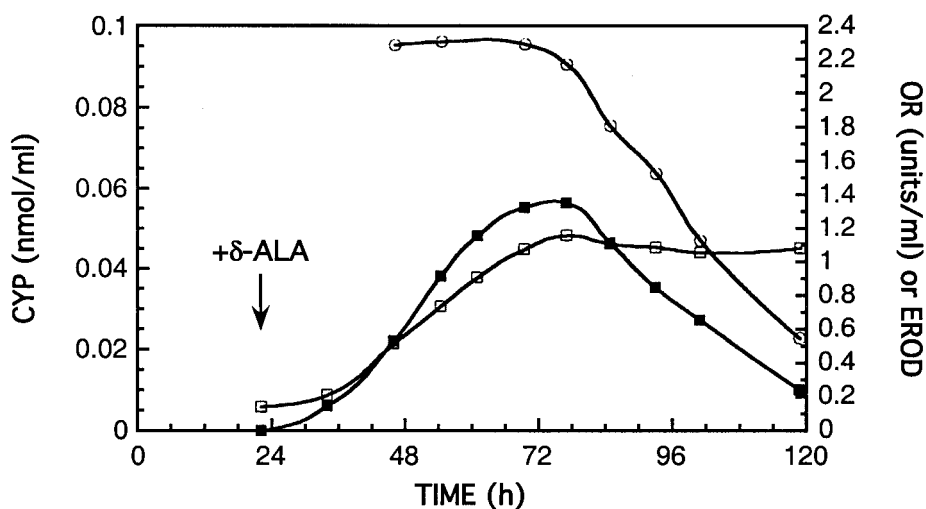


Figure 2. Time-course of co-expression in Sf9 cells. CYP1A1 holoenzyme content (■), OR activity (□) and EROD activity, given in $\text{nmol min}^{-1} \text{nmol}^{-1}$ CYP1A1 (○). Cells in spinner cultures were infected with 3.2 pfu/cell of bvCYP1A1.1 and 1.05 pfu/cell of bvOR, fortified with δ -amino levulinic acid (+ δ -ALA) at 22 h p.i. 14-ml aliquots of the culture were taken at the indicated times and analysed as described in the Materials and methods. EROD activity was determined as described in the legend of figure 1. Data are the average of two determinations.

Characterization of microsomes containing co-expressed CYP1A1 variants and OR

Co-infection of Sf9 cells resulted in expression of CYP1A1 and OR in the microsomal membrane. Electrophoretic analysis of the microsomes showed one major band >72 kDa (figure 3 A, lanes 2-4) which comigrated with OR in control microsomes (lanes 5 and 7). No such bands were detected in microsomes prepared from uninfected cells (lane 6). A similarly intense band, characteristic of CYP1A1, is not visible in co-expressed microsomes because the specific content of co-expressed CYP is too low. However, immunoblot analysis of microsomes prepared from insect cells proved expression of all CYP1A1 variants and OR, as revealed by simultaneous probing the blot with polyclonal goat anti-rat CYP1A1/1A2 and goat anti-rat OR antibodies (figure 3B, lanes 2-4). The same antibodies did not detect any immunoreactive proteins in microsomes from uninfected Sf9 cells as negative control (lane 6). As expected the antibodies showed strong immunoreactivity with bv-expressed wild-type CYP1A1 in CYP1A1 supersomes (lane 7, positive control) and microsomes expressing solely CYP1A1.1 (lane 1), as well as with OR in microsomes expressing exclusively OR (lane 5).

Quantitative analysis revealed that the expression level of apo-CYP1A1 was 50-60% less when co-expressed with OR than when expressed alone. The protein levels were determined by the intensity of the bands after immunoblotting by image analysis. Holoenzyme expression of CYP1A1 in the presence of OR (determined by CO-reduced difference spectra) was still lower compared with the holoenzyme expressed alone (only 10-16%). These data confirm previous results obtained for the co-expression by co-infection of CYP2A1 and CYP2A6, both with OR (Tamura *et al.* 1992, Chen *et al.* 1997). As discussed in these papers, it is assumed that OR expression directly influenced the CYP holoenzyme level,

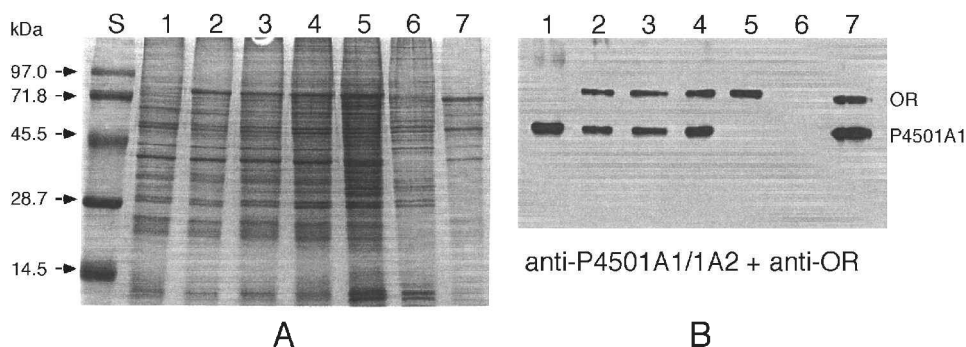


Figure 3. SDS-PAGE analysis (12.5%) (A) and immunoblots (B) of microsomes prepared from Sf9 cells co-expressing human CYP1A1 variants and human OR. Lanes 1, microsomes containing solely CYP1A1; 2-4, co-expressed microsomes containing CYP1A1.1, CYP1A1.2 and CYP1A1.4 each with OR; 5, microsomes containing solely OR; 6, control microsomes prepared from uninfected Sf9 cells; 7, CYP1A1-supersomes; S, molecular weight standards, indicated at the left (in kDa). (B) 1.8, 1.6, 1.6, 1.9, 4.4, 1.8 and 0.7 μ g microsomal protein was loaded onto lanes 1-7, respectively, and was subjected to SDS-PAGE followed by transfer to nitrocellulose membranes. Membranes were incubated overnight simultaneously with an antibody mixture of 1:1000 dilution of goat anti-rat CYP1A1/1A2 and 1:10 000 dilution of goat anti-rat OR. Blots were developed using 1:10 000 dilution of peroxidase-labelled anti-goat IgG as the secondary antibody and detected using the BM chemiluminescence blotting system with horseradish peroxidase-labelled secondary antibodies.

probably via haem degradation. Since a similar decrease in active CYP content has been noted for the co-expression of CYP2D6 and OR using an expression vector containing both genes (CYP2D6 and OR) (Paine *et al.* 1996), the observation that high levels of OR have an adverse effect on P450 expression may be a general property of co-expression in Sf9 cells.

Post-translational stabilization of CYP1A1

Degradation of the expressed holo-CYP1A1 can be circumvented, at least partially, by addition of haem ligands or inhibitors during co-expression. It was observed that imidazole and α -naphthoflavone supplementation during cell culture increased the holoenzyme level nearly 2-fold probably by protection of the enzyme from haem degradation initiated by OR expression (data not shown). This finding is in agreement with recent reports that the cellular level of enzymes of the CYP1A subfamily are regulated at the post-translational level (Werlinder *et al.* 1998), as are those of CYP2E1 and CYP3A1 (Zhukov and Ingelman-Sundberg 1999).

Co-expressed microsomes showed a characteristic CO-ferrous P450 spectrum with peak absorption at 446.5 nm, almost no cytochrome P420 absorption (data not shown) and cytochrome c reductase activity. Typical data for CYP and microsomal protein content, OR activity and the molar ratio of CYP to OR in microsomal preparations are shown in table 1.

Catalytic activities and inhibition by monoclonal antibodies

The expressed CYP1A1 variants were enzymatically active with EROD as substrate. Table 2 shows that the turnover rates were slightly but significantly ($p < 0.05$) increased for CYP1A1.2 and .4, respectively, compared with the

Table 1. Characteristics of microsomes prepared from Sf9 insect cells co-expressing human CYP1A1 variants and human OR^a.

	CYP1A1.1	CYP1A1.2	CYP1A1.4
CYP content (nmol ml ⁻¹)	0.36 ± 0.05	0.44 ± 0.05	0.47 ± 0.05
Protein content (mg ml ⁻¹)	20.0 ± 2.0	26.0 ± 3.0	15.0 ± 2.0
OR activity (units ml ⁻¹)	17.0 ± 2.0	14.6 ± 2.0	21.6 ± 2.5
CYP/OR ^b (mol mol ⁻¹)	1 : 25	1 : 18	1 : 24

^a CYP holoenzyme content, microsomal protein, and activity of OR and EROD were determined as described in the Materials and methods. Data are means ± SD from at least three determinations.

^b OR content was determined by cytochrome c reduction using a specific activity of purified OR = 1.87 units nmol⁻¹ OR (Tamura *et al.* 1992).

Table 2. EROD and PROD activity of microsomes prepared from cells co-expressing human CYP1A1 variants and human NADPH-PH450 reductase.

	CYP1A1.1	CYP1A1.2	CYP1A1.4
EROD ^a (nmol min ⁻¹ nmol ⁻¹ CYP1A1)	16.0 ± 1.4	20.5 ± 1.8	22.5 ± 2.0
PROD ^b (nmol min ⁻¹ nmol ⁻¹ CYP1A1)	1.5 ± 0.3	1.8 ± 0.4	1.5 ± 0.3

^a Rates were determined at 37°C and are means ± SD as determined by ANOVA based on three different determinations. Activity was determined with a 7-ER concentration of 4.3 µM.

^b Activity was determined with 7-pentoxoresorufin concentration of 5 µM. Data are means ± SD of three determinations.

wild-type enzyme. The differences between CYP1A1.2 and .4 were not significant ($p > 0.05$). In general, these are relatively minor differences confirming previous data for the *E. coli* expressed (Zhang *et al.* 1996) and the yeast expressed CYP1A1.1 and CYP1A1.2 variants (Persson *et al.* 1997). PROD activities of the three variants were not significantly different ($p > 0.05$). As expected, they were substantially lower as compared with EROD activities confirming results obtained for bv-expressed wild-type human CYP1A1 (Buters *et al.* 1995) and rat CYP1A1 (Burke and Mayer 1983).

Whilst the optimized expression conditions gave comparable CYP:OR ratios for all variants it might be that for a single variant a different CYP:OR ratio is required for optimal catalytic activity. Pre-incubations of microsomes with purified human OR at a stoichiometry of purified OR:CYP = 5 (mol mol⁻¹) for 5 min at 37°C before starting the reaction did not lead to increased EROD activity proving that the concentration of OR in the co-expressing microsomes was saturating for all CYP1A1 variants.

The specificity of EROD activity by the different CYP1A1 variants was substantiated by incubations with an inhibitory monoclonal antibody against human CYP1A1. Figure 4 demonstrates that the antibody inhibited resorufin formation catalysed by each of the CYP1A1 variants completely (> 90%).

One objective of future studies is to analyse the role of CYP1A1 polymorphism in the bioactivation of widely distributed environmental carcinogens such as B[a]P. Therefore, the three polymorphic CYP1A1 variants were assayed using B[a]P as substrate. Because it is known that epoxide hydrolase has a large influence on metabolism (Holder *et al.* 1974), all assays were undertaken in the presence of epoxide hydrolase. All B[a]P metabolites (table 3) were well separated by HPLC and the respective formation rates were determined. Incubations with control microsomes not expressing CYP1A1 and OR were negative for B[a]P metabolism, as were assays performed without NADPH (data not shown).

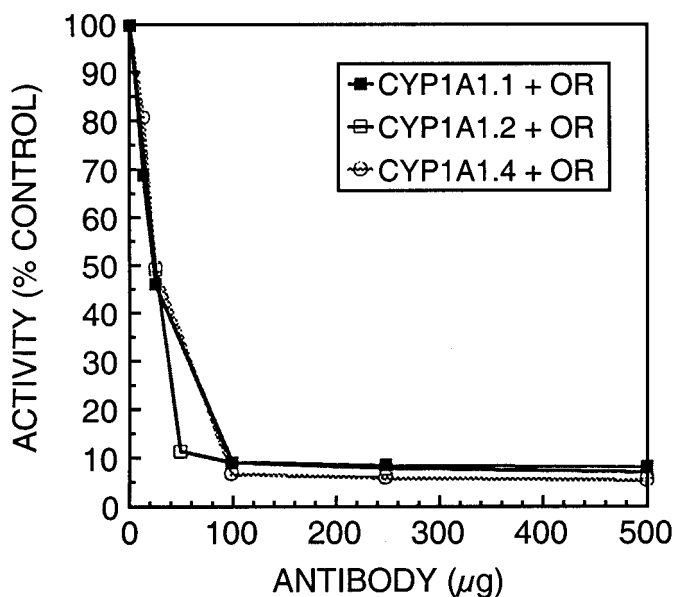


Figure 4. Inhibition of CYP1A1-catalysed EROD activity by monoclonal antibodies to human CYP1A1; ■, CYP1A1.1; □, CYP1A1.2; ○, CYP1A1.4. Antibodies were pre-incubated with 10 pmol of the respective CYP1A1 variant (in microsomes) as described in the Materials and methods. 7-ER concentration: 0.43 μM. Control: activities in the formation of resorufin for CYP1A1.1, CYP1A1.2 and CYP1A1.4 without antibodies were 9.0, 11.7 and 13.9 nmol min⁻¹ nmol⁻¹ CYP1A1, respectively. Data are the average of three determinations.

Total metabolism of B[a]P was highest with the wild-type CYP1A1. CYP1A1.4 produced metabolites at a rate of almost 60% of the wild-type and CYP1A1.2 of only ~40%. The metabolism of B[a]P by wild-type human CYP1A1 has been studied extensively (Shimada *et al.* 1989, Shou *et al.* 1994, Bauer *et al.* 1995, Gautier *et al.* 1996) whereby differences between the studies in the formation rates of B[a]P metabolites were reported, which are probably due to the use of

Table 3. Formation rates of B[a]P metabolites by human CYP1A1 variants co-expressed with NADPH-cytochrome P450 reductase^a.

	CYP1A1.1	CYP1A1.2	CYP1A1.4
3-Hydroxy-B[a]P	1.57 ± 0.15	0.55 ± 0.07	0.74 ± 0.10
9-Hydroxy-B[a]P	0.78 ± 0.10	0.27 ± 0.06	0.40 ± 0.06
B[a]P-7,8-dihydrodiol	0.70 ± 0.10	0.18 ± 0.08	0.41 ± 0.06
B[a]P-9,10-dihydrodiol	0.70 ± 0.10	0.24 ± 0.08	0.47 ± 0.06
B[a]P-4,5-dihydrodiol	0.13 ± 0.03	0.01 ± 0.01	0.12 ± 0.03
B[a]P-1,6-dione	0.03 ± 0.01	0.04 ± 0.01	0.13 ± 0.03
B[a]P-3,6-dione	0.84 ± 0.12	0.58 ± 0.07	0.48 ± 0.06
B[a]P-6,12-dione	0.80 ± 0.10	0.35 ± 0.09	0.48 ± 0.06
Total	5.57	2.22	3.23

^a Microsomal preparations of CYP1A1 variants were incubated with epoxide hydrolase in the presence of 25 μM B[a]P, as described in the Materials and methods. Metabolites were analysed by HPLC as described in the Materials and methods. Rates are means ± SD of three analyses and are expressed as nmol min⁻¹ nmol⁻¹ CYP1A1.

different expression systems and assay conditions. Catalytic activities of insect cell-expressed human wild-type CYP1A1 were previously obtained using reconstitution with purified OR based on both Sf9 membranes and purified enzyme (Buters *et al.* 1995). Comparison of our activities after co-expression with those from reconstitution showed that all major metabolites were formed in all systems. Our total metabolic rates were intermediary between those determined for reconstituted membranes and reconstituted purified CYP1A1 (Buters *et al.* 1995). Likewise, for commercially available CYP1A1-supersomes, absolute and relative rates of metabolite formation roughly comparable with ours were reported, considering that these values were measured in the presence of 10 μ M B[a]P (Kim *et al.* 1998). Further, the present results are in agreement with the only study which reported rates for the CYP1A1.2 variant for metabolites associated with the carcinogenicity of B[a]P (Zhang *et al.* 1996). Zhang *et al.* found for reconstituted, purified *E. coli*-expressed CYP1A1 rates for formation of B[a]P-7,8-dihydrodiol and B[a]P-9,10-dihydrodiol which were $\sim$ 2-3-fold higher for the wild-type enzyme compared with the CYP1A1.2 variant. Data for CYP1A1.4, to our knowledge, have not been reported until now. Taken collectively, all three CYP1A1 variants co-expressed with OR were active in producing all B[a]P metabolites with high rates.

In conclusion, the three CYP1A1 variants could be each co-expressed with OR as functionally active unmodified, native enzymes in the microsomal membrane allowing studies of protein-protein interaction and catalytic properties in a natural membrane environment. Expression was optimized to yield catalytically active systems with similar characteristics, particularly the stoichiometry of the enzymes. The results have shown that the co-infection approach and the use of simple microsomal preparations provides a good basis to elucidate possible differences in the catalytical properties of the various CYP1A1 variants without the need for subsequent purification.

Acknowledgements

The authors are grateful to Dr F. J. Gonzalez for providing CYP1A1 cDNA and virus for OR expression and to Dr H. V. Gelboin for monoclonal antibodies (both at National Cancer Institute, NIH, Bethesda, MD). The authors thank Dr A. Chernogolov and C. Andrée for gel electrophoresis and Western blotting, R. Zummach (Max Delbrueck Centre for Molecular Medicine, Berlin-Buch) for assistance in HPLC, and A. Sternke for skilful cell culture. The studies were supported by grants of the German Research Foundation DFG to I. R. and D. S. (RO 1287/2-1+ 2) and the Volkswagen-Stiftung to D. S. (I/75 468).

References

- BAUER, E., GUO, Z., UENG, Y. F., BELL, L. C., ZELDIN, L. and GUENGERICH, F. P., 1995, Oxidation of benzo[a]pyrene by recombinant human cytochrome P450 enzymes. *Chemical Research in Toxicology*, **8**, 136-142.
- BURKE, M. D. and MAYER, R. T., 1983, Inherent specificities of purified cytochromes P450 and P448 toward biphenyl hydroxylation and ethoxyresorufin deethylation. *Chemical and Biological Interactions*, **45**, 243-258.
- BUTERS, J. T. M., SHOU, M., HARDWICK, J. P., KORZEKWA, K. R. and GONZALEZ, F. J., 1995, cDNA-directed expression of human cytochrome P450 CYP1A1 using baculovirus. *Drug Metabolism and Disposition*, **23**, 696-701.

- CASCORBI, I., BROCKMOLLER, J. and ROOTS, I., 1996, A C4887A polymorphism in exon 7 of human CYP1A1: population frequency, mutation linkages, and impact on lung cancer susceptibility. *Cancer Research*, **56**, 4965-4969.
- CHEN, L., BUTERS, J. T. M., HARDWICK, J. P., TAMURA, S., PENMAN, T. W., GONZALEZ, F. J. and CRESPI, C. L., 1997, Coexpression of cytochrome P450A6 and human NADPH-P450 oxidoreductase in the baculovirus system. *Drug Metabolism and Disposition*, **25**, 399-405.
- GAUTIER, J. C., LECOEUR, S., COSME, J., PERRET, A., URBAN, P., BEAUNE, P. and POMPON, D., 1996, Contribution of human cytochrome P450 to benzo[a]pyrene and benzo[a]pyrene-7,8-dihydrodiol metabolism, as predicted from heterologous expression in yeast. *Pharmacogenetics*, **6**, 489-499.
- GONZALEZ, F. J. and KORZEKWA, K. R., 1995, Cytochromes P450 expression systems. *Annual Review of Pharmacology and Toxicology*, **35**, 369-390.
- GUENGERICH, F. P. and SHIMADA, T., 1998, Activation of procarcinogens by human cytochrome P450 enzymes. *Mutation Research*, **400**, 201-213.
- HAYASHI, S., WATANABE, J., NAKACHI, K. and KAWAJIRI, K., 1991, Genetic linkage of lung cancer-associated *Msp*I polymorphisms with amino acid replacement in the heme binding region of the human cytochrome P450IA1 gene. *Journal of Biochemistry*, **110**, 407-411.
- HOLDER, G., YAGI, H., DANSETTE, P., JERINA, D. M., LEVIN, W., LU, A. Y. H. and CONNEY, A. H., 1974, Effects of inducers and epoxide hydrolase on the metabolism of benzo[a]pyrene by liver microsomes and a reconstituted system: Analysis by high-pressure liquid chromatography. *Proceedings of the National Academy of Sciences, USA*, **72**, 4356-4360.
- HOULSTON, R. S., 2000, CYP1A1 polymorphisms and lung cancer risk: a meta-analysis. *Pharmacogenetics*, **10**, 105-114.
- HUANG, C., CHERN, H., CHANG, K., CHENG, C., HSU, S. and SHEN, C., 1999, *Cancer Research*, **59**, 4870-4875.
- INGELMAN-SUNDBERG, M., DALY, A. K., OSCARSON, M. and NEBERT, D. W., 2000, Human cytochrome P450 (CYP) genes: recommendations for the nomenclature of alleles. *Pharmacogenetics*, **10**, 91-93.
- JAISWAL, A. K., GONZALEZ, F. J. and NEBERT, D. W., 1985, Human dioxin-inducible cytochrome P₁-450: complementary DNA and amino acid sequence. *Science*, **228**, 80-83.
- KIM, J. H., STANSBURY, K. H., WALKER, N. J., TRUSH, M. A., STRICKLAND, P. T. and SUTTER, T. R., 1998, Metabolism of benzo[a]pyrene and benzo[a]pyrene-7,8-diol by human cytochrome P450 1B1. *Carcinogenesis*, **19**, 1847-1853.
- NEBERT, D. W., INGELMAN-SUNDBERG, M. and DALY, A. K., 1999, Genetic epidemiology of environmental toxicity and cancer susceptibility: human allelic polymorphisms in drug-metabolizing enzyme genes, their functional importance, and nomenclature issues. *Drug Metabolism Reviews*, **31**, 467-487.
- ONG, C. E., MINERS, J. O., BIRKETT, D. J. and BHASKER, C. R., 1998, Baculovirus-mediated expression of cytochrome P450C8 and human NADPH-cytochrome P450 reductase: optimization of protein expression. *Xenobiotica*, **28**, 137-152.
- PAINE, M. J. I., GILHAM, D., ROBERTS, G. C. K. and WOLF, C. R., 1996, Functional high level expression of cytochrome P450D6 using baculoviral expression systems. *Archives of Biochemistry and Biophysics*, **328**, 143-150.
- PERSSON, I., JOHANSSON, I. and INGELMAN-SUNDBERG, M., 1997, *In vitro* kinetics of two human CYP1A1 variant enzymes suggested to be associated with interindividual differences in cancer susceptibility. *Biochemical and Biophysical Research Communications*, **231**, 227-230.
- SATO, M., SATO, T., IZUMO, T. and AMAGASA, T., 1999, Genetic polymorphism of drug-metabolizing enzymes and susceptibility to oral cancer. *Carcinogenesis*, **20**, 1927-1931.
- SCHWARZ, D., KISSELEV, P., SCHUNCK, W. H., CHERNOGOLOV, A., BODOL, W., CASCORBI, I. and ROOTS, I., 2000, Allelic variants of human cytochrome P450 1A1 (CYP1A1): effect of T461N and I462V substitutions on steroid hydroxylase specificity. *Pharmacogenetics*, **10**, 519-530.
- SELKIRK, J. K., CROY, R. G. and GELBOIN, H. V., 1974, Benzo[a]pyrene metabolites: efficient and rapid separation by high-pressure liquid chromatography. *Science*, **184**, 169-171.
- SHIMADA, T., MARTIN, M. V., PRUESS-SCHWARTZ, D., MARNETT, L. J. and GUENGERICH, F. P., 1989, Roles of individual human cytochrome P450 enzymes in the bioactivation of benzo[a]pyrene, 7,8-dihydroxy-7,8-dihydrobenzo[a]pyrene, and other dihydrodiol derivatives of polycyclic aromatic hydrocarbons. *Cancer Research*, **49**, 6304-6312.
- SHOU, M., KORZEKWA, K. R., CRESPI, C. L., GONZALEZ, F. J. and GELBOIN, H. V., 1994, The role of 12 cDNA-expressed human, rodent, and rabbit cytochromes P450 in the metabolism of benzo[a]pyrene and benzo[a]pyrene *trans*-7,8-dihydrodiol. *Molecular Carcinogenesis*, **10**, 159-168.
- TAMURA, S., KORZEKWA, K. R., KIMURA, S., GELBOIN, H. V. and GONZALEZ, F. J., 1992, Baculovirus-mediated expression and functional characterization of human NADPH-P450 oxidoreductase. *Archives of Biochemistry and Biophysics*, **293**, 219-223.

- TAN, Y., PATTEN, C. J., SMITH, T. and YANG, C. S., 1997, Competitive interactions between cytochromes P450 2A6 and 2E1 for NADPH-cytochrome P450 oxidoreductase in the microsomal membranes produced by a baculovirus expression system. *Archives of Biochemistry and Biophysics*, **342**, 82-91.
- WANG, M. H., PATTEN, C. J., YANG, G. Y., PARANAWITHANA, S. R., TAN, Y. and YANG, C. S., 1996, Expression and coupling of human cytochrome P450 2E1 and NADPH-cytochrome P450 oxidoreductase in dual expression and co-infection systems with baculovirus in insect cells. *Archives of Biochemistry and Biophysics*, **334**, 380-388.
- WERLINDER, V., ZHUKOV, A. and INGELMAN-SUNDBERG, M., 1998, Posttranslational regulation of cytochromes P4501A1 and 1A2. Paper presented at the 12th International Symposium on Microsomes and Drug Oxidations, 20-24 July, Montpellier, abst. 370.
- YASUKOCHI, Y. and MASTERS, B. S. S., 1976, Some properties of a detergent-solubilized NADPH-cytochrome c (cytochrome P-450) reductase purified by biospecific affinity chromatography. *Journal of Biological Chemistry*, **251**, 5337-5344.
- ZHANG, Z. Y., FASCO, M. J., HUANG, L., GUENGERICH, F. P. and KAMINSKY, L. S., 1996, Characterization of purified human recombinant cytochrome P4501A1-Ile⁴⁶² and -Val⁴⁶²: assessment of a role for the rare allele in cancerogenesis. *Cancer Research*, **56**, 3926-3933.
- ZHUKOV, A. and INGELMAN-SUNDBERG, M., 1999, Relationship between cytochrome P450 catalytic cycling and stability: fast degradation of ethanol-inducible cytochrome P450 2E1 (CYP2E1) in hepatoma cells is abolished by inactivation of its electron donor NADPH-cytochrome P450 reductase. *Biochemical Journal*, **340** (Pt 2), 453-458.

Disposition of propargyl alcohol in rat and mouse after intravenous, oral, dermal and inhalation exposure

K. J. DIX[‡], D. P. COLEMAN[‡], J. E. FOSSETT[‡],
N. F. GAUDETTE, JR[‡], A. P. STANLEY[‡], B. F. THOMAS[‡]
and A. R. JEFFCOAT^{‡*}

[‡]Research Triangle Institute, PO Box 12194, 3040 Cornwallis Road, Research Triangle Park, NC 27709, USA

Received 11 January 2001

1. The disposition of propargyl alcohol (PAL) radiolabelled with carbon-14 ([2,3-¹⁴C]PAL) was determined in the F344 rat and B6C3F₁ mouse following intravenous (i.v.), oral, inhalation and dermal exposure.

2. By 72 h following an i.v. (1 mg kg⁻¹) or oral (50 mg kg⁻¹) dose, 76-90% of the dose was excreted. Major routes of excretion by rat were urine (50-62%), CO₂ (19-26%) and faeces (6-14%). Major routes of excretion by mouse were urine (30-40%), CO₂ (22-26%) and faeces (10-20%). Less than 6% of the dose remained in tissues at 72 h. Biliary excretion of radioactivity by rat (62% in 4 h) was much greater than elimination in faeces (6% in 72 h), indicating that PAL metabolites underwent extensive enterohepatic recycling.

3. Dermal exposure studies demonstrated that dermal absorption of PAL was minimal due to its inherent volatility.

4. In the inhalation studies (1, 10 or 100 ppm for 6 h), 23-68% of the radioactivity to which animals were exposed was absorbed. The primary route of excretion was urine (23-53%), and a significant portion was exhaled as volatile organics (15-30%).

5. PAL was extensively metabolized by both species. One metabolite was identified as 3,3-bis[(2-(acetylamino)-2-carboxyethyl)thio]-1-propanol, which is consistent with Banijamali *et al.* (1999).

Introduction

Propargyl alcohol (2-propyn-1-ol, PAL) (figure 1) is an acetylenic alcohol, high-production volume chemical used as a chemical/pharmaceutical intermediate, a corrosion inhibitor, and a solvent stabilizer (Dalton *et al.* 1963, HSDB 2000). At room temperature, PAL is a moderately volatile liquid, and there is potential human occupational exposure to PAL by inhalation and dermal contact.

The toxicity of PAL has been evaluated in *in vitro* and *in vivo* assays. The oral LD₅₀ of PAL is 50-60 mg kg⁻¹ for the female and 100-120 mg kg⁻¹ for the male Sprague-Dawley rat (Archer 1985). The inhalation (1 h) LC₅₀ of PAL is 970-1120 ppm for the female and 1180-1220 ppm for the male Sprague-Dawley rat (Vernot *et al.* 1977). Repeated oral exposure to PAL resulted in liver and kidney toxicity in rat (Rubinstein *et al.* 1989, Lington and Bevan 1994). PAL is negative to weakly positive in Ames tests for mutagenicity, positive in the Chinese hamster ovary

[‡]Present address: Lovelace Respiratory Research Institute, PO Box 5890, Albuquerque, NM 87185, USA.

*Author for correspondence; e-mail: arj@rti.org

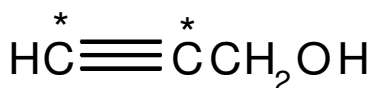


Figure 1. Structure of propargyl alcohol. *Location of radiolabel.

assay for chromosome aberrations, and negative in the mouse bone marrow micronucleus assay (Blakely *et al.* 1994).

PAL is metabolized to a very reactive α,β -unsaturated aldehyde, 2-propyn-1-al, by liver catalase *in vitro* (DeMaster *et al.* 1994) and by alcohol oxidase isolated from yeast (Nichols and Cromartie 1980). This reactive aldehyde may be responsible for the inhibition of liver aldehyde dehydrogenase (DeMaster *et al.* 1986). Furthermore, unlike allylic alcohols, PAL is a poor substrate for alcohol dehydrogenase (DeMaster *et al.* 1994). The metabolism of [1,2,3- ^{13}C 2,3- ^{14}C]PAL by the male Sprague-Dawley rat has been reported by Banijamali *et al.* (1999). About 56% of an orally administered dose (40 mg kg $^{-1}$) was excreted in urine by 96 h after dose administration. The major urinary metabolites resulted from the conjugation of one or more glutathione molecules across the triple bond. Reported urinary metabolites are 3-(carboxymethylthio)-2-propenoic acid, 3-(methylsulphinyl)-2-(methylthio)-2-propenoic acid, 3,3-bis[(2-(acetylamino)-2-carboxyethyl)thio]-1-propanol, 3-[[2-(acetylamino)-2-carboxyethyl]thio]-3-[[2-amino-2-carboxyethyl]thio]-1-propanol, and 3-[[2-(acetylamino)-2-carboxyethyl]sulphinyl]-3-[[2-(acetylamino)-2-carboxyethyl]thio]-1-propanol.

The objectives of the current study were to evaluate the disposition of PAL in rat and mouse following oral, intravenous, dermal and inhalation exposure.

Materials and methods

Chemicals

Non-radiolabelled PAL (99%) was obtained from Aldrich Chemical Company, Inc. (Milwaukee, WI, USA). [2,3- ^{14}C]PAL (2.33 mCi mmol $^{-1}$) was received from New England Nuclear (Boston, MA, USA). The radiochemical purity of [^{14}C]PAL was determined prior to each use and ranged from 90 to 95%. At least two minor radiolabelled impurities eluted prior to and one following [^{14}C]PAL. Acetonitrile (HPLC grade) and sodium hydroxide (50% w/w) were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Methyl *t*-butyl ether (MTBE) (high-purity solvent) was purchased from Burdick & Jackson, Inc. (Muskegon, MI, USA). Magnesium sulphate (anhydrous powder), hydrogen peroxide (30%) and trifluoroacetic acid (TFA) (reagent grade) were purchased from J. T. Baker (Phillipsburg, NJ, USA). Perchloric acid was purchased from EM Science (Gibbstown, NJ, USA). Soluene-350 tissue solubilizer and Ultima Gold scintillation cocktail were purchased from Packard Instrument Company, Inc. (Meriden, CT, USA). Sodium chloride (0.9% aqueous) was purchased from Baxter Healthcare Corporation (Deerfield, IL, USA). Absolute ethanol (200 proof) was purchased from McCormick Distilling Co., Inc. (Weston, MO, USA) and from Aaper Alcohol Chemical Co. (Shelbyville, KY, USA).

Animals

All studies were conducted in accordance with US federal guidelines for the care and use of laboratory animals and were approved by the RTI Institutional Animal Care and Use Committee. Male Fischer 344 (F344) rats (10–16 weeks old on study; 201–264 g on dose day) and male B6C3F $_1$ mice (10–16 weeks old on study; 21.1–33.8 g on dose day) were purchased from Charles River Laboratories (Raleigh, NC, USA). The number of animals used in each study is summarized in table 1. Animals were held in quarantine for at least 1 week prior to use in a study and were allowed feed (Certified Purina Rodent Chow 5002) and tap water *ad libitum*. Animal quarters were maintained at 64–79°F and 30–70% relative humidity. Light/darkness was cycled at 12-h intervals. Animals used for bile collection were anaesthetized and kept on heating pads to maintain body temperature throughout the experiment. In

Table 1. Summary of experimental treatments.

Study design ^a	Dose route	Target dose or atmosphere concentration	Number of animals	Actual dose (mg kg ⁻¹) or atmosphere concentration (ppm) ^b (mean ± SD)
Male F344 rat				
TD	i.v.	1 mg kg ⁻¹	28 ^c	1.00 ± 0.04
BE	i.v.	1 mg kg ⁻¹	4	1.01 ± 0.03
MB	i.v.	1 mg kg ⁻¹	5	0.94 ± 0.06
MB	oral	50 mg kg ⁻¹	5	49.9 ± 0.2
MB	dermal	5 mg kg ⁻¹	4	6.41 ± 1.35
MB	inhalation	1 ppm	4	0.910 ± 0.190
MB	inhalation	10 ppm	4	10.0 ± 1.54
MB	inhalation	100 ppm	4	101 ± 8.74
Male B6C3F ₁ mouse				
TD	i.v.	1 mg kg ⁻¹	28 ^c	0.976 ± 0.057
MB	i.v.	1 mg kg ⁻¹	5	1.06 ± 0.05
MB	oral	50 mg kg ⁻¹	4	49.9 ± 0.1
MB	inhalation	1 ppm	4	0.910 ± 0.190
MB	inhalation	10 ppm	4	10.0 ± 1.54
MB	inhalation	100 ppm	4	101 ± 8.74

^aTD, tissue distribution study; BE, biliary excretion study; MB, mass balance study.

^bAverage atmosphere concentration determined chromatographically over the 6-h exposure.

^cN = 4 per species per time point for each of seven time points.

mass balance studies, animals were housed individually in all-glass metabolism cages that provided for the separate collection of urine, faeces, CO₂ and expired volatile organics. Animals were acclimated to the metabolism cages 1 day prior to dosing. For inhalation studies, animals were acclimated to Battelle® restrainers (Geneva) for 2 h a day on the 2 days prior to exposure and restrained during the 6-h exposure.

Anaesthesia and euthanasia. Anaesthesia was used to avoid undue pain or distress. Rats were anaesthetized with ketamine/xylazine (7:1, 50–80 mg kg⁻¹ or to effect) prior to preparation of the dermal dose site and at the time the dermal appliance was removed. Rats used in the biliary excretion study were anaesthetized with sodium pentobarbital orally (35 mg kg⁻¹) and intraperitoneally (45 mg kg⁻¹) prior to surgery and intraperitoneally (5 mg kg⁻¹) as needed to maintain a state of anaesthesia during the bile collection period. At the end of the studies, conscious rats were anaesthetized with ketamine/xylazine followed by cardiac puncture. Anaesthetized and exsanguinated rats were euthanized by section of the diaphragm. Mice were anaesthetized with CO₂ and euthanized by cervical dislocation following cardiac puncture.

Preparation of animals for biliary excretion study. Rats used in the biliary excretion study were surgically prepared in-house. Animals were anaesthetized for the duration of the study. A midline incision was made from the xiphoid process to the inguinal region. The bile duct was ligated at its entrance to the duodenum, and a small cut was made in the duct anterior to the ligature. The bevelled end of a small length of PE-50 tubing was inserted into the duct and secured with silk sutures. The cannula was exteriorized and the abdominal incision closed with sutures.

Preparation of animals for dermal studies. About 24 h prior to dosing, rats used in dermal dose studies were anaesthetized as described above. The back and shoulders of each animal were then clipped to remove the fur. The clipped area was washed with a soap solution, rinsed with water and inspected for cuts or nicks. If an animal had damaged skin in the area intended for dosing, it was removed from the study and replaced with another. The area to which the dose was applied (~ 3.0 × 4.0 cm or 12 cm²) was outlined with a waterproof marker, and the animal was allowed to recover overnight.

Dose preparation and administration

Actual administered PAL doses are summarized in table 1. Intravenous (i.v.) dose formulations contained [¹⁴C]PAL in a 9:1 (v/v) preparation of 0.9% aqueous sodium chloride and ethanol. They were

administered into a lateral tail vein from a 1-ml glass disposable syringe (GlasPak[®], Becton-Dickenson Co.) equipped with a 27-gauge $\times$ 1/2 inch needle for rat and a 100- μ l gas-tight Hamilton syringe equipped with a 30-gauge $\times$ 1/2 inch needle for mouse. The target i.v. dose was 1.0 mg PAL kg⁻¹ body weight in a dose volume of 1 ml kg⁻¹.

Oral dose formulations contained [¹⁴C]PAL and non-radiolabelled PAL in water. The target oral dose was 50 mg PAL kg⁻¹ body weight in a dose volume of 5 ml kg⁻¹. The dose was administered from either a 2.5-ml disposable syringe equipped with a 16-gauge $\times$ 2 inch gavage needle with a 3-mm ball tip (rat) or a 250- μ l Leur-lock syringe equipped with a 18-gauge $\times$ 1.5 inch gavage needle with a 2.25-mm ball tip (mouse).

The dermal dose formulations contained [¹⁴C]PAL in ethanol at a target concentration of 6.25 mg PAL ml⁻¹ formulation. The target dose was $\sim$ 5 mg kg⁻¹, which is about one-tenth the dermal LD₅₀ determined for rabbit (Vernot *et al.* 1977). Before dosing, a non-occlusive protective appliance was attached to the animal just outside of the dose site with medical adhesive (Hollister, Inc., Libertyville, IL, USA). This protective appliance consisted of a 1-cm high frame of polyurethane foam with an outer covering of charcoal-impregnated material cut from a dust/mist respirator (Cat. 9913; 3M, St Paul, MN, USA). Immediately after dosing, a cover fashioned of the same charcoal-impregnated material was placed over the dose site and secured to the top of the frame to trap [¹⁴C]PAL that volatilized from the dose site. The dermal dose was administered in a volume of $\sim$ 200 μ l over a surface area of 12 cm², which corresponded to a total dose of $\sim$ 1.25 mg/rat. The dose formulation was applied to the dose site with a 1-ml glass disposable syringe equipped with a 16-gauge, 3-mm ball-tipped needle and was spread as evenly as possible over the entire dose site.

Generation and delivery of PAL vapour

Nose-only inhalation experiments were conducted using an apparatus constructed at the Research Triangle Institute as described (Slauter *et al.* 1994). All surfaces contacting PAL vapours prior to inspiration by study animals were stainless steel, glass or Teflon[®].

Inlet air flow entered a glass vapour-generation column (60 cm $\times$ 11 mm i.d.) filled with 3-mm glass beads and maintained at $\sim$ 86°C. A feedstock solution that contained water (low dose only), radiolabelled PAL and non-radiolabelled PAL was metered into the bead bed through small-bore Teflon[®] tubing using an infusion pump. The proper infusion rate was determined experimentally during preliminary trials.

Target atmosphere concentrations for inhalation exposures were 1, 10 and 100 ppm. Actual average atmosphere concentrations are summarized in table 1.

Analysis of exposure atmosphere

PAL vapour concentrations in inhalation exposure atmospheres were measured by capillary GC on an EC-WAX Econo-Cap column (0.53 mm i.d. $\times$ 30 m; Alltech Associates, Inc., Deerfield, IL, USA). Standard curves were constructed using solvent standards containing the mass of PAL expected in 1 ml vapour at PAL concentrations ranging from 0.5 to 1.5 ppm ($r^2 = 0.999$, CV < 4.5% at all concentrations) for the 1-ppm exposure, from 5 to 15 ppm ($r^2 = 0.998$, CV < 2.9% at all concentrations) for the 10-ppm exposure, and from 70 to 118 ppm ($r^2 = 1.000$, CV < 2.2% at all concentrations) for the 100-ppm exposure.

Tissue distribution time-course studies

Animals ($n = 28$ rat, 28 mouse) received a single i.v. dose via a lateral tail vein. The average doses were 1.00 ± 0.04 mg kg⁻¹ for rat and 0.976 ± 0.057 mg kg⁻¹ for mouse. Groups of four animals of each species were sacrificed at 0.25, 0.5, 1, 2, 4, 8 and 24 h post-dosing, and the following tissues were harvested: blood, skin, muscle, adipose, kidney, liver, spleen, lung, testis, bladder, heart, brain, stomach (with contents), small intestine (with contents), caecum (with contents) and large intestine (with contents).

Biliary excretion study

Anaesthetized and bile duct cannulated rats ($n = 4$) received a single i.v. dose (1.01 ± 0.03 mg kg⁻¹) via a lateral tail vein. Following dose administration, bile was collected at 30-min intervals for up to 4 h into tarred vials.

Mass balance studies

Mass balance studies were conducted after i.v., dermal, oral and inhalation exposure to [¹⁴C]PAL. Animals were housed individually in all-glass metabolism cages throughout the studies except during (1) dose administration and (2) removal of the protective appliance and unabsorbed PAL in dermal administration studies. Urine and faeces were collected over dry ice in timed fractions. Radiolabelled volatile organics and carbon dioxide were collected in cryogenic ethanol and sodium hydroxide traps,

respectively. Urine, faeces, volatile organics and carbon dioxide were collected in timed fractions ending at 6 (except faeces), 12 and 24 h post-dosing in all mass balance studies. Additional collections were made at 48 and 72 h post-dosing for the i.v. and oral dose studies. At necropsy, urine was removed from the urinary bladder and added to the final urine collection. Metabolism cages were rinsed at the end of the experiment with water and ethanol. Animals were killed at 24 (inhalation and dermal exposures) or 72 h (i.v. and oral exposures) post-dosing. The following tissues were harvested except in the dermal and inhalation exposure studies: blood (cardiac puncture), skin (ears), muscle (hind leg and abdominal), adipose tissue (epididymal and perirenal), kidney, liver, spleen, lung, testis, bladder, heart, brain, stomach (with contents), small intestine (with contents), caecum (with contents) and large intestine (with contents). In i.v. administration studies, animals ($n = 5$ rats, 5 mice) received average doses of 0.94 ± 0.06 mg kg⁻¹ (rat) and 1.06 ± 0.05 mg kg⁻¹ (mouse). In oral administration studies, animals ($n = 5$ rats, 4 mice) received average doses of 49.9 ± 0.2 mg kg⁻¹ (rat) and 49.9 ± 0.1 mg kg⁻¹ (mouse). In addition to evaluating mass balance, a second objective of the oral dose study was to obtain sufficient masses of excreted metabolites for identification. Hence, the oral dose was high relative to the i.v. dose.

In dermal administration studies, rat ($n = 4$) received an average dose of 6.41 ± 1.35 mg kg⁻¹. After application of the dose, animals were placed in individual metabolism cages for the duration of the 6-h exposure. At the end of the exposure period, the animals were anaesthetized, appliances removed, dose sites washed to remove any unabsorbed PAL, then the animals were returned to their metabolism cages for the remainder of the study. PAL trapped by the charcoal-impregnated filters was extracted with ethanol. At the end of the study, the animals were again anaesthetized and the dose sites gently scrubbed with gauze soaked in water. The animals were then euthanized, and the dose sites were excized and repeatedly rinsed with water. The unabsorbed dose was determined by summing the radioactivity recovered from the protective appliance, ethanol extracts of charcoal-impregnated traps, and the dose site wash. The absorbed dose was determined by summing the radioactivity recovered from excreta, expired breath traps, cage rinse, blood, carcass, and dose site.

In inhalation studies, animals ($n = 4$ rats, 4 mice per exposure concentration) were exposed to PAL vapours at concentrations of 0.910 ± 0.190 , 10.0 ± 1.54 or 101 ± 8.74 ppm. Individual animal doses were calculated based on the total mass of PAL absorbed by each animal during exposure. Absorbed PAL (mg) was calculated by dividing the total radiolabel recovered in the carcass, exhaled breath and excreta for each animal (μ Ci) by the specific activity of [¹⁴C]PAL in the feedstock solution used for vapour generation (μ Ci mg⁻¹ PAL). Absorbed doses were 0.0816 ± 0.0053 , 0.901 ± 0.089 and 4.93 ± 0.39 mg for rat in the 1-, 10- and 100-ppm exposure groups, respectively. For mouse, absorbed doses were 0.0314 ± 0.0086 , 0.284 ± 0.088 and 1.06 ± 0.35 mg for animals in the 1-, 10- and 100-ppm exposure groups.

The percentage of inhaled PAL absorbed over the exposure duration was calculated to determine the effect of exposure concentration on absorption. Absorbed doses (mg) were divided by the total PAL mass inhaled over the exposure duration to determine the percentage of inhaled PAL absorbed (per cent absorption). Rat and mouse respiratory minute volumes (RMVs) were multiplied by the time-weighted average exposure concentration and exposure duration to calculate the total PAL mass inhaled during exposure. RMVs used for these calculations were 174 and 54 ml min⁻¹ for rat and mouse, respectively (Chang *et al.* 1983, Brown, 1997).

Liquid scintillation spectrometry (LSS)

Samples were assayed for total radioactivity by LSS either directly (urine, bile, ethanol trapping solution, sodium hydroxide trapping solution, dermal appliance, charcoal-impregnated filter extracts, skin-wash gauze) or following solubilization in Soluene-350 (faeces, tissues) or ethanolic sodium hydroxide (stomach, caecum, small and large intestines, dermal dose site, residual carcass). Control samples of urine, faeces, ethanol trapping solution, sodium hydroxide trapping solution and bile were collected prior to dose administration and analysed for radiochemical content to determine background counts.

High-pressure liquid chromatography (HPLC)

All liquid chromatographic analyses were conducted using two Waters model 510 pumps (Milford, MA, USA), a Rheodyne model 7125 injector (Alltech Associates, Inc., Deerfield, IL, USA), a variable wavelength UV detector set at 210 or 254 nm (Applied Biosystems 757 Absorbance Detector, Aston, PA, USA), and a flow-through radioactivity detector (β -RAM with 500- μ l glass cell; IN/US Instruments, Tampa, FL, USA). All mobile phases were prepared by volume, and all gradients were linear. Various columns and mobile phase systems were used for different tasks and are described below. Except where noted, mobile phases were mixtures of 0.1% TFA in acetonitrile (A) and 0.1% TFA in water (B).

Radiochemical purity in the dose preparations was determined using a Partisil PAC column (250×4.6 mm, 10 μ m; Whatman, Clifton, NJ or Phenomenex, Torrance, CA, USA) and an isocratic mobile phase that consisted of MTBE:acetonitrile 99:1 at a flow rate of 1 ml min⁻¹. For chromatographic analysis of the oral dose preparations, an aliquot of the dose preparation was mixed with

MTBE, and the solution dried over anhydrous magnesium sulphate. Prior to injection on the HPLC, mixtures were vortexed and then centrifuged at 16 000g for ~10 min.

Radiolabelled components in urine and bile were profiled using a Zorbax ODS analytical column (250 × 4.6 mm, 5 µm; Hewlett Packard Co., Wilmington, DE, USA). The flow rate of the mobile phase was 1 ml min⁻¹ for 35 min, 1–1.5 ml min⁻¹ over 1 min, then 1.5 ml min⁻¹ for the remainder of the run time. Mobile phase composition was: 0% A (100% B) for 3 min, 0–12% A (3–33 min), 12–100% A (33–35 min), then 100% A (35–42 min). To quantitate the various radiolabelled metabolites, fractions of each run were collected and analysed for carbon-14 content by LSS.

Radiolabelled metabolites of PAL were purified on a Phenomenex Luna C18 semipreparatory column (250 × 10 mm, 10 µm). The mobile phase composition was the same as that used to profile metabolites, but the flow rate of the mobile phase was 4 ml min⁻¹ for 38 min, 4–5.5 ml min⁻¹ over 2 min, and 5.5 ml min⁻¹ for the remainder of the run. The isolated fractions were neutralized with 10 µl TEA in acetonitrile (4.2 ml TEA plus 7.2 ml acetonitrile).

Metabolite identification

One purified metabolite was analysed by liquid chromatography/mass spectrometry (LC/MS) using a Finnigan LCQ quadrupole ion trap mass spectrometer and an electrospray atmospheric pressure interface (Finnigan, San Jose, CA, USA). A Phenomenex Aqua C18 reverse-phase HPLC column (250 × 4.6 mm, 5 µm; Torrance, CA, USA) was used with a mobile phase flow rate of 0.5 ml min⁻¹. Initially the column was equilibrated with 5% A. After sample injection, the mobile phase remained at 5% A for 5 min, then changed over 3 min to a final concentration of 30% A where it remained until the completion of the run. The mass spectrometer was set to scan in the positive-ion mode and scanned the *m/z* range 115–1000. When the base ion in the full scan reached > 10 000 abundance, a 'data dependent scan' was programmed to occur wherein the ion was isolated and a MS/MS scan performed on the daughter ions.

Statistics

Summary statistics (mean ± SD) only are presented.

Results

Tissue distribution time-course studies in rat and mouse

Results of tissue distribution time-course studies in rat and mouse receiving 1-mg kg⁻¹ i.v. doses are shown in tables 2 and 3, respectively. Throughout the entire time-course, the highest concentrations of radioactivity were observed in the organs of excretion (liver, kidney, urinary bladder). Radioactivity in other tissues, with the exception of adipose, was fairly evenly distributed. Overall, trends in the

Table 2. Time-course of radioactivity in tissues following a 1 mg kg⁻¹ i.v. dose of [¹⁴C]PAL to the male F344 rat.

Tissue	ng-equivalents PAL g ⁻¹ tissue ^a						
	0.25 h	0.5 h	1 h	2 h	4 h	8 h	24 h
Adipose	142 ± 15	126 ± 54	139 ± 87	87.1 ± 36.9	61.4 ± 16.4	98.0 ± 55.0	89.1 ± 47.0
Bladder	1020 ± 114	1080 ± 358	7660 ± 5960	4770 ± 2580	962 ± 566	210 ± 41	133 ± 10
Blood	1130 ± 75	805 ± 71	418 ± 95	223 ± 19	165 ± 11	122 ± 5	83.6 ± 3.5
Brain	509 ± 46	304 ± 77	207 ± 25	183 ± 8	149 ± 12	74.8 ± 6.3	46.5 ± 7.1
Heart	911 ± 85	678 ± 64	482 ± 72	287 ± 27	177 ± 19	82.3 ± 9.4	66.4 ± 2.8
Kidney	2430 ± 152	3750 ± 676	3890 ± 105	2730 ± 283	1290 ± 111	541 ± 60	325 ± 18
Liver	2390 ± 146	2130 ± 304	1310 ± 264	798 ± 76	669 ± 66	577 ± 80	238 ± 26
Lung	859 ± 135	617 ± 89	354 ± 38	222 ± 34	171 ± 18	150 ± 8	127 ± 7
Muscle	796 ± 46	462 ± 113	240 ± 108	143 ± 78	78.3 ± 14.1	44.3 ± 7.7	27.3 ± 1.7
Skin	691 ± 57	538 ± 96	358 ± 68	233 ± 110	147 ± 27	101 ± 27	93.5 ± 1.7
Spleen	758 ± 44	584 ± 93	500 ± 47	367 ± 16	253 ± 17	185 ± 13	147 ± 23
Testis	756 ± 58	457 ± 69	300 ± 41	182 ± 22	100 ± 9	69.2 ± 5.2	42.0 ± 3.2

^aMean ± SD, *n* = 4.

Table 3. Time-course of radioactivity in tissues following a 1 mg kg⁻¹ i.v. dose of [¹⁴C]PAL to male B6C3F₁ mouse.

Tissue	ng-equivalents PAL g ⁻¹ tissue ^a						
	0.25 h	0.5 h	1 h	2 h	4 h	8 h	24 h
Adipose	176 ± 113	121 ± 61	191 ± 103	106 ± 44	154 ± 155	73.8 ± 62.6	26.7 ± 12.9
Bladder	942 ± 492	722 ± 289	3940 ± 4630	4040 ± 3230	2150 ± 1720	1860 ± 946	371 ± 60
Blood	793 ± 52	452 ± 23	239 ± 26	138 ± 30	97.4 ± 20.0	55.5 ± 3.1	34.6 ± 5.5
Brain	365 ± 52	192 ± 4	145 ± 26	107 ± 17	129 ± 38	43.1 ± 14.3	24.7 ± 5.5
Heart	620 ± 103	365 ± 23	234 ± 47	148 ± 27	115 ± 29	55.5 ± 17.2	29.2 ± 8.2
Kidney	1870 ± 193	2250 ± 433	2730 ± 705	1170 ± 239	1060 ± 519	312 ± 41	151 ± 25
Liver	2280 ± 376	895 ± 149	616 ± 134	338 ± 49	413 ± 76	248 ± 55	103 ± 20
Lung	671 ± 122	392 ± 29	261 ± 39	174 ± 39	179 ± 82	95.0 ± 12.6	52.7 ± 7.1
Muscle	477 ± 85	216 ± 2	116 ± 26	94.2 ± 39.4	64.2 ± 16.6	34.2 ± 6.9	17.2 ± 4.7
Skin	549 ± 105	332 ± 43	190 ± 51	142 ± 49	100 ± 41	58.3 ± 11.0	45.5 ± 14.8
Spleen	491 ± 92	358 ± 60	347 ± 60	236 ± 39	354 ± 157	158 ± 104	64.5 ± 10.3
Testis	438 ± 68	212 ± 37	238 ± 53	85.5 ± 13.3	111 ± 40	102 ± 76	26.2 ± 3.4

^aMean ± SD, *n* = 4.

per cent dose in tissues over time were very similar for rat and mouse. In most tissues, excluding kidney and urinary bladder, the highest radioactivity content was observed at 0.25 h following dosing. The concentration of radioactivity in rat kidney and bladder peaked at 1 h. In mouse, the concentration of radioactivity in the kidney and bladder peaked at 1 and 2 h, respectively. By 24 h following dosing, only 3-6% of the dose remained in the tissues.

A significant amount of radioactivity was present in the gastrointestinal tract of both rat and mouse after the i.v. dose (table 4). Radioactivity present in the small intestine (including lumen contents) reached a maximum of 30% of administered dose for rat (1-h time point) and 35% for mouse (0.5-h time point), then quickly dropped to 3-4% by 4 h following dosing for both species. As the per cent dose in the small intestine decreased, radioactivity in the caecum and large intestine increased but by a substantially lesser amount. This is likely due to enterohepatic circulation.

Biliary excretion study in rat

About 62% of the intravenously administered radioactivity was excreted in bile by 4 h post-dosing (figure 2). Excretion of radioactivity in bile was essentially complete by 2.5 h following dosing with ~59% of the dose being excreted in bile during this period.

Intravenous and oral dose mass balance studies in rat and mouse

Results of the i.v. dose mass balance studies are shown in table 5. Rats rapidly excreted PAL-derived radioactivity, with 83.9% of the dose excreted in the first 24 h after dosing. By 72 h, 88.4% of the dose was recovered in excreta, primarily in urine (62.3%) and as CO₂ (19.1%). Less than 1% of the dose was recovered in volatile organics traps, and 6.4% was excreted in faeces. About 5% of the administered dose remained in the body at 72 h.

Table 4. Time-course of radioactivity in the gastrointestinal tract of the male F344 rat and B6C3F₁ mouse following a 1 mg kg⁻¹ i.v. dose of [¹⁴C]PAL.

Tissue	% Dose recovered ^a						
	0.25 h	0.5 h	1 h	2 h	4 h	8 h	24 h
Rat							
Stomach	1.82 ± 1.51	1.34 ± 0.80	0.753 ± 0.436	0.357 ± 0.103	0.159 ± 0.043	0.0940 ± 0.0069	0.375 ± 0.524
Small intestine	13.1 ± 1.9	19.9 ± 5.8	29.9 ± 1.2	25.5 ± 3.3	4.29 ± 0.81	1.04 ± 0.04	0.469 ± 0.034
Caecum	1.24 ± 0.12	1.27 ± 0.27	1.61 ± 0.32	3.91 ± 2.27	11.9 ± 2.3	4.20 ± 0.79	0.429 ± 0.158
Large intestine	0.780 ± 0.114	0.657 ± 0.215	0.748 ± 0.068	0.453 ± 0.169	1.90 ± 0.77	1.71 ± 1.02	0.344 ± 0.109
Total GI tract	16.9 ± 1.3	23.2 ± 6.1	33.0 ± 1.4	30.3 ± 2.8	18.3 ± 2.7	7.04 ± 1.56	1.62 ± 0.11
Mouse							
Stomach	2.64 ± 1.28	1.42 ± 0.94	0.840 ± 0.382	0.237 ± 0.078	0.263 ± 0.080	0.289 ± 0.214	0.0460 ± 0.0028
Small intestine	17.9 ± 2.1	34.8 ± 8.2	33.8 ± 4.9	7.99 ± 2.35	2.75 ± 1.04	1.22 ± 0.60	0.365 ± 0.096
Caecum	0.881 ± 0.227	0.965 ± 0.124	2.75 ± 2.66	14.4 ± 2.9	4.53 ± 2.81	1.62 ± 0.13	0.133 ± 0.022
Large intestine	0.605 ± 0.139	0.651 ± 0.182	0.779 ± 0.335	4.07 ± 2.21	2.56 ± 0.78	1.17 ± 0.51	0.0808 ± 0.0374
Total GI tract	22.0 ± 2.1	37.9 ± 8.4	38.2 ± 2.5	26.7 ± 4.0	10.1 ± 2.6	4.30 ± 0.54	0.624 ± 0.087

^aMean ± SD, *n* = 4.

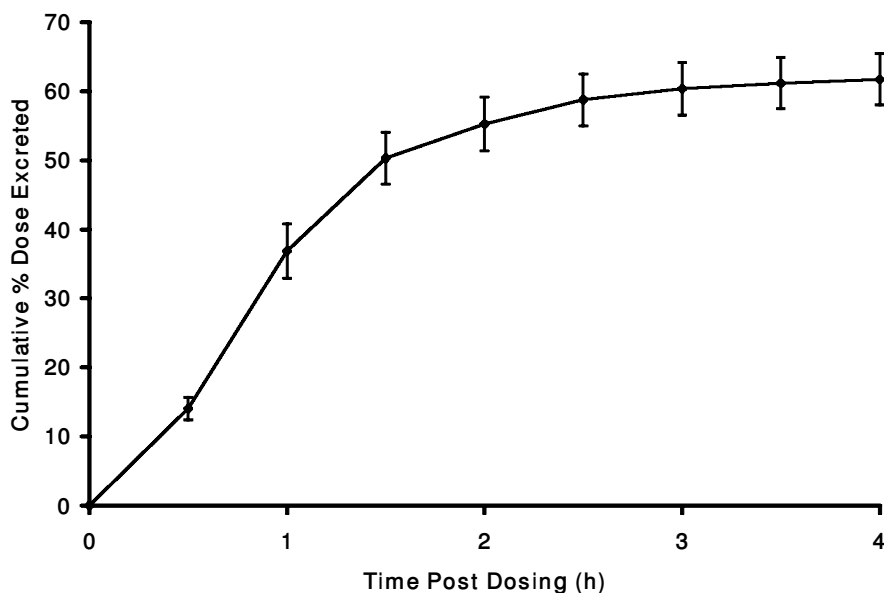


Figure 2. Cumulative biliary excretion of radioactivity after a single i.v. dose of [^{14}C]PAL (1 mg kg $^{-1}$) to the male F344 rat. Mean $\pm$ SEM ($n = 4$).

Mouse excreted intravenously administered radioactivity slower than did rat, with 67.1% of the dose excreted by 24 h after dosing, and 76.0% of the dose by 72 h. As with rat, the largest percentages of the administered dose were excreted in urine (40.0%) and exhaled as CO $_2$ (25.6%). An additional 9.6% of the dose was recovered in faeces and 0.85% as exhaled volatile organics. An overall average of about 84% of the administered dose was recovered with $\sim$ 3.4% of the dose remaining in the body at 72 h.

Table 5. Excretion of radioactivity following intravenous administration of 1 mg [^{14}C]PAL kg $^{-1}$ to the male F344 rat and B6C3F $_1$ mouse.

End of collection	% Dose recovered ^a						
	Urine	Faeces	Volatile organics	CO $_2$	Tissues	Cage rinse	Total
Rat							
6 h	40.2 $\pm$ 5.9	NA ^b	0.3 $\pm$ 0.2	11.6 $\pm$ 0.9	NA	NA	52.2 $\pm$ 5.6
12 h	54.2 $\pm$ 3.4	1.4 $\pm$ 1.2	0.4 $\pm$ 0.2	16.4 $\pm$ 1.0	NA	NA	72.3 $\pm$ 2.9
24 h	60.1 $\pm$ 3.0	5.5 $\pm$ 0.6	0.4 $\pm$ 0.2	17.8 $\pm$ 1.0	NA	NA	83.9 $\pm$ 1.7
48 h	61.9 $\pm$ 2.8	6.3 $\pm$ 0.6	0.5 $\pm$ 0.2	18.6 $\pm$ 1.0	NA	NA	87.3 $\pm$ 1.5
72 h	62.3 $\pm$ 2.6 ^c	6.4 $\pm$ 0.6	0.5 $\pm$ 0.2	19.1 $\pm$ 1.1	5.3 $\pm$ 0.2	0.3 $\pm$ 0.1	94.1 $\pm$ 1.3
Mouse							
6 h	8.8 $\pm$ 12.5	NA	0.65 $\pm$ 0.20	20.8 $\pm$ 3.1	NA	NA	30.2 $\pm$ 14.0
12 h	30.0 $\pm$ 10.0	5.3 $\pm$ 5.1	0.71 $\pm$ 0.22	22.7 $\pm$ 4.1	NA	NA	58.6 $\pm$ 8.4
24 h	35.5 $\pm$ 9.9	7.2 $\pm$ 2.0	0.78 $\pm$ 0.24	23.6 $\pm$ 4.1	NA	NA	67.1 $\pm$ 7.3
48 h	38.9 $\pm$ 9.6	8.4 $\pm$ 2.5	0.83 $\pm$ 0.26	25.3 $\pm$ 3.6	NA	NA	73.4 $\pm$ 6.7
72 h	40.0 $\pm$ 9.4 ^c	9.6 $\pm$ 2.8	0.85 $\pm$ 0.28	25.6 $\pm$ 3.6	3.4 $\pm$ 0.3	4.2 $\pm$ 2.8	83.6 $\pm$ 4.0

^aMean $\pm$ SD, $n = 5$.

^bNot applicable. The first faeces collection was at 0-12 h. Tissues and cage rinse were obtained at 72 h only. Tissues include sampled tissues and residual carcass.

^c72-h urine collection includes urine present in the bladder at study termination.

Results of the oral dose mass balance study are shown in table 6. There appeared to be no adverse effects of the high oral dose to mouse. However, ~10 min following dosing, the eyes of the rat started tearing, which resulted in excessive blinking by the animals. After ~30-45 min, rat remained still with their eyes closed. By 2 h following dosing, all rat appeared normal.

Similar to the i.v. study, PAL-derived radioactivity from oral doses was rapidly excreted, and rat excreted radioactivity faster than mouse. By 24 h after dosing, 73.2 versus 53.7% of the dose was excreted by rat and mouse, respectively. At 72 h, rat excreted 90% of the dose, whereas only 72.7% of the dose was recovered in mouse excreta. As in the i.v. study, major routes of excretion by both species were urine and CO₂. The amount of faecal excretion was greater after an oral compared with an i.v. dose and was likely due to incomplete absorption of the orally administered radioactivity. Very little radioactivity (2.4-5.3%) remained in the tissues at 72 h.

Dermal dose mass balance studies in rat

Recovery of the radiolabelled dose (absorbed and unabsorbed) in the dermal dose mass balance study is shown in table 7. An average of 14.6% of the administered dose was recovered in excreta by 24 h following dosing. An average of 3.0% of the administered dose was recovered in urine with ~0.5 and 0.7% recovered in faeces and CO₂, respectively. About 10% of the dose was recovered in volatile organics traps, with most of that collected during the 6-h exposure period. Despite attempts to contain PAL that volatilized from the dose site, our methods may not have been completely foolproof; it is possible that the radioactivity recovered in the volatile organics traps may have escaped the dermal appliance and should be considered unabsorbed. Hence, the majority of the dose (at least 85.9%) was not

Table 6. Recovery of radioactivity following oral administration of 50 mg [¹⁴C]PAL kg⁻¹ to the male F344 rat and B6C3F₁ mouse.

End of collection	% Dose recovered ^a						
	Urine	Faeces	Volatile organics	CO ₂	Tissues	Cage rinse	Total
Rat							
6 h	20.1 ± 1.0	NA ^b	0.12 ± 0.02	5.9 ± 0.4	NA	NA	26.1 ± 1.0
12 h	31.0 ± 1.4	2.7 ± 2.2	0.15 ± 0.03	15.7 ± 1.0	NA	NA	49.5 ± 2.4
24 h	41.3 ± 3.2	10.7 ± 1.6	0.17 ± 0.04	21.0 ± 2.2	NA	NA	73.2 ± 5.8
48 h	49.1 ± 2.3	13.6 ± 1.2	0.19 ± 0.04	25.2 ± 1.3	NA	NA	88.1 ± 0.4
72 h	49.8 ± 2.5 ^c	14.0 ± 1.3	0.20 ± 0.04	26.0 ± 1.3	5.3 ± 0.4	0.3 ± 0.1	95.7 ± 0.6
Mouse							
6 h	4.3 ± 6.3	NA	0.17 ± 0.08	7.4 ± 5.1	NA	NA	11.9 ± 10.0
12 h	8.2 ± 6.7	6.5 ± 2.5	0.28 ± 0.11	13.5 ± 6.8	NA	NA	28.5 ± 8.1
24 h	20.0 ± 10.7	13.5 ± 8.7	0.41 ± 0.14	19.8 ± 2.3	NA	NA	53.7 ± 4.5
48 h	27.9 ± 14.0	18.0 ± 8.5	0.51 ± 0.18	21.6 ± 2.0	NA	NA	67.9 ± 4.6
72 h	29.9 ± 14.0 ^c	20.2 ± 9.3	0.58 ± 0.19	22.1 ± 2.1	2.4 ± 0.2	10.4 ± 4.0	85.5 ± 2.2

^aMean ± SD; n = 5 rats or 4 mice.
^bNot applicable. The first faeces collection was at 0-12 h. Tissues and cage rinse were obtained at 72 h only. Tissues include sampled tissues and residual carcass.
^c72-h urine collection includes urine present in the bladder at study termination.

Table 7. Recovery of radioactivity following dermal administration of 5 mg [^{14}C]PAL kg^{-1} to the male F344 rat.^a

End of collection	Cumulative % of applied dose excreted				
	Urine	Faeces	Volatile organics ^b	CO ₂	Total ^c
3 h	NA ^d	NA	5.3 ± 0.9	NA	5.3 ± 0.9 (NA)
6 h	1.3 ± 0.6	NA	8.1 ± 2.1	0.3 ± 0.1	9.7 ± 2.5 (1.6 ± 0.6)
24 h	3.0 ± 0.7	0.5 ± 0.2	10.4 ± 0.8	0.7 ± 0.1	14.6 ± 1.7 (4.3 ± 1.0)

% of Applied dose			
Absorbed dose		Unabsorbed dose	
Excreta ^e	4.3 ± 1.0	Protective appliance	1.2 ± 0.3
Blood	0.01 ± 0.00	First charcoal trap extract	68.7 ± 1.6
Dose site skin	0.3 ± 0.1	Second charcoal trap extract	4.7 ± 0.4
Carcass	0.5 ± 0.1	Dose site wash	0.8 ± 0.2
		Volatile organics	10.4 ± 0.8
Total % dose absorbed	5.2 ± 1.1	Total % dose unabsorbed	85.9 ± 1.3

^aMean ± SD, $n = 4$.^bIt is likely that this is not radioactivity that was absorbed and exhaled, rather radioactivity that escaped the dermal appliance.^cNumbers in parentheses exclude volatile organics; see note b.^dNot applicable. The first urine and CO₂ collections were at 0-6 h; the first faeces collection was at 0-24 h.^eExcludes radioactivity in the volatile organics traps; see note b.

absorbed, and most of that was recovered in the charcoal-impregnated trapping material that surrounded the dermal appliance.

Inhalation exposure mass balance studies in rat and mouse

Inhaled and absorbed doses for rat and mouse are shown in table 8. Inhaled doses (estimated as described in the Materials and methods) were 0.148, 1.45 and 14.8 mg for rat in the 1-, 10- and 100-ppm exposure groups. Mice inhaled 0.0459,

Table 8. Absorption of inhaled [^{14}C]PAL by rat and mouse.

Parameter	Rat	Mouse
1 ppm exposure		
Inhaled dose (mg)	0.148	0.0459
Absorbed dose (mg) ^a	0.0816 ± 0.0053	0.0314 ± 0.0086
% Absorbed ^a	55.2 ± 3.6	68.4 ± 18.7
10 ppm exposure		
Inhaled dose (mg)	1.45	0.451
Absorbed dose (mg) ^a	0.901 ± 0.089	0.284 ± 0.088
% Absorbed ^a	62.1 ± 6.2	63.0 ± 19.5
100 ppm exposure		
Inhaled dose (mg)	14.8	4.59
Absorbed dose (mg) ^a	4.93 ± 0.39	1.06 ± 0.35
% Absorbed ^a	33.3 ± 2.6	23.1 ± 7.6

^aMean ± SD, $n = 4$. See the Materials and methods for the calculation of inhaled and absorbed dose.

0.451 and 4.59 mg in the 1-, 10- and 100-ppm exposure groups, respectively. Within a species, the per cent of inhaled PAL that was absorbed was similar for the 1- and 10-ppm exposures (55.2 and 62.1% for rat, 68.4 and 63.0% for mouse), then dropped precipitously in the 100-ppm exposure (33.3% for rat, 23.1% for mouse).

Recovery of absorbed radioactivity in the inhalation studies is shown in table 9. Consistent with results obtained from i.v. (table 5) and oral (table 6) studies, a majority of the absorbed dose was eliminated over 24 h by rat and mouse (82-87%) following each of the three inhalation exposures. Primary routes of excretion were urine and as CO₂. In contrast to the i.v. and oral studies, a relatively large per cent of the dose was excreted as volatile organics during the first 6 h after exposure, which may have been exhaled PAL. At 24 h, 13-17 and 8-11% of the dose was recovered in rat and mouse tissues, respectively. It should be noted that the reported recovery of radioactivity in mouse faeces may be higher than what was actually excreted by this route due to contamination of the faeces with urine. Similarly, reported recovery of radioactivity in mouse urine may be lower than what was actually excreted by this route because some of the excreted urine was present in the faecal samples. In our experience, this is typical in mouse studies.

Table 9. Recovery of radioactivity following inhalation exposure of 1, 10 or 100 ppm [¹⁴C]PAL to the male F344 rat and B6C3F₁ mouse.

End of collection ^b	Absorbed dose recovered ^a						
	Urine	Faeces	Volatile organics	CO ₂	Tissues	Cage rinse	Total
Rat (1 ppm)							
6 h	9.40 ± 4.62	NA ^c	-	-	-	-	9.40 ± 4.62
12 h	42.4 ± 3.9	1.59 ± 0.69	10.7 ± 7.8	7.25 ± 1.27	-	-	61.9 ± 6.0
24 h	52.6 ± 2.2	6.34 ± 0.72	16.8 ± 4.2	9.40 ± 1.40	13.0 ± 5.8	1.77 ± 0.96	100
Rat (10 ppm)							
6 h	2.64 ± 5.24	NA ^b	-	-	-	-	2.64 ± 5.24
12 h	30.5 ± 5.7	1.70 ± 1.42	15.1 ± 4.4	11.0 ± 0.4	-	-	58.3 ± 5.4
24 h	42.1 ± 5.2	8.05 ± 1.46	17.6 ± 4.9	13.7 ± 1.0	16.6 ± 2.9	1.68 ± 0.41	100
Rat (100 ppm)							
6 h	9.15 ± 6.61	NA ^b	-	-	-	-	9.15 ± 6.61
12 h	26.3 ± 7.1	0.711 ± 0.447	18.0 ± 3.4	13.8 ± 2.8	-	-	58.7 ± 9.5
24 h	37.6 ± 4.9	7.22 ± 1.86	20.5 ± 3.2	17.6 ± 2.4	14.3 ± 2.7	2.95 ± 1.89	100
Mouse (1 ppm)							
6 h	NA ^c	NA ^c	-	-	-	-	-
12 h	19.1 ± 13.7	10.5 ± 2.4	14.0 ± 3.9	11.0 ± 4.7	-	-	54.6 ± 17.2
24 h	42.6 ± 16.3	16.3 ± 6.4	15.3 ± 4.3	12.9 ± 5.4	8.35 ± 4.21	4.61 ± 2.63	100
Mouse (10 ppm)							
6 h	NA ^c	NA ^b	-	-	-	-	-
12 h	24.1 ± 8.6	5.46 ± 4.16	26.7 ± 10.0	6.91 ± 1.03	-	-	63.2 ± 9.1
24 h	32.1 ± 6.7	13.1 ± 5.3	29.5 ± 11.5	8.18 ± 1.27	9.57 ± 2.74	7.45 ± 6.59	100
Mouse (100 ppm)							
6 h	NA ^c	NA ^b	-	-	-	-	-
12 h	17.0 ± 11.6	19.0 ± 4.5	16.3 ± 5.9	13.2 ± 1.1	-	-	65.5 ± 3.6
24 h	23.4 ± 11.2	24.4 ± 4.9	17.4 ± 6.7	16.5 ± 1.2	10.6 ± 2.1	7.68 ± 3.06	100

^aMean ± SD; *n* = 4 rats or 4 mice. A dash indicates that no sample was collected at that time point.

^bUrine and faeces were collected over intervals ending at 6 (during exposure), 12 and 24 h. Traps for collection of volatile organics and CO₂ were changed at 12 and 24 h. Tissues and cage rinse were obtained at 24 h only. Tissues include sampled tissues and residual carcass.

^cNo sample was produced for collection during exposure.

Biliary and urinary metabolite profiles

No parent PAL was found in either urine or bile.

Chromatographic analysis of bile samples obtained from four rats after an i.v. dose of PAL revealed a striking peculiarity. Peaks eluting in the 10-12 min time interval were missing in two of four rats (figure 3). This difference was consistent for each bile collection period. The reason for the observed variability is unknown. The presence of six to 11 distinct peaks in the chromatogram indicates that PAL was extensively metabolized.

Analysis of urine from rat and mouse further indicated that PAL was extensively metabolized. Representative chromatograms of radiolabelled metabolites in rat and mouse urine are shown in figure 4. Chromatographic profiles of rat urine obtained after inhalation exposure were qualitatively similar to those observed after i.v. and oral exposure (data not shown). Likewise, the chromatographic profiles of radiolabelled metabolites in mouse urine were similar after inhalation and i.v. exposure (data not shown). At least 10 metabolites were present in rat urine. The metabolite profile in mouse urine was somewhat different from the profile in rat in that there were additional early-eluting peaks in the region of 8-12 min into the chromatographic run (figure 4).

Urine obtained from rat after an oral dose of PAL was used to isolate radiolabelled metabolites for identification. For this work, a semipreparative column was used, which resulted in better resolution of peaks such that as many as 22 peaks were detected in some urine samples (data not shown). Individual metabolite fractions were collected from the semipreparative column, then neutralized and concentrated under a gentle stream of nitrogen. Recovery of radioactivity was determined at each step. Overall recovery of radioactivity was 73-100% in all but two metabolite fractions in which only 42-44% of the original radioactivity was recovered. These two metabolites are suspected to be volatile.

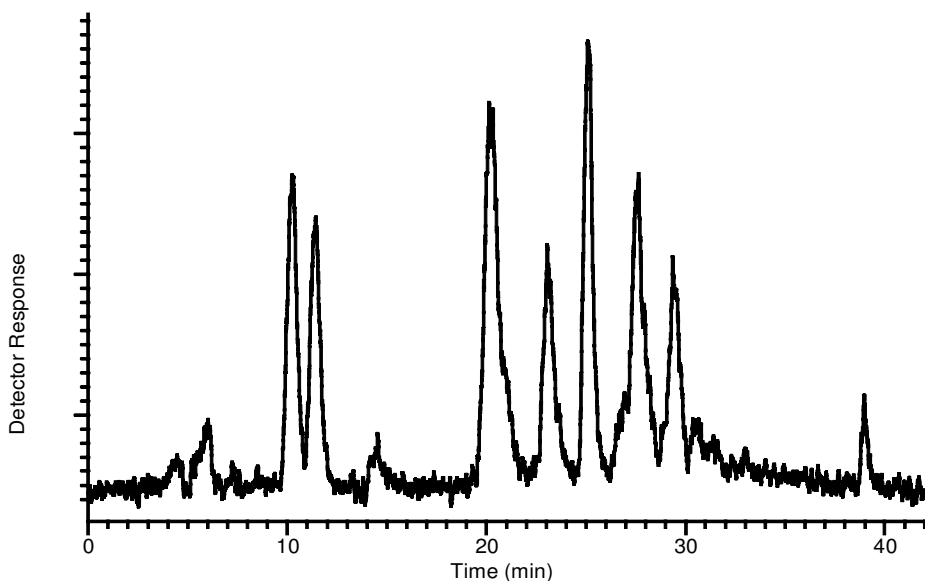


Figure 3. Representative radiochromatogram of bile collected after a single i.v. exposure of [^{14}C]PAL to the male F344 rat. Peaks eluting at 10-12 min were not present in two of four rats.

With the exception of the peak that eluted at ~ 36 min (identified as peak T_{sp} in figures 5 and 6), we could not identify the radiolabelled metabolites. For some of the other metabolites, purification resulted in too little mass for mass and NMR spectrometric analysis. In other cases, residual TFA and/or TEA prohibited us from obtaining definitive spectra. Mass spectrometric analysis of peak T_{sp} using electrospray ionization resulted in the production of a prominent $M+H$ ion at m/z 383 (figure 6A). Ion-neutral interactions between the non-charged molecule and

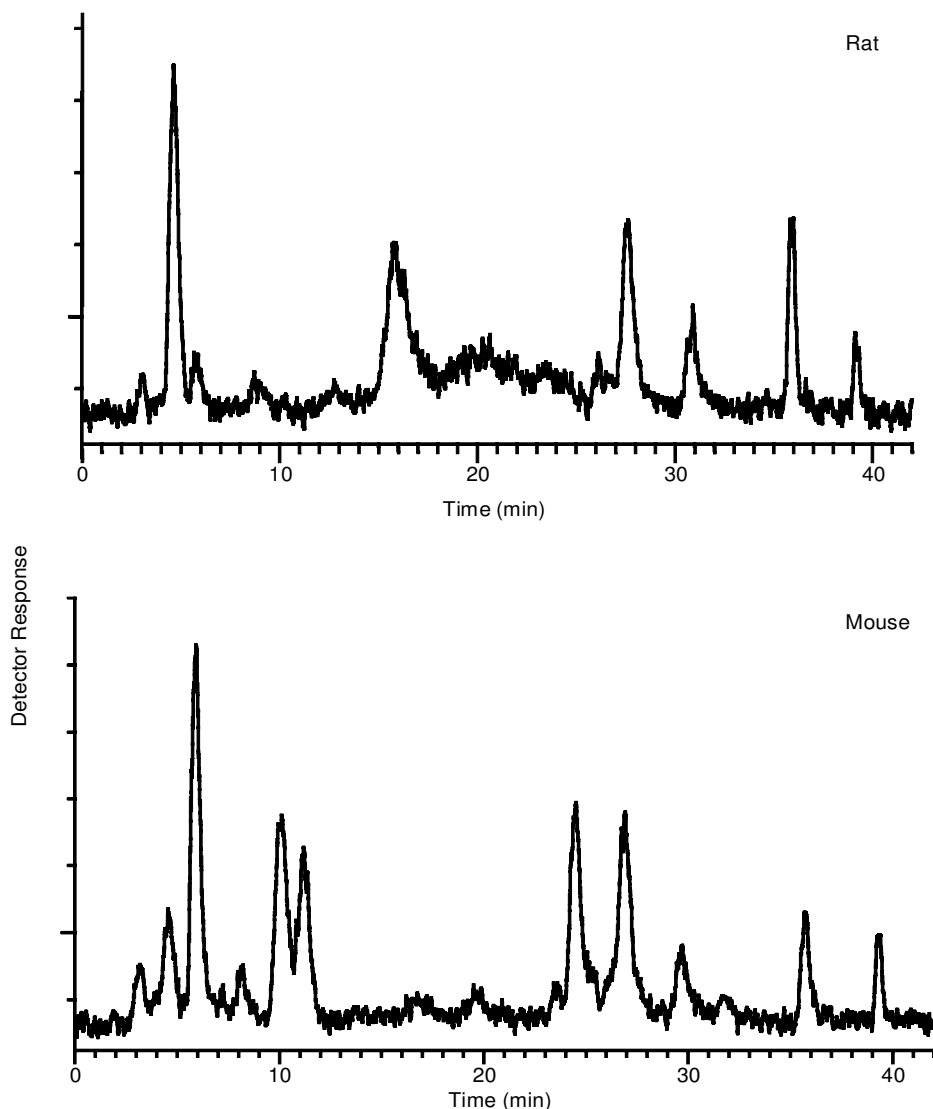
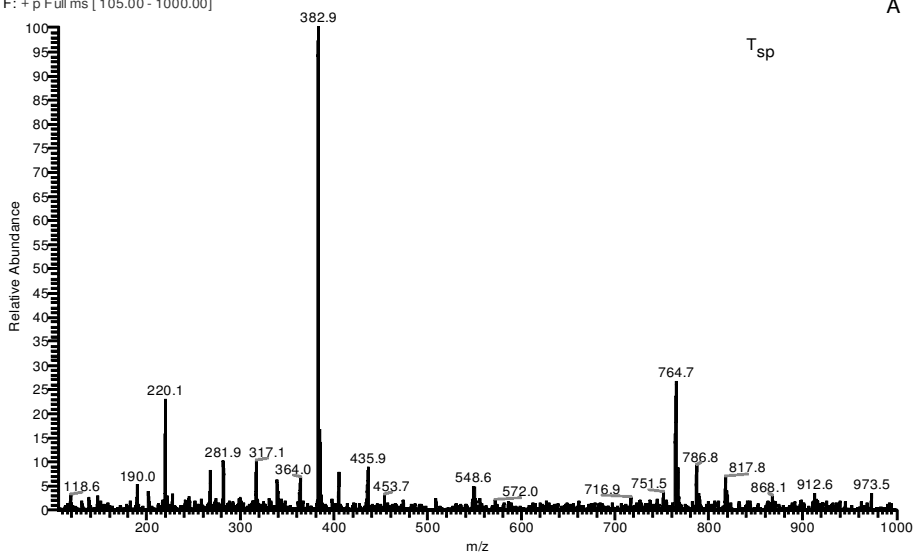


Figure 4. Representative radiochromatograms of urine samples collected after a single inhalation exposure of $[^{14}\text{C}]\text{PAL}$ to the male F344 rat and male B6C3F₁ mouse. Chromatograms of urine samples collected from rat after i.v. and oral administration were qualitatively similar to those from rat after inhalation exposure. Likewise, chromatograms of urine samples collected from mouse after i.v. exposure were qualitatively similar to those from mouse after inhalation exposure.

S#: 548-550 RT: 18.88-18.94 AV: 2 SB: 20 16.87-17.35, 15.98-16.22 NL: 2.11E5
F: + p Full ms [105.00 - 1000.00]

A



S#: 549-551 RT: 18.92-18.97 AV: 2 NL: 1.05E5
F: + p d Full ms2 382.93

B

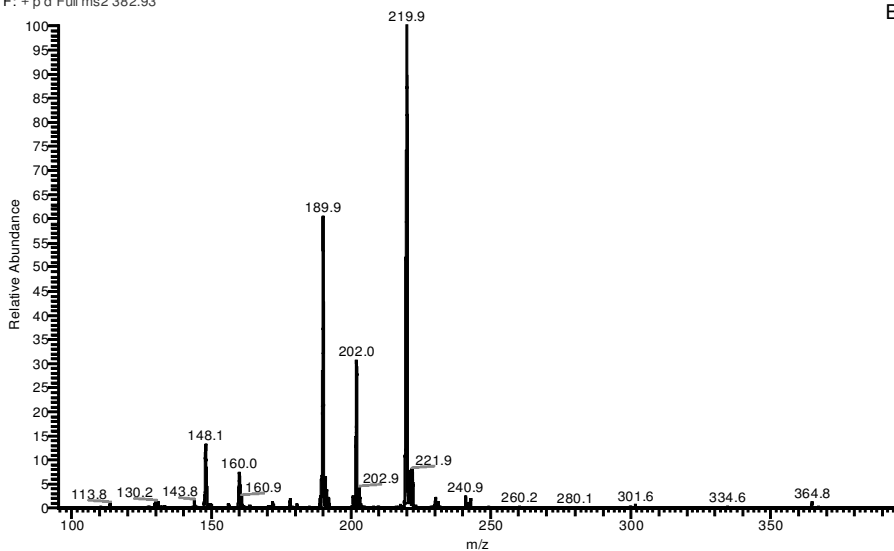


Figure 5. Mass spectral analysis of peak T_{sp} (peak eluting at ~ 36 min in figure 4). (A) Positive-ion full-mass spectrum of peak T_{sp} . (B) Full-scan spectrum obtained from MS/MS analysis of m/z 383 from (A).

its protonated form resulted in the production of an additional ion at m/z 765. An additional ion with relative intensity $> 20\%$ was seen at m/z 220. Since this ion was the base-ion produced upon MS/MS analysis of the ion at m/z 383, it appears that this ion at m/z 220 is the result of thermally induced fragmentation of the parent compound. Isolation and MS/MS analysis of the ion at m/z 383 resulted in the production of daughter ions at m/z 220, 202, 190, 160 and 148 as shown in figure 6B. This fragmentation pattern is consistent with the structure of 3,3-bis[(2-

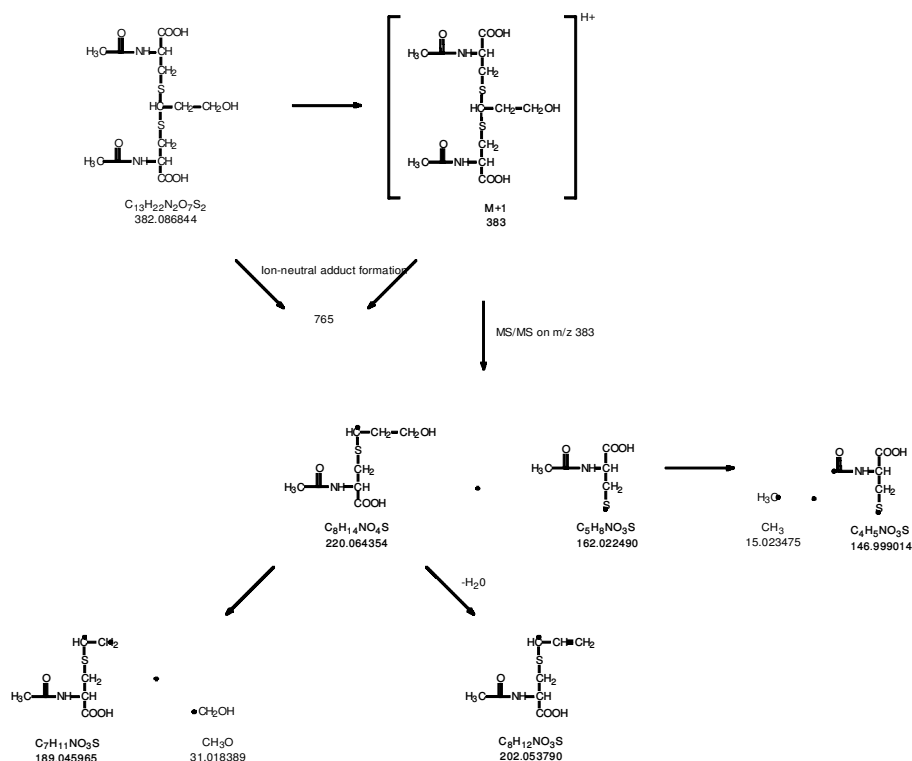


Figure 6. Possible ionization and fragmentation pathways for the production of the ions seen in the full scan and MS/MS analyses of peak T_{sp} shown in figure 5.

(acetylamino)-2-carboxyethylthio]-1-propanol, a urinary metabolite of PAL reported by Banijamali *et al.* (1999). These daughter ions could be rationalized in part by the cleavage patterns shown in figure 6. While the simple fragmentation patterns provided in figure 8 in some instances result in ions that differ by one mass unit (or in the case of m/z 162, two units) from those observed, it is likely that the fragmentation pathways shown produced the observed ions, albeit with the addition of a proton transfer or a more complex intramolecular rearrangement.

Discussion

These studies were undertaken to investigate the disposition of PAL, an alkyne alcohol, in rat and mouse. PAL is quickly distributed and excreted by both rat and mouse following an i.v., oral, or inhalation exposure. Primary routes of excretion are urine and as exhaled CO_2 , indicating extensive metabolism. Rats excreted the i.v. and oral doses faster and to a greater extent than did mouse. After the oral dose, both species excreted more radioactivity in faeces compared with the i.v. dose, which could have been due to the oral dose not being entirely absorbed.

The presence of a large percentage of the i.v. dose in the gastrointestinal tract suggested extensive biliary excretion. Biliary excretion and mass balance studies in rat corroborated these findings and demonstrated significant enterohepatic circulation of PAL metabolites; 62% of an i.v. dose was excreted in bile, whereas only

6% was recovered in faeces. Not only was PAL excreted to a high degree in bile, it was excreted as a number of metabolites. The biliary metabolite profile contained at least six radiolabelled peaks.

The primary routes of occupational exposure to PAL are dermal contact and inhalation. Given that PAL is moderately volatile, there is some question as to the internal dose one might receive from dermal exposure to PAL. Hence, we investigated the disposition of dermally applied PAL in rat and mouse. Owing to the inherent volatility of PAL, a non-occlusive dermal appliance that could trap PAL vapours was devised. Our studies demonstrated that at least 75% of the applied dermal dose volatilized from the surface of the skin and was unavailable for absorption. It is possible that the 8-10% of the dose recovered in the traps for volatile organics may have been PAL that was not absorbed and escaped the dermal appliance, making nearly 85% of the applied dose unavailable for absorption. Thus, it is unlikely that humans would receive a significant internal dose of PAL upon dermal exposure unless the skin was in contact with liquid PAL for a prolonged period. It is likely that the route of exposure more relevant to humans is inhalation rather than dermal.

Inhalation studies were conducted in rat and mouse exposed to PAL vapours at concentrations of 1, 10 and 100 ppm. These exposure concentrations were selected based on proposed concentrations for toxicity studies. We found that absorption of inhaled PAL (% absorbed) by both rat and mouse decreased at the 100-ppm exposure concentration relative to the 1- and 10-ppm exposures. Zissu (1995) reported the observation that inhalation exposure to PAL (~25 and 88 ppm) results in a decrease in respiratory rate in the Swiss mouse. Respiratory rate was not measured in the current studies, and the amount of PAL inhaled was assumed to be the theoretical amount inhaled during the 6-h exposure based on a normal respiratory rate. If we assume that the respiratory rate was decreased in animals exposed to 100-ppm PAL compared with animals in the 1- and 10-ppm groups, then the per cent absorbed could be the same for all three exposure groups. A decreased respiratory rate in the 100-ppm group may explain the lower absorbed dose. Indeed, when one considers the absolute mass of PAL recovered from the animals, there is a linear increase with exposure concentration for both species ($r^2 > 97\%$ for rat and mouse), but the increase is not dose-proportional.

As observed in the i.v. and oral dose mass balance studies, PAL was rapidly eliminated over 24 h by both rat and mouse following a single 6-h nose-only inhalation exposure to PAL vapours. About 85% of the absorbed dose was eliminated in this interval. Urinary excretion accounted for the largest portion of the dose eliminated over 24 h by rat and mouse at all three exposure concentrations. Exposure concentration-related differences in the routes of excretion were observed in both rat and mouse. Species-related differences in the routes of excretion of [^{14}C]PAL-derived radioactivity were observed in rat and mouse for each inhalation exposure concentration. Rat excreted more of the dose in urine compared with mouse, and mouse excreted more of the dose in faeces compared with rat. No significant accumulation of dose-derived radioactivity was observed in any sampled tissues or organs in either species at either exposure concentration.

The presence of numerous metabolites in urine (rat and mouse) and bile (rat) coupled with the high degree of elimination as CO_2 indicate that PAL was extensively metabolized. The urinary metabolite profiles for the two species as well as the rat biliary metabolite profiles were qualitatively similar. One metabolite

was analysed by LC/MS/MS. The fragmentation pattern for this metabolite is consistent with the structure of 3,3-bis[(2-(acetylamino)-2-carboxyethyl)thio]-1-propanol (Banijamali *et al.* 1999), the result of the addition of two glutathione molecules across the carbon-carbon triple bond, possibly via Michael addition to an aldehyde intermediate.

Summary and conclusions

PAL was extensively metabolized and rapidly eliminated upon i.v., oral, dermal and inhalation exposure. Biliary excretion of PAL-derived radioactivity was significant, with relatively little faecal excretion, indicating a high degree of enterohepatic circulation. Owing to the volatility of PAL, the risk to humans from dermal exposure is likely to be minimal unless dermal contact is prolonged.

Acknowledgements

The authors acknowledge Drs H. B. Matthews and L. T. Burka of NIEHS for technical discussions and critical review of the manuscript. They are also indebted to Ms Melody Gower and Ms Yun-Lan Yueh for excellent technical assistance, and Dr Jason P. Burgess for his involvement in the NMR spectrometry experiments. This work was supported by NIEHS contract N01-ES-75407.

References

- ARCHER, T. E., 1985, Acute oral toxicity as LD₅₀ (mg/kg) of propargyl alcohol to male and female rats. *Journal of Environmental Science and Health*, **B20**, 593-596.
- BANIJAMALI, A. R., XU, Y., STRUNK, R. J., GAY, M. H., ELLIS, M. C., PUTTERMAN, G. J. and SUMNER, S. J., 1999, Identification of metabolites of [1,2,3-¹³C]propargyl alcohol in rat urine by ¹³C NMR and mass spectrometry. *Journal of Agriculture and Food Chemistry*, **47**, 1717-1729.
- BLAKELY, D. H., MAUS, K. L., BELL, R., BAYLEY, J., DOUGLAS, G. R. and NESTMANN, E. R., 1994, Mutagenic activity of 3 industrial chemicals in a battery of *in vitro* tests. *Mutation Research*, **320**, 273-283.
- BROWN, R. P., DELP, M. D., LINDSTEDT, S. L., RHOMBERG, L. R. and BELILES, R. P., 1997, Physiological parameter values for physiologically based pharmacokinetic models. *Toxicology and Industrial Health*, **13**, 407.
- CHANG, J. C. F., GROSS, E. A., SWENBERG, J. A. and BARROW, C. S., 1983, Nasal cavity deposition, histopathology, and cell proliferation after single or repeated formaldehyde exposures in B6C3F1 mice and F-344 rats. *Toxicology and Applied Pharmacology*, **68**, 161-176.
- DALTON, P. B., HAHNEL, E. C., BRUSIE, J. P. and HORT, E. V., 1963, Alcohols, unsaturated. In A. Standen (ed.), *Kirk-Othmer Encyclopedia of Chemical Technology*, 2nd edn, vol. 1 (New York: Wiley), pp. 598-618.
- DEMASTER, E. G., DAHLSEID, T. and REDFERN, B., 1994, Comparative oxidation of 2-propyn-1-ol with other low molecular weight unsaturated and saturated primary alcohols by bovine liver catalase *in vitro*. *Chemical Research in Toxicology*, **7**, 414-419.
- DEMASTER, E. G., SHIROTA, F. N. and NAGASAWA, H. T., 1986, Role of propionaldehyde and other metabolites in the pargyline inhibition of rat liver aldehyde dehydrogenase. *Biochemical Pharmacology*, **35**, 1481-1489.
- HAZARDOUS SUBSTANCE DATA BANK, 2000, U.S. National Library of Medicine, <http://chem.sis.nlm.nih.gov/hsdb>.
- LINGTON, A. W. and BEVAN, C., 1994, Alcohols. In G. D. Clayton and F. E. Clayton (eds), *Patty's Industrial Hygiene and Toxicology*, 4th edn, vol. II, Part D (New York: Wiley), pp. 2585-2759.
- NATIONAL RESEARCH COUNCIL, 1996, *Guide for the Care and Use of Laboratory Animals* (Washington, DC: National Academy Press).
- NICHOLS, C. S. and CROMARTIE, T. H., 1980, Irreversible inactivation of the flavoenzyme alcohol oxidase with acetylenic alcohols. *Biochemical and Biophysical Research Communication*, **97**, 216-221.

- RUBINSTEIN, R., IRENE, S., SONOWANE, B., DEROSA, C. and BATHIJA, A., 1989, 30 and 90 day rat oral toxicity of propargyl alcohol. *Toxicologist*, **9**, 248.
- SLAUTER, R. W., COLEMAN, D. P., GAUDETTE, N. F., MCKEE, R. H., MASTEN, L. W., GARDINER, T. H., STROTHER, D. E., TYLER, T. R. and JEFFCOAT, A. R., 1994, Disposition and pharmacokinetics of isopropanol in F-344 rats and B6C3F1 mice. *Fundamental and Applied Toxicology*, **23**, 407-420.
- VERNOT, E. H., MACEWEN, J. D., HAUN, C. C. and KINKEAD, E. R., 1977, Acute toxicity and skin corrosion data for some organic and inorganic compounds and aqueous solutions. *Toxicology and Applied Pharmacology*, **42**, 417-423.
- ZISSU, D., 1995, Histopathological changes in the respiratory tract of mice exposed to ten families of airborne chemicals. *Journal of Applied Toxicology*, **15**(3), 207-213.

High-resolution magic angle spinning ^1H -NMR spectroscopy studies on the renal biochemistry in the bank vole (*Clethrionomys glareolus*) and the effects of arsenic (As^{3+}) toxicity

J. L. GRIFFIN^{†*}, L. WALKER[‡], R. F. SHORE[‡] and J. K. NICHOLSON[†]

[†]Biological Chemistry, Division of Biomedical Sciences, The Sir Alexander Fleming Building, Imperial College, Exhibition Road, London SW7 2AZ, UK

[‡]NERC Centre for Ecology & Hydrology, Monks Wood, Huntingdon PE28 2LS, UK

Received 11 January 2001

1. High-resolution magic angle spinning (MAS) ^1H -NMR spectroscopy was used to study renal metabolism and the toxicity of As^{3+} , a common environmental contaminant, in the bank vole (*Clethrionomys glareolus*), a wild species of rodent.

2. Following a 14-day exposure to an environmentally relevant dose of As_2O_3 (28 mg kg^{-1} feed), voles displayed tissue damage at autopsy. MAS ^1H spectra indicated abnormal lipid profiles in these samples.

3. Tissue necrosis was also evident from measurements of the apparent diffusion coefficient of water in the intact tissue using MAS ^1H diffusion-weighted spectroscopy, its first application to toxicology.

4. Comparison of renal tissue from the wood mouse (*Apodemus sylvaticus*) exposed to identical exposure levels of As^{3+} suggested that the bank vole is particularly vulnerable to As^{3+} toxicity.

Introduction

Conventional NMR has been applied extensively to the study of toxicological insults in laboratory animals and humans (Sanins *et al.* 1992, Shockcor *et al.* 1996, Holmes *et al.* 1997). Solution state ^1H -NMR spectroscopy allows the simultaneous observation of a range of metabolites, but tissue extracts often reflect the solubility of a particular metabolite rather than its actual concentration *in vivo*. Whereas spectroscopy *in vivo* is possible, the resultant spectra are poorly resolved.

High-resolution magic angle spinning (HRMAS) ^1H -NMR spectroscopy of intact tissue produces spectra that are comparable in resolution and spectral quality with those produced by conventional solution-state NMR. By spinning solid material at the magic angle (54.7°), both macro- and microscopic field inhomogeneities and dipolar-dipolar coupling effects are averaged (Andrew and Eades 1953). This is further aided by the semiliquid nature of biological tissue (Tomlins *et al.* 1998). The technique is especially useful for studying lipids within cells and tissue (Cheng *et al.* 1997, Millis *et al.* 1997, Weybright *et al.* 1998, Griffin *et al.* 2000a), and may, in turn, be particularly effective for observing the long-term toxic effects of chronic exposure to environmental toxins. This is because the lipid composition of biomembranes reflect long-term dietary intake.

* Author for correspondence; e-mail: j.griffin@ic.ac.uk

Whereas most toxicology studies have involved laboratory animals, we are currently investigating the impact of environmental toxins on wild mammals (Griffin *et al.* 2000a). Here, HRMAS ^1H -NMR was used to study the renal tissue of the bank vole (*Clethrionomys glareolus*), a species that commonly occurs on arsenic-contaminated sites (Ismail and Roberts 1992, Erry *et al.* 1999, 2000), and the effects that an environmentally realistic oral exposure to As^{3+} has on the kidney. As^{3+} is readily transported by erythrocytes with renal tissue being one of the initial target organs of this toxin (Oehme 1972). Proteinuria, glycosuria and haematuria reflect damage to the membrane of renal tubular cells and inhibition of renal tissue enzyme caused by As^{3+} (St Petery *et al.* 1970). Also presented is the first use of MAS diffusion-weighted spectroscopy as a tool to monitor necrosis in intact tissue. Tissue damage in the vole was not detected in the wood mouse (*Apodemus sylvaticus*), another rodent commonly found on contaminated sites (Ismail and Roberts 1992, Erry *et al.* 1999, 2000), suggesting that renal tissue from the bank vole may be particularly susceptible to As^{3+} -induced damage.

Materials and methods

Bank voles from laboratory colonies (Centre for Ecology & Hydrology, Monks Wood, Huntingdon, UK) were maintained on standard laboratory feed (RM1, Special Dietary Services, Witham, UK) prior to the study. They were housed under constant environmental conditions (15.5 h light/8.5 h dark at 22°C).

Male voles (20.6 ± 0.9 (SE) g body weight, $n = 10$) were divided randomly into two groups of five. One group was fed for 14 days on RM1 laboratory feed (control diet), the other on the same feed contaminated with $28 \text{ mg kg}^{-1} \text{ As}_2\text{O}_3 \text{ kg}^{-1}$ diet. This dietary arsenic concentration was within the range of concentrations measured in the stomach contents of the free-living vole on moderately and heavily As-contaminated sites (Erry *et al.* 2000). Voles were killed by cervical dislocation and a visual autopsy was performed. The left kidney was dissected and a 15-mg section of the inner renal cortex was taken for MAS ^1H spectroscopy. Additional renal tissue was used to investigate the biochemical structure of the kidney.

To compare HRMAS ^1H -NMR spectra with conventional solution state spectra, inner cortical tissue from the right kidney was also excized and frozen for conventional aqueous extraction. Frozen tissue was pulverized and dissolved in 50% acetonitrile/50% D_2O . Unlike the preparation of perchloric acid extraction, this method prevents the loss of amino acids by avoiding precipitation. The supernatant was lyophilized and reconstituted in D_2O . The remaining pellet underwent lipid extraction using 75% $\text{CDCl}_3/25\% \text{ CD}_3\text{OH}$. Solutions were pipetted into 5-mm NMR tubes and analysed using a Bruker DRX 600 spectrometer interfaced to a 14.1 Tesla magnet. ^1H -NMR spectra were acquired using a presaturation pulse sequence with a 2-s interpulse delay (Nicholson *et al.* 1995).

MAS ^1H -NMR was performed at 14.1 Tesla on the above spectrometer. Tissue soaked in D_2O with 10 mm TSP (sodium trimethyl silyl propionate) was placed in a 4 mm o.d. zirconium oxide rotor and spun at 5000 Hz at 300 K. A modified Carr-Purcell-Meiboom-Gill spin-echo pulse sequence $[90 - (\pi - 180 - \pi)_n]$ -acquisition) was used as a T_2 filter to remove the effects of lipids and large macromolecules on the spectra obtained (Garrod *et al.* 1999) (typically $n = 20$; total T_2 filter (2 nt) = 40 ms). Spectral assignments were confirmed by two-dimensional ^1H - ^1H COSY (Aue *et al.* 1976) and ^1H - ^{13}C HSQC experiments (Bax and Morris 1981). To calculate apparent diffusion coefficients (ADCs), diffusion-weighted spectra were acquired using a variable magnetic field gradient (maximum $100 \text{ gauss cm}^{-1}$) applied along the magic angle axis to attenuate metabolite resonances using a standard LED bipolar pulse program (Bruker, Germany). Spectra were acquired for 32 fractional increments of the gradient field with a 50-ms gradient delay (Δ), gradient pulse length of 1 ms (δ) and a 100- μs gradient recovery time (τ). ADCs were calculated according to the Stejskal-Tanner equation as described by Wu *et al.* (1995):

$$\ln(A_g) = \ln(A_0) - g^2 \delta^2 \gamma^2 D (\Delta - \delta/3 - \tau/2), \quad (1)$$

where g is the gradient strength, and A_g and A_0 are the resonance integrals at gradient strength g and during no gradient attenuation, respectively. A plot of $\ln(A_g)$ versus g^2 yields a linear plot of gradient $\gamma^2 \delta^2 D (\Delta - \delta/3 - \tau/2)$ for a one-component system from which D was calculated (Excel, Microsoft, CA, USA), assuming diffusion to be Gaussian in nature. For multicomponent systems a curved plot was produced consisting of a linear portion from the slow-moving environment and an area of curvature

containing contributions from both slow and fast ADC environments. ADCs were calculated from the first linear portion to assess the slowest diffusing environment and this contribution was subtracted from the attenuation curve to assess the diffusion of the other pool of water (assuming any exchange between the pools is minor). The accuracy of the gradient calibration was assessed using the ADC of free water.

Metabolite resonance integrals were compared between voles fed contaminated and control diet using a Student's *t*-test with Welch correction. Results are means $\pm$ SD. For line and curve fits, linear regression was applied with fits being considered significant for $R^2 > 0.95$.

To investigate whether voles were particularly susceptible to arsenic-induced renal tissue damage, two sets of five male wood mice (20.9 ± 1.8 g body weight) were fed either uncontaminated or 28 mg kg^{-1} As_2O_3 kg^{-1} contaminated feed in an identical manner to voles. Following sacrifice, tissue from the left kidney was examined both visually and using the CPMG pulse program as previously described.

Results

Despite the amount of tissue required for conventional solution-state NMR being six times more than for HRMAS ^1H -NMR, the two techniques produced comparable spectra for low molecular weight metabolites (figure 1). Some resonances such as those from creatine, glutamate and glutamine were more clearly resolved in the aqueous extract, but this arose largely from peak overlap with lipids and proteins in MAS spectra. The phosphocholine resonance (δ 3.26) dominated the choline-containing metabolites in aqueous extracts, but notably it was the smallest of the three choline derivatives in the MAS ^1H -NMR spectra of both

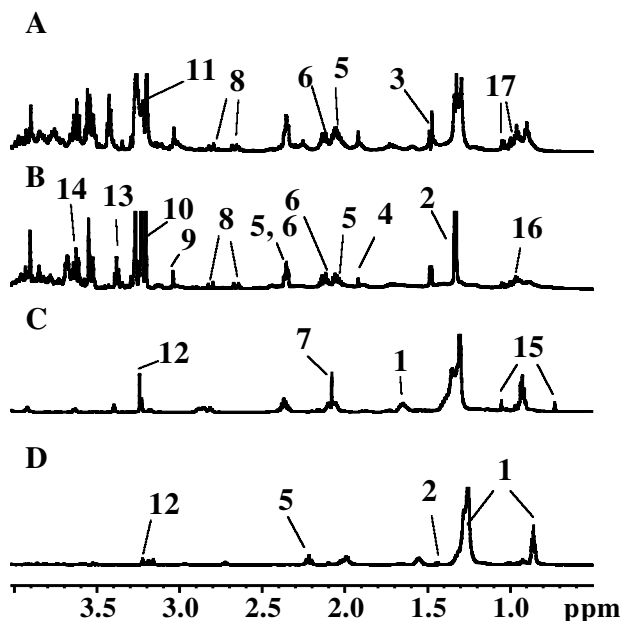


Figure 1. Spectra demonstrating the different chemical environments a number of metabolites are contained within: CPMG ^1H -HRMAS spectrum acquired on a piece of inner cortex from bank vole kidney (A), aqueous extract of another piece of inner cortex (B), chloroform/methanol extract of the resulting pellet after the aqueous extract (C) and CPMG ^1H -HRMAS spectrum of the final pellet remaining after the two extractions (D). 1, lipid triglycerides; 2, lactate; 3, alanine; 4, acetate; 5, glutamate/*N*-acetyl glutamate; 6, glutamine; 7, methionine; 8, citrate; 9, creatine; 10, choline; 11, phosphocholine/glycerophosphocholine; 12, phosphatidylcholine; 13, taurine; 14, myo-inositol; 15, non-esterified cholesterol esters; 16, leucine; 17, valine.

whole tissue and the pellet remaining after tissue extraction. Despite prior aqueous extraction, a number of aqueous soluble metabolites such as glutamate and alanine were still detected in the pellet remaining after extraction (figure 1).

To examine the chemical environment of the metabolites in vole tissue measured by HRMAS ^1H -NMR, a series of CPMG spectra was acquired with a variety of spin-echo delays ranging from 40 to 160 ms (figure 2). On increasing the spin-echo delay in the pulse sequence, signals from CH_2 lipid triglyceride resonances (with short T_2 relaxation times) were more greatly attenuated in relation to resonances from phosphatidylcholine, phosphocholine, choline and amino acids (having longer T_2 's). However, the relative intensity of the CH_2 triglyceride resonance decreased with respect to the CH_3 signal intensity on increasing the spin-echo delay from 80 to 160 ms.

Following sacrifice of the animals, a visual autopsy was performed on the kidney, liver, gut, lung and heart. In three of the voles exposed to As^{3+} , mild haemorrhagic changes were apparent in the renal cortex. At autopsy no other gross changes were observed in the voles. HRMAS ^1H -NMR spectra from the vole

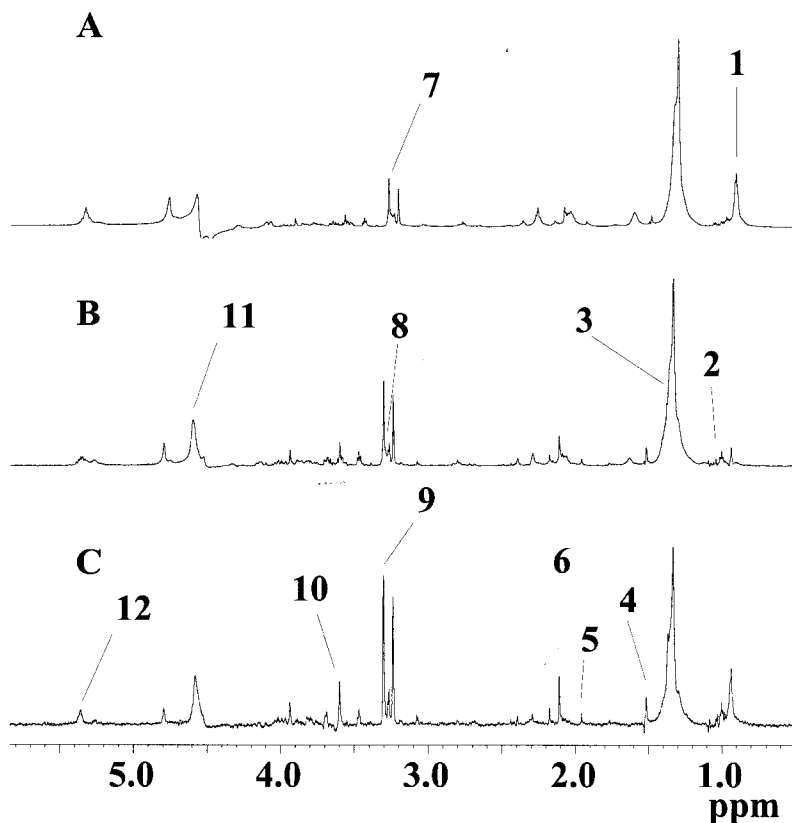


Figure 2. CPMG spectra of bank vole kidney tissue (inner cortex) acquired with spin-echo delays of 40 (A), 80 (B) and 160 ms (C). 1, CH_3 lipid resonance; 2, leucine/valine; 3, CH_2 lipid resonance; 4, alanine (deutero alanine following exchange); 5, acetate; 6, methionine; 7, choline; 8, phosphocholine; 9, phosphatidylcholine/glycerophosphocholine; 10, glycine; 11, water (partially saturated); 12, olefinic fat groups.

spectra indicated a general broadening of $(CH_2)_n$ lipid triglyceride resonances in the kidneys of the three voles with haemorrhagic damage (figure 3). The triplet resonances of taurine at δ 3.42 were decreased in these spectra. Considering all the spectra and comparing the integral regions between δ 1.22 and 1.34 that made up the $(CH_2)_n$ lipid triglyceride resonance directly, the mean resonance was clearly altered in pattern in tissue exposed to As^{3+} . For the integral region δ 1.38-1.42 the signal was decreased following exposure (control = 52.5 ± 10.3 ; all As^{3+} -dosed voles = 22.5 ± 2.6 relative to the total spectral intensity set to 1000 arbitrary intensity units; $p < 0.05$, $n = 5$) but increased for δ 1.26-1.30 (control = 47.5 ± 7.9 ; all As^{3+} -dosed voles = 83.3 ± 14.1 ; $p < 0.05$, $n = 5$). During the acquisition of spectra, it was noted that magnetic field homogeneity was inferior within the tissue as determined by sample line-shapes for samples with haemorrhagic damage, probably due to the presence of metahaemoglobin. However, the TSP in the D_2O added to the sample as a lock and chemical shift reference had a line width of < 2 Hz in all samples. No biochemical differences were detected between the two groups for the aqueous extracts examined.

To examine further the level of cellular disruption in the renal tissue, ADCs were calculated for intra- and extracellular water (figure 4). The ADC of intracellular water was increased in the group exposed to As^{3+} ($6.27 \pm 1.95 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$, $n = 5$ compared with control tissue ($4.05 \pm 0.74 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$, $p = 0.042$). The faster diffusing water environment (extracellular) had similar ADCs for both tissue types but lower than that of free water (all animals exposed

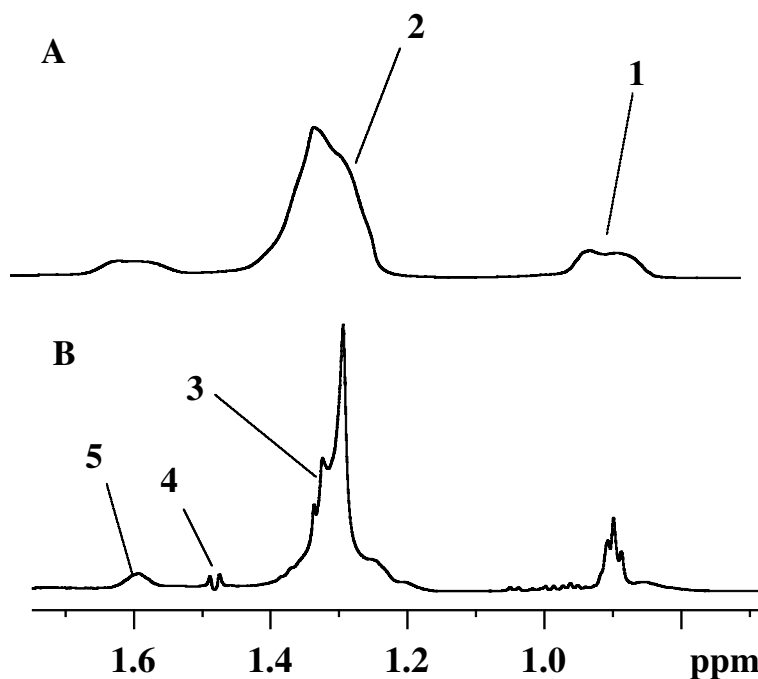


Figure 3. CH_3 and CH_2 lipid triglyceride region of two CPMG spectra from the vole fed control (B) and As^{3+} -contaminated (A) diets. A decrease in spectral resolution in spectrum (A) was also accompanied by a change in lipid profile. 1, CH_3 lipid triglycerides; 2, CH_2CH_2 and CH_3CH_2 lipid triglycerides; 3, lactate; 4, alanine; 5, $COCH_2CH_2$ lipid triglycerides.

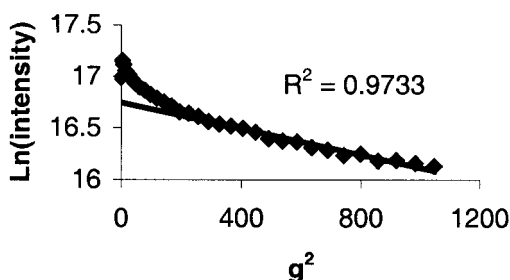


Figure 4. Plot of $\log_e$ [attenuation] against gradient strength ($\text{g Gauss}^{-1} \text{cm}^{-1}$) squared for water in renal tissue from an As^{3+} -exposed vole. The trend line indicates the fit of the final 19 gradient strengths used in the diffusion-weighted spectrum. Deviation from linearity indicates multi-compartmentation of water. R^2 for least-squares fit for trendline and final 19 points.

to As^{3+} : $10.7 \pm 2.7 \times 10^{-6} \text{cm}^2 \text{s}^{-1}$, $n = 5$; control tissue $8.0 \pm 1.3 \times 10^{-6} \text{cm}^2 \text{s}^{-1}$; free water $22.29 \times 10^{-6} \text{cm}^2 \text{s}^{-1}$; Luo *et al.* 1999).

To investigate whether bank vole renal tissue was particularly prone to As^{3+} -induced damage, the wood mice similarly exposed to As_2O_3 were killed and autopsied. No gross anatomical changes were evident. No changes were also evident in HRMAS ^1H -NMR spectra of renal tissue (data not shown).

Discussion

The HRMAS ^1H -NMR analysis carried out here demonstrates that conventional aqueous and organic extraction, normally carried out when tissue metabolites are examined using liquid-state NMR, may be incomplete, even for water-soluble metabolites. In this study, the pellet remaining after aqueous and lipid extractions contained not only lipid constituents, but also amino acids, including alanine and glutamate. While an acetonitrile/water extraction process was used, Weybright *et al.* (1998) also observed a similar effect with adipocytes using methanol/water extraction and suggested that these aqueous soluble metabolites were encapsulated in lipid bilayers preventing their extraction. These bilayers may be associated with vesicles used to transport chemicals, including xenobiotics, around the cell (Rothman 1994). MAS ^1H -NMR spectroscopy provides the only method capable of simultaneously investigating many of the 'motionally constrained' compartments found in a tissue.

An important benefit of using NMR spectroscopy to investigate complex biological materials is that different NMR experiments can be tailored to investigate the chemical environments in which molecules are found. By varying the relaxation delay in the CPMG spin-echo experiments, spectra are obtained that are dependent on the spin-spin relaxation properties (T_2) of the molecules detected, and hence the local molecular environment. In this study, increasing the relaxation delay from 40 to 160 ms greatly attenuated the CH_2 resonance associated with lipid triglycerides in kidney tissue, whereas the signal intensities of protons on phosphatidylcholine, phosphocholine, glycerophosphocholine and choline were less attenuated. Similar effects have been observed in human liposarcoma (Millis *et al.* 1999), indicating that choline derivatives have a high degree of fluidity compared with lipid triglycerides. Unsurprisingly, as the relaxation delay was increased, the

signal from small, highly mobile amino acid and polyol resonances became more visible (possessing long T_2 's). However, the relative attenuation of the resonance associated with CH_3 lipid triglycerides first decreased and then increased relative to the signal intensity of CH_2 triglyceride protons. This is consistent with lipid triglycerides existing in at least two major NMR-detectable environments. Weybright *et al.* (1998) also noted two pools of lipids when they were examining intact adipocytes, measuring diffusion coefficients an order of magnitude apart depending on the environment.

After being exposed to As^{3+} , three of five voles showed signs of haemorrhage within the renal tissue. MAS ^1H spectra obtained from the renal tissue identified an altered CH_2 profile in these samples, with the resonance associated with CH_2CH_2 and CH_3CH_2 groups at δ 1.30 being particularly broad. This may in part result from leakage of blood into renal tissue, with methaemoglobin causing resonance line broadening. However, given the specific increase in size and shape of the resonance at $\sim$ 1.3 ppm, this suggests other pathophysiological changes occurred in the vole renal tissue. In blood plasma, the $(CH_2)_n$ resonance is a composite signal with LDL lipids contributing to the lower ppm region ($\delta \sim$ 1.22–1.30) of the resonance (upfield) and VLDL to the upper ppm region ($\delta \sim$ 1.30–1.40) (Nicholson *et al.* 1995), while in spectra of skeletal muscle the $(CH_2)_n$ resonance of intra- and extracellular myocellular lipids have been found to centre around δ 1.28 and 1.50, respectively (Schick *et al.* 1993, Boesch *et al.* 1997). This suggests either a higher proportion of LDL lipid but less VLDL lipid in the blood within the tissue or a change in the proportion of intra- and extracellular lipids within the renal tissue following As^{3+} exposure. The lipid composition of blood plasma depends on diet and lipid homeostasis (Newsholme and Leech 1983). As^{3+} toxicity alters lipid constituents, as target sites include the gastrointestinal tract and the bile duct in the liver (Goyer 1995), both salient points in lipid metabolism. Thus, by altering lipid absorption into the body, As^{3+} toxicity may have changed both the profile of lipids in blood plasma and the ratio of intra- and extracellular lipids in peripheral tissues such as the kidney to produce some of the changes seen.

To investigate cellular disruption within renal tissue, the ADCs of water were measured using a magic angle field gradient to attenuate the resonance intensity of water. This technique has been applied to ADC measurement in cells using both high-resolution solution state and MAS NMR techniques (Pfeuffer *et al.* 1998, Torres *et al.* 1998, Garcia-Perez *et al.* 1999), but not, to our knowledge, in intact tissues as a high-resolution diagnostic tool. An increase in the ADC of the slowest diffusing pool of water was recorded following As^{3+} exposure. This pool represents intracellular water and the change detected suggests either an increase in cell size or increased rupture of smaller cells, as might occur if As^{3+} toxicity induced necrosis in epithelial cells.

Renal tissue from wood mice exposed to As^{3+} did not exhibit any visible or NMR spectral abnormalities, suggesting bank voles are particularly susceptible to As^{3+} toxicity. We have previously demonstrated that bank vole renal tissue is susceptible Cd^{2+} toxicity (Griffin *et al.* 2000a) and vole renal tissue may be especially vulnerable to toxic insults. However, molecular signposts for necrotic damage in wood mouse renal tissue may be particularly hard to identify using HRMAS ^1H -NMR spectroscopy, as renal tissue contains relatively large proportion of fatty acids, obscuring many other metabolites (Griffin *et al.* 2000b). This

preliminary study into the response of wild rodents to environmental contaminants does highlight the physiological differences between species, suggesting that several species should be sampled in order to estimate a xenobiotic's impact on an ecosystem.

In conclusion, this study demonstrates that by using spectral editing in conjunction with MAS ^1H -NMR spectroscopy, such as T_2 and diffusion weighting pulse programs, it becomes possible to examine the environment in which these metabolites occur, further improving the diagnostic capabilities of this technique.

Acknowledgements

The work was supported by a Natural Environment Research Council-funded Environmental Diagnostics award. J. L. G. is funded by a Royal Society University Research Fellowship.

References

- ANDREW, E. R. and EADES, R. G., 1953, Removal of dipolar broadening of NMR spectra of solids by spectral rotation. *Nature*, **183**, 1802.
- AUE, W. P., BARTHOLDI, E. and ERNST, R. R., 1976, Two dimensional spectroscopy. Application to nuclear magnetic resonance. *Journal of Chemical Physics*, **64**, 2229.
- BAX, A. and MORRIS, G. A., 1981, An improved method for heteronuclear chemical shift correlation by two-dimensional NMR. *Journal of Magnetic Resonance*, **42**, 501-505.
- BOESCH, C., SLOTBOOM, H., HOPPELER, H. and KRIES, R., 1997, *In vivo* determination of intramyocellular lipids in human muscle by means of localized ^1H -MR-spectroscopy. *Magnetic Resonance Medicine*, **37**, 484-494.
- CHENG, L. L., MA, M. J., BECERRA, L., HALE, T., TRACEY, I., LACKNER, A. and GONZALEZ, R. G., 1997, Quantitative neuropathology by high resolution magic angle spinning proton magnetic resonance spectroscopy. *Proceedings of the National Academy of Sciences, USA*, **94**, 6408-6413.
- ERRY, B. V., MACNAIR, M. R., MEHARG, A. A. and SHORE, R. F., 1999, Seasonal variation in dietary and body organ arsenic concentrations in wood mice (*Apodemus sylvaticus*) and bank voles (*Clethrionomys glareolus*). *Bulletin of Environmental Contamination and Toxicology*, **63**, 567-574.
- ERRY, B. V., MACNAIR, M. R., MEHARG, A. A. and SHORE, R. F., 2000, Arsenic contamination in wood mice (*Apodemus sylvaticus*) and bank voles (*Clethrionomys glareolus*) on abandoned mine sites in south-western Britain. *Environmental Pollution*, **110**, 179-187.
- GARCIA-PEREZ, A. I., LOPEZ-BELTRAN, E. A., KLUNER, P., LUQUE, J., BALLESTEROS, P. and CERDAN, S., 1999, Molecular crowding and viscosity as determinants of translational diffusion of metabolites in subcellular organelles. *Archives of Biochemistry and Biophysics*, **362**, 329-338.
- GARROD, S., HUMPFER, E., SPRAUL, M., CONNER, S. C., POLLEY, S., CONNELLY, J., LINDON, J. C., NICHOLSON, J. K. and HOLMES, E., 1999, High-resolution magic angle spinning ^1H NMR spectroscopic studies on intact rat renal cortex and medulla. *Magnetic Resonance in Medicine*, **41**, 1108-1118.
- GOYER, R. A., 1995, Toxic effects of metals. In K. Klassen (ed.), *Casarett & Doull's Toxicology*, 5th edn. (Singapore: McGraw-Hill).
- GRIFFIN, J. L., WALKER, L. A., GARROD, S., HOLMES, E., SHORE, R. F. and NICHOLSON, J. K., 2000b, NMR spectroscopy based metabolomic studies on the comparative biochemistry of the kidney and urine of the bank vole (*Clethrionomys glareolus*), wood mouse (*Apodemus sylvaticus*), white toothed shrew (*Crocidura suaveolens*) and the laboratory rat. *Comparative Biochemistry and Physiology, Part B*, **127**, 357-367.
- GRIFFIN, J. L., WALKER, L. A., TROKE, J., OSBORN, D., SHORE, R. F. and NICHOLSON, J. K., 2000a, The initial pathogenesis of cadmium induced renal toxicity. *FEBS Letters*, **478**, 147-150.
- HOLMES, E., FOXALL, P. J., SPRAUL, M., FARRANT, R. D., NICHOLSON, J. K. and LINDON, J. C., 1997, 750 MHz ^1H NMR spectroscopy characterisation of the complex metabolic pattern of urine from patients with inborn errors of metabolism: 2-hydroxyglutaric aciduria and maple syrup urine disease. *Journal of Pharmacology and Biomedical Analysis*, **15**, 1647-1659.
- ISMAIL, A. and ROBERTS, R. D., 1992, Arsenic in small mammals. *Environmental Technology*, **13**, 1091-1095.

- LUO, R. S., LIU, M. L. and MAO, X. A., 1999, NMR diffusion and relaxation study of drug-protein interaction. *Spectrochimica Acta, Part A*, **55**, 1897-1901.
- MILLIS, K., MAAS, E., CORY, D. G. and SINGER, S., 1997, Gradient high resolution magic angle spinning nuclear magnetic resonance spectroscopy of human adipocyte tissue. *Magnetic Resonance in Medicine*, **38**, 399-403.
- MILLIS, K., WEYBRIGHT, P., CAMPBELL, N., FLETCHER, J. A., FLETCHER, C. D., CORY, D. G. and SINGER, S., 1999, Classification of human liposarcoma and lipoma using *ex vivo* proton nmr spectroscopy. *Magnetic Resonance in Medicine*, **41**, 257-267.
- NEWSHOLME, E. A. and LEECH, A. R., 1983, Catabolism of lipids. In E. A. Newsholme and A. R. Leech (eds.), *Biochemistry for the Medical Sciences* (Chichester: Wiley) pp. 336-356.
- NICHOLSON, J. K., FOXALL, P. J., SPRAUL, M., FARRANT, R. D. and LINDON, J. C., 1995, 750 MHz ^1H and ^1H - ^{13}C NMR spectroscopy of human blood plasma. *Analytical Chemistry*, **67**, 793-811.
- OEHME, F. W., 1972, Mechanisms of heavy metal toxicities. *Clinical Toxicology*, **5**, 151-167.
- PFEUER, J., FLOGEL, U., DREHER, W. and LEIBFRITZ, D., 1998, Restricted diffusion and exchange of intracellular water: theoretical modelling and diffusion time dependence of ^1H NMR measurements on perfused glial cells. *NMR in Biomedicine*, **11**, 19-31.
- ROTHMAN, J. E., 1994, Mechanisms of intracellular protein transport. *Nature*, **372**, 55-63.
- SANINS, S., TIMBRELL, J. A., ELCOMBE, C. and NICHOLSON, J. K., 1992, Proton NMR spectroscopic studies on the metabolism and biochemical effects of hydrazine *in vivo*. *Archives of Toxicology*, **66**, 489-495.
- SCHICK, F., EISMANN, B., JUNG, W. I., BONGERS, H., BUNSE, M. and LUTZ, O., 1993, Comparison of localized proton NMR signals of skeletal muscle and fat tissue *in vivo*: two lipid compartments in muscle tissue. *Magnetic Resonance in Medicine*, **29**, 158-167.
- SHOCKCOR, J. P., UNGER, S. E., WILSON, I. D., FOXALL, P. J. D., NICHOLSON, J. K. and LINDON, J. C., 1996, Combined HPLC, NMR spectroscopy and ion-trap mass spectrometry with application to the detection and characterization of xenobiotics and endogenous metabolites in human urine. *Analytical Chemistry*, **68**, 4431-4435.
- ST PETERY, J., RENNERT, O. M., CHOI, H. and WOLFSON, S., 1970, Arsenic poisoning in childhood. *Clinical Toxicology*, **3**, 519-526.
- TOMLINS, A., FOXALL, P. J. D., LINDON, J. C., LYNCH, M. J., SPRAUL, M., EVERETT, J. and NICHOLSON, J. K., 1998, High resolution magic angle spinning ^1H nuclear magnetic resonance analysis of intact prostatic hyperplastic and tumour tissues. *Analytical Communications*, **35**, 113-115.
- TORRES, A. M., MICHNIEWICZ, R. J., CHAPMAN, B. E., YOUNG, G. A. R. and KUCHEL, P. W., 1998, Characterisation of erythrocyte shapes and sizes by NMR diffusion-diffraction of water: correlations with electron micrographs. *Magnetic Resonance Imaging*, **16**, 423-434.
- WEYBRIGHT, P., MILLIS, K., CAMPBELL, N., CORY, D. G. and SINGER, S., 1998, Gradient, high resolution, magic angle spinning ^1H nuclear magnetic resonance spectroscopy of intact cells. *Magnetic Resonance in Medicine*, **39**, 337-344.
- WU, D., CHEN, A. and JOHNSON, C. S., 1995, An improved diffusion-ordered spectroscopy experiment incorporating bipolar-gradient pulses. *Journal of Magnetic Resonance (Series A)*, **115**, 260-264.

Characterization of moclobemide *N*-oxidation in human liver microsomes

J. HOSKINS^{†*}, G. SHENFIELD[†], M. MURRAY[‡] and A. GROSS^{†§}

[†] Department of Clinical Pharmacology, Royal North Shore Hospital, St Leonards, NSW 2065, Australia

[‡] School of Physiology and Pharmacology, University of New South Wales, Randwick NSW 2052, Australia

[§] Present address: James Lance GlaxoSmithKline Medicines Research Unit, Parkes 10 E, Prince of Wales Hospital, Randwick NSW 2031, Australia

Received 11 January 2001

1. Moclobemide undergoes morpholine ring *N*-oxidation to form a major metabolite in plasma, Ro12-5637.

2. The kinetics of moclobemide *N*-oxidation in human liver microsomes (HLM) ($n = 6$) have been investigated and the mixed-function oxidase enzymes catalysing this reaction have been identified using inhibition, enzyme correlation, altered pH and heat pretreatment experiments.

3. *N*-oxidation followed single enzyme Michealis-Menten kinetics (0.02–4.0 mM). $K_{m \text{ app}}$ and V_{max} ranged from 0.48 to 1.35 mM (mean $\pm$ SD 0.77 ± 0.34 mM) and 0.22 to 2.15 nmol $\text{mg}^{-1} \text{ min}^{-1}$ (1.39 ± 0.80 nmol $\text{mg}^{-1} \text{ min}^{-1}$), respectively.

4. The *N*-oxidation of moclobemide strongly correlated with benzydamine *N*-oxidation, a probe reaction for flavin-containing monooxygenase (FMO) activity, (0.1 mM moclobemide, $r_s = 0.81, p < 0.005$; 4 mM moclobemide, $r_s = 0.94, p = 0.0001$). Correlations were observed between moclobemide *N*-oxidation and specific cytochrome P450 (CYP) activities at both moclobemide concentrations (0.1 mM moclobemide, CYP2C19 $r_s = 0.66, p < 0.05$; 4 mM moclobemide, CYP2E1 $r_s = 0.56, p < 0.05$).

5. The general P450 inhibitor, *N*-benzylimidazole, did not affect the rate of Ro12-5637 formation (0% inhibition versus control) at 1.3 mM moclobemide. Furthermore, the rate of Ro12-5637 formation in HLM was unaffected by inhibitors or substrates of specific P450s ($< 10\%$ inhibition versus control).

6. Heat pretreatment of HLM in the absence of NADPH (inactivating FMOs) resulted in 97% inhibition of Ro12-5637 formation. *N*-oxidation activity was greatest when incubated at pH 8.5. These results are consistent with the reaction being FMO mediated.

7. In conclusion, moclobemide *N*-oxidation activity has been observed in HLM *in vitro* and the reaction is predominantly catalysed by FMOs with a potentially small contribution from cytochrome P450 isoforms.

Introduction

Moclobemide is a reversible selective inhibitor of monoamine oxidase-A (MAO-A) that is widely prescribed for the treatment of depression. It undergoes extensive first-pass metabolism and following oral administration $< 0.5\%$ of the parent drug is excreted unchanged in the urine (Jauch *et al.* 1990). Moclobemide undergoes *N*- and *C*-oxidation to form its two major metabolites in plasma, Ro12-5637 and Ro12-8095, respectively (Jauch *et al.* 1990) (figure 1). Ro12-5637 is a

* Author for correspondence: Department of Pharmacology, Bosch Building D05, University of Sydney, NSW 2006, Australia; e-mail: janelle@med.usyd.edu.au

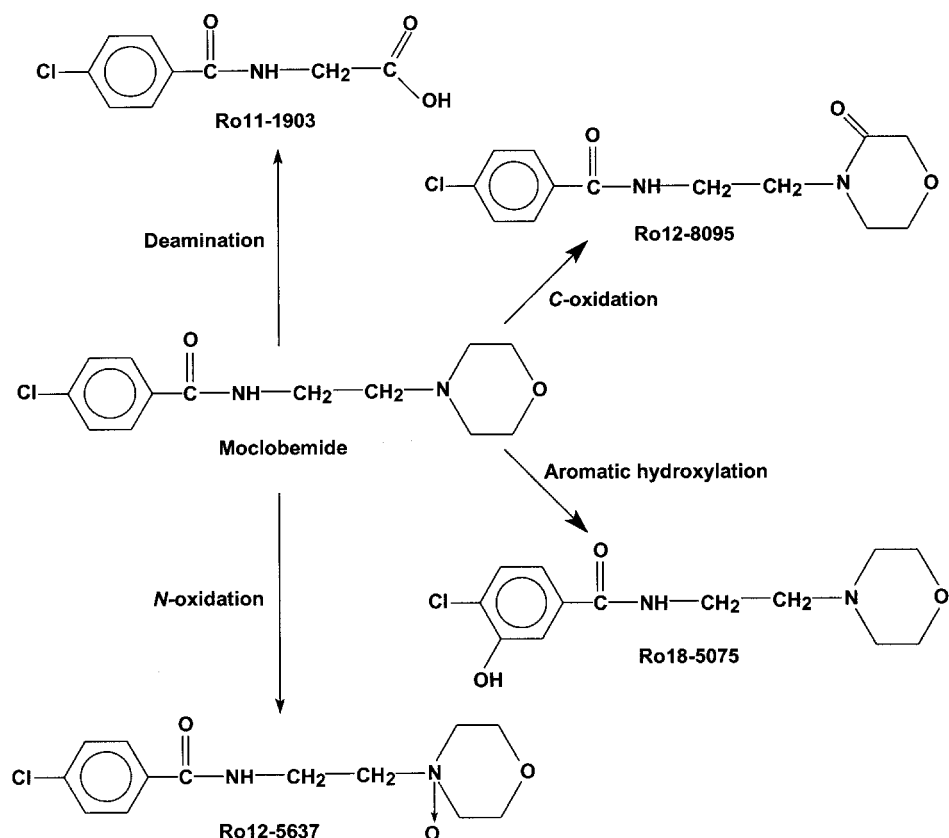


Figure 1. Primary metabolic pathways of moclobemide.

weak inhibitor of MAO-A, whereas Ro12-8095 has no MAO-A inhibitory activity (Da Prada *et al.* 1989). About 35% of an orally administered dose of moclobemide is recovered in urine as Ro12-5637, whereas Ro12-8095 accounts for <1% of the dose (Mayersohn and Guentert 1995).

The enzymes catalysing moclobemide oxidation have not been characterized. *In vivo* evidence suggests that the C-oxidation pathway is partially mediated by cytochrome P450 2C19 (Gram *et al.* 1995). *N*-oxidation of the tertiary amines of many psychoactive drugs, including imipramine, clozapine and olanzapine, is predominantly catalysed by FMOs rather than by cytochrome P450s (Lemoine *et al.* 1990, Ring *et al.* 1996, Tugnait *et al.* 1997). *N*-oxidation of moclobemide occurs at the tertiary amine of the morpholine ring suggesting that this reaction may be FMO rather than P450 mediated.

Cytochrome P450s and FMOs are present in the microsomal fraction prepared by differential centrifugation of homogenized hepatic cells. The hepatic oxidation of a drug can be characterized *in vitro* using microsomes prepared from human liver samples. Straightforward experiments can be employed to determine whether a reaction is FMO- or P450-mediated (Grothusen *et al.* 1996) and enzyme correlation and chemical inhibition experiments can be performed to identify the specific P450 isoforms involved (Chang *et al.* 1994, Newton *et al.* 1995, Bourrie

et al. 1996, Ono *et al.* 1996). The objective of the current study was to characterize moclobemide N-oxidation in human liver microsomes (HLM) *in vitro* and to identify the mixed-function oxidase(s) that catalyse(s) this reaction.

Materials and methods

Chemicals

Dextromethorphan hydrobromide, coumarin, α -naphthoflavone, phenacetin, quinidine, salicylic acid, tolbutamide, troleandomycin (TAO) and β -NADP⁺ (β -nicotinamide adenine dinucleotide phosphate, potassium salt) were purchased from Sigma Chemical Co. (St Louis, MO, USA). *S*-mephenytoin and ($\pm$)-4'-hydroxymephenytoin were purchased from Salford Ultrafine Chemicals and Research Ltd (Manchester, UK) and didiethylcarbamate (DDC) and *N*-benzylimidazole from Aldrich Chemical Co. (Milwaukee, WI, USA). Other drugs and drug metabolites were generously provided as follows: moclobemide, Ro12-8095, Ro12-5637 and dextrorphan tartrate from Roche Pty Ltd (Sydney, NSW, Australia), ketoconazole from Janssen-Cilag Pty Ltd (North Ryde, NSW, Australia), sulphaphenazole from Ciba-Geigy Australia Ltd (Pendle Hill, NSW, Australia), and benzydamine and benzydamine *N*-oxide from 3M Pharmaceuticals Pty Ltd (Sydney, NSW, Australia). Glucose 6-phosphate and glucose 6-phosphate dehydrogenase were purchased from Boehringer Mannheim (Sydney, NSW, Australia). Methanol and triethylamine were HPLC grade and purchased from BDH (Kilsyth, VIC, Australia). Potassium phosphate salts, 70% perchloric acid, 85% orthophosphoric acid and magnesium chloride were analytical grade and purchased from Ajax Chemicals (Auburn, NSW, Australia). Methanolic solutions were prepared of moclobemide and inhibitors and stored at -20°C . Ro12-5637 was prepared in double-distilled water.

Ro12-5637 high-performance liquid chromatography (HPLC) assay

The reversed-phase HPLC method of Geschke *et al.* (1987) was modified to measure moclobemide and Ro12-5637 in microsomal incubates. A mobile phase consisting of methanol:triethylamine:33.5 mM potassium dihydrogen orthophosphate (25:0.1:74.9 v/v/v), adjusted to pH 3 using orthophosphoric acid, was pumped at a flow rate of 1.3 ml min^{-1} using an LC-10AT pump (Shimadzu Oceania Pty Ltd, Rydalmere, NSW, Australia) with a back pressure of $150\text{--}180\text{ kg cm}^{-2}$. An SIL-10AXL Autoinjector (Shimadzu Oceania) was used to inject 0.13 ml of the incubates and calibration standards onto a Spherisorb 5 μm C6 column $150 \times 4.6\text{ mm i.d.}$ (Phenomenex, Torrance, CA, USA). An SPD-10A UV-Vis detector (Shimadzu Oceania) set at 240 nm detected moclobemide and Ro12-5637. The system was controlled and chromatograms integrated using CLASS LC10 (v.1.60) software (Shimadzu Oceania). Calibration standards of 0.04–12.3 μM Ro12-5637 in 0.2 ml 0.1 M potassium phosphate buffer (pH 7.4) and 10 μl 17.5% perchloric acid were prepared in 1.7 ml centrifuge tubes (Edwards Instrument Co., Narellan, NSW, Australia). Standards were vortexed and centrifuged for 8 min at 6800g. Standard curves were estimated by least-squares linear regression of Ro12-5637 concentration versus peak height. Concentrations of Ro12-5637 in the microsomal incubates were calculated from the regression lines.

Microsomal preparation

Microsomes from HL2-32 ($n = 11$) were prepared as described by Sutton *et al.* (Sutton *et al.* 1997). HLM1-6 were prepared from healthy hepatic tissue obtained at biopsy that would otherwise have been discarded as surgical waste. After tissue removal it was immediately placed into liquid nitrogen and stored at -70°C . The cytochrome P450 content and protein concentration were determined as previously described (Rieutord *et al.* 1995).

Moclobemide N-oxidation microsomal assay

Methanolic solutions of moclobemide and inhibitor solutions (prepared in methanol and stored at -20°C) were added to 10 ml polypropylene tubes (Sarstedt Australia Pty Ltd, Technology Park, SA, Australia) and the methanol was evaporated using a Speedivac Rotary Evaporator (Savant Instruments, Inc., Farmingdale, NY, USA) prior to incubation. A typical incubation (final volume of 0.2 ml) contained moclobemide, potassium phosphate buffer (0.1 M, pH 7.4) and an NADPH-generating system (final concentrations: 10 mM glucose 6-phosphate, 10 mM MgCl_2 , 1 mM NADP⁺, 0.4 U glucose 6-phosphate dehydrogenase). Samples were pre-incubated for 5 min at 37°C in a constant-temperature SW-20 $^{\circ}\text{C}$ agitated water bath (Julabo Labortechnik, Seelbach, Germany) and reactions were initiated by adding HLM. The reaction proceeded without a lid so that the incubate was in equilibrium with

oxygen in the air. The reaction was stopped by adding 10 μ l 17.5% perchloric acid and samples were then vortexed and centrifuged for 8 min at 6800g.

The relationship between rate of *N*-oxide formation and HLM protein concentration was assessed from 0.025 to 0.3 mg ml⁻¹ at 37°C for 60 min. The relationship between rate of formation and incubation time was assessed from 5 to 120 min at 37°C at a protein concentration of 0.1 mg ml⁻¹. To determine the reproducibility of the microsomal assay, moclobemide was incubated with HLM2 in duplicate on four separate occasions to assess between-day reproducibility and in quadruplicate on a single occasion to assess within-day reproducibility. The conditions selected for investigation were a protein concentration of 0.1 mg ml⁻¹ and an incubation time of 60 min. For kinetic studies, samples from HLM1, 2-6 and HL27 were incubated with increasing concentrations of moclobemide (0.02–4.0 mM). For activity correlations the rate of Ro12-5637 formation was measured at 0.1 and 4 mM moclobemide in 11 HLM samples. All other experiments were performed at 1.3 mM moclobemide (average apparent K_m). To investigate the contribution of FMO to the *N*-oxidation of moclobemide, microsomes (HLM2) were pre-incubated with and without the NADPH-regenerating system at 50°C for 1 min and stored on ice for <10 min before being incubated with moclobemide. To assess the effect of pH on moclobemide *N*-oxidation, the rate of Ro12-5637 formation was measured at pHs 7.0, 7.4, 8.0, 8.5, 9.0 and 9.5 in HLM2. For these incubations, 0.2 M Tris-HCl was prepared and adjusted to the appropriate pH by the addition of 0.1 M HCl. All incubations were performed in duplicate and the average value is reported.

Activity of specific P450 isoforms and FMOs

Eleven HLM samples were assessed for the following activities: testosterone 6 β -hydroxylation (CYP3A4; 50 μ M), 7-ethoxyresorufin-*O*-deethylation (CYP1A2; 2.5 μ M), *N*-dimethylnitrosoamine *N*-demethylation (CYP2E1; 4 mM), aniline 4-hydroxylation (CYP2E1; 5 mM) and tolbutamide 4-hydroxylation (CYP2C9; 0.3 mM) as described (Sutton *et al.* 1997). Dextromethorphan *O*-demethylation (CYP2D6; 40 μ M) (Kerry *et al.* 1993), *S*-mephenytoin 4'-hydroxylation (CYP2C19; 0.2 mM) (Chiba *et al.* 1993) and benzydamine *N*-oxidation (FMO; 0.5 mM) (Baldock *et al.* 1990, Lang *et al.* 1998) were also assessed.

Chemical inhibition experiments

Potential chemical inhibitors were pre-incubated with moclobemide as described above and the reactions were initiated by adding HLM2. The mechanistic inhibitors, TAO and DDC, were pre-incubated with HLM and an NADPH-regenerating system for 30 min at 37°C and the moclobemide *N*-oxidation reaction initiated by adding moclobemide in 0.1 M phosphate buffer (pH 7.4). The final concentrations of putative P450 inhibitors and substrates were 1 mM *N*-benzylimidazole (general P450 inhibitor), 10 and 25 μ M coumarin (CYP2A6), 10 and 50 μ M DDC (CYP2E1), 2 and 5 μ M ketoconazole (CYP3A4/5), 50 and 250 μ M *S*-mephenytoin (CYP2C19), 0.1 and 1.0 μ M α -naphthoflavone (CYP1A1/2), 50 and 250 μ M phenacetin (CYP1A1/2), 0.5 and 5 μ M quinidine (CYP2D6), 10 and 20 μ M sulphaphenazole (CYP2C9), 0.2 and 0.5 mM tolbutamide (CYP2C9), and 10 and 50 μ M TAO (CYP3A4). Salicylic acid (0.25 mM) was used as a negative control. The final concentrations were selected on the basis of literature reports of each compound's maximum inhibition selectivity (Grothusen *et al.* 1996) and the K_m of the substrates (Chiba *et al.* 1993, Chang *et al.* 1994, Newton *et al.* 1995, Bourrie *et al.* 1996, Ono *et al.* 1996).

Data analysis

The rate of metabolite formation (v) was calculated as nanomoles of Ro12-5637 formed per milligram of protein per min (nmol mg⁻¹ min⁻¹). Apparent K_m and V_{max} derived from Eadie-Hofstee plots were used as initial estimates for fitting the kinetic data to the single-enzyme Michaelis-Menten equation using extended least-squares non-linear regression. The relationships between moclobemide *N*-oxidation and the activities of FMOs and specific P450s were assessed by calculating the magnitude and significance of the Spearman rank correlation coefficients.

Results

Using the HPLC technique described, the retention times of moclobemide and Ro12-5637 were 5.0 and 6.9 min, respectively, with a final run time of 20 min. A representative chromatogram of moclobemide incubated with HLM and an NADPH-regenerating system is shown in figure 2A. The calibration curve was linear for the measurement of Ro12-5637 over the concentration range studied with regression coefficients > 0.99. A typical equation for the Ro12-5637 calibra-

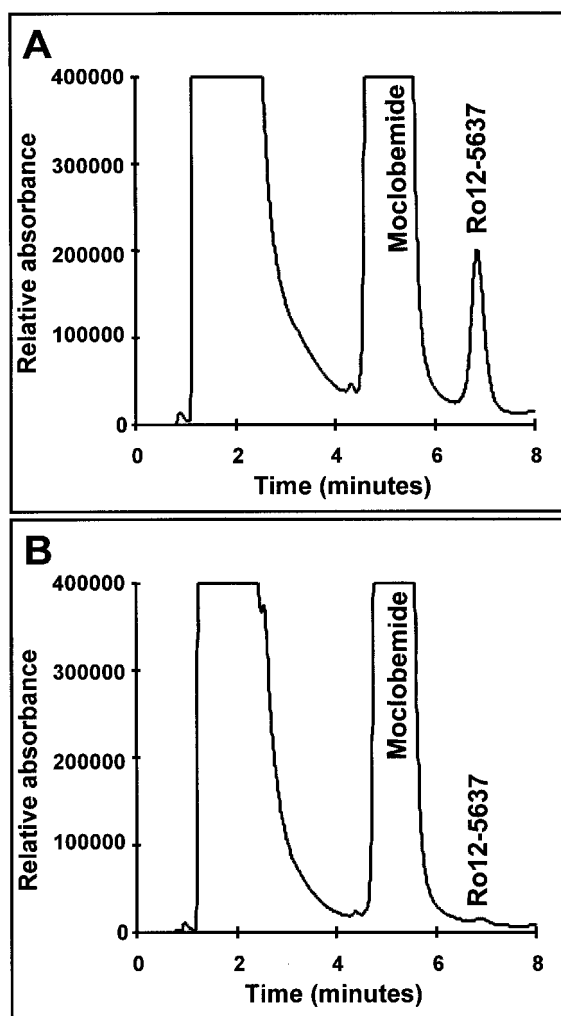


Figure 2. Representative chromatograms of moclobemide incubated with human liver microsomes ($0.1 \text{ mg protein ml}^{-1}$) at 37°C for 60 min. Chromatogram (A) is an incubation of moclobemide (1.3 mM) with HLM2 and (B) is an incubation of moclobemide (1.3 mM) with HLM2 pre-incubated at 50°C for 1 min in the absence of a NADPH-regenerating system. FMOs are unstable at 50°C in the absence of NADPH.

tion curve was $y = 5555x - 540$. The limit of quantification for the Ro12-5637 assay was $0.04 \mu\text{M}$.

Formation of Ro12-5637 was observed in the presence of an NADPH-regenerating system but not in the absence of NADPH under the same conditions (table 1). Over the range of moclobemide concentrations studied, $<10\%$ of the parent drug was metabolized. At a moclobemide concentration of 1.3 mM the within-day coefficient of variation of Ro12-5637 formation ($n = 4$) was $<4.0\%$. The between-day coefficient of variation for the incubation assay determined at 0.1 mM moclobemide on four separate occasions was $<4.0\%$.

Ro12-5637 formation was linear with time from 5 to 120 min and was also linear with protein concentration from 0.025 to 0.3 mg ml^{-1} (data not shown). The

Table 1. Velocity of moclobemide *N*-oxidation to Ro12-5637 under different experimental conditions. Moclobemide (1.3 mM) was incubated with human liver microsomes (HLM2) for 60 min at 37°C.

HLM pre-incubation conditions	Incubation co-factor ⁺	Velocity (nmol mg ⁻¹ min ⁻¹) [‡]
No preincubation	+ NADPH	1.26
No preincubation	–NADPH	0.00
Heat [§]	+ NADPH	0.03
Heat, NADPH [§]	+ NADPH	1.12

⁺Cofactor was an NADPH regenerating system consisting of glucose-6-phosphate, MgCl₂, NADP⁺ and glucose-6-phosphate dehydrogenase.

[‡]Each value represents the average of duplicate measurements.

[§]Microsomal protein was preincubated with moclobemide for 1 min at 50°C in the presence or absence of NADPH regenerating system and stored on ice <10 min before being incubated with moclobemide as per usual.

rate of Ro12-5637 formation was subsequently determined over the concentration range of 0.02-4 mM moclobemide. Eadie-Hofstee plots for metabolite formation were monophasic for all six livers tested so kinetic data was fitted to the Michaelis-Menten equation for a single enzyme system (figure 3). The apparent *K_m* varied 2.8-fold from 0.48 to 1.35 mM (mean ± SD = 0.77 ± 0.34 mM) and *V_{max}* varied 9.8-fold from 0.22 to 2.15 nmol mg⁻¹ min⁻¹ (mean ± SD = 1.39 ± 0.80 nmol mg⁻¹ min⁻¹).

In a bank of 11 HLM samples, large interindividual variability in moclobemide *N*-oxidation was observed at both 0.1 mM (26-fold) and 4 mM (65-fold). One

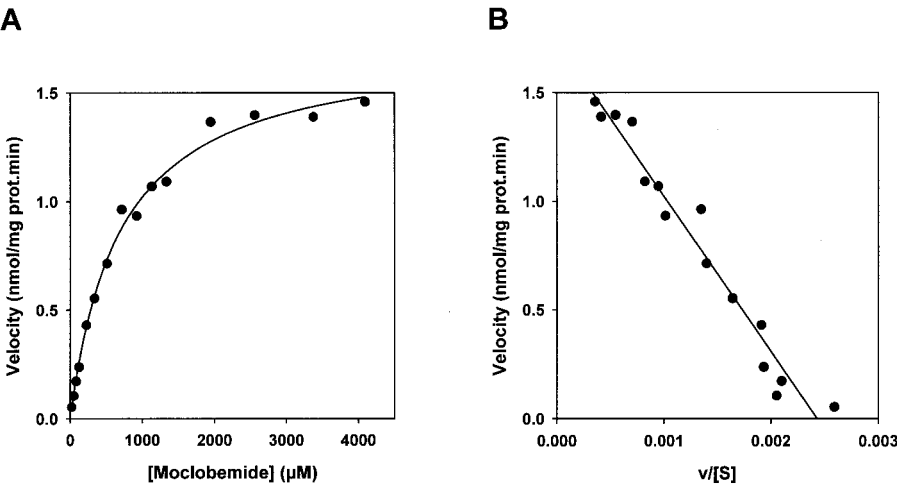


Figure 3. Moclobemide (0.02-4.0 mM) incubated with human liver microsomes (HLM6; 0.1 mg protein ml⁻¹) at 37°C for 60 min. Data are presented as Michaelis-Menten (A) and Eadie-Hofstee (B) plots. Each point represents the average of duplicate measurements.

sample had very low *N*-oxidation activity (moclobemide 0.1 mM, $v = 0.007 \text{ nmol mg}^{-1} \text{ min}^{-1}$; 4 mM, $0.023 \text{ nmol mg}^{-1} \text{ min}^{-1}$) relative to other HLM samples and so was considered an outlier and excluded from further analysis. The activities of the 10 human liver preparations varied 4-fold at 0.1 mM moclobemide and 6-fold at 4 mM. The Spearman rank correlations between moclobemide *N*-oxidation and isoform specific P450 and FMO activities are presented in table 2. At both moclobemide concentrations high correlations were observed between the rate of Ro12-5637 formation and the *N*-oxidation of benzydamine, a probe reaction for FMO activity. At 0.1 mM moclobemide, but not 4.0 mM, a weaker correlation was observed between moclobemide *N*-oxidation and *S*-mephenytoin 4'-hydroxylation. At the high moclobemide concentration a weak correlation was observed between moclobemide *N*-oxidation and one of the CYP2E1 probe reactions, aniline 4-hydroxylation, but not for another CYP2E1 mediated reaction, *N*-dimethylnitrosoamine *N*-demethylation. No significant relationships between moclobemide *N*-oxidation and 7-ethoxyresorufin *O*-deethylation, dextromethorphan *O*-demethylation, tolbutamide 4-hydroxylation and testosterone 6 β -hydroxylation were observed suggesting that CYP1A2, CYP2D6, CYP2C9 and CYP3A4 respectively do not mediate this reaction at the moclobemide concentrations tested.

At 1.3 mM moclobemide, the general P450 inhibitor, *N*-benzylimidazole, had no effect on Ro12-5637 formation by HLM. In addition, all of the isoform specific inhibitors and substrates tested produced <10% inhibition of *N*-oxidation (data not shown). Heat treatment of HLM in the absence of an NADPH-regenerating system (Grothusen *et al.* 1996) decreased Ro12-5637 formation to 2% of control in HLM (table 1). The NADPH-generating system protected the reaction from thermal inactivation (88% of control activity) (table 1). At pH 7.4 the rate of moclobemide *N*-oxidation was greater for moclobemide incubated with HLM in Tris-HCl buffer rather than phosphate buffer (1.14 versus $1.00 \text{ nmol mg}^{-1} \text{ min}^{-1}$, respectively). Ro12-5637 formation was greater at pH > 7.4 and maximal at pH 8.5 ($2.10 \text{ nmol mg}^{-1} \text{ min}^{-1}$) (figure 4).

Table 2. Correlations (r_s) between moclobemide *N*-oxidation and flavin-containing monooxygenase (FMO) activity and cytochrome P450 (CYP) isoform-specific activities in a panel of human liver microsomal preparations ($n = 10$).

Isoform-selective activity	Correlation coefficient (r_s) with moclobemide <i>N</i> -oxidation	
	0.1 mM	4 mM
Benzydamine <i>N</i> -oxidation (FMO)	0.806**	0.939***
7-Ethoxyresorufin <i>O</i> -deethylation (CYP1A2)	0.527	0.394
Tolbutamide 4-hydroxylation (CYP2C9)	0.358	0.503
<i>S</i> -mephenytoin 4'-hydroxylation (CYP2C19)	0.661*	0.249
Dextromethorphan <i>O</i> -demethylation (CYP2D6)	0.376	-0.394
<i>N</i> -dimethylnitrosamine <i>N</i> -demethylation (CYP2E1)	-0.134	0.146
Aniline 4-hydroxylation (CYP2E1)	0.430	0.564*
Testosterone 6 β -hydroxylation (CYP3A4)	0.394	0.297

* $p < 0.05$; ** $p < 0.005$; *** $p < 0.0005$.

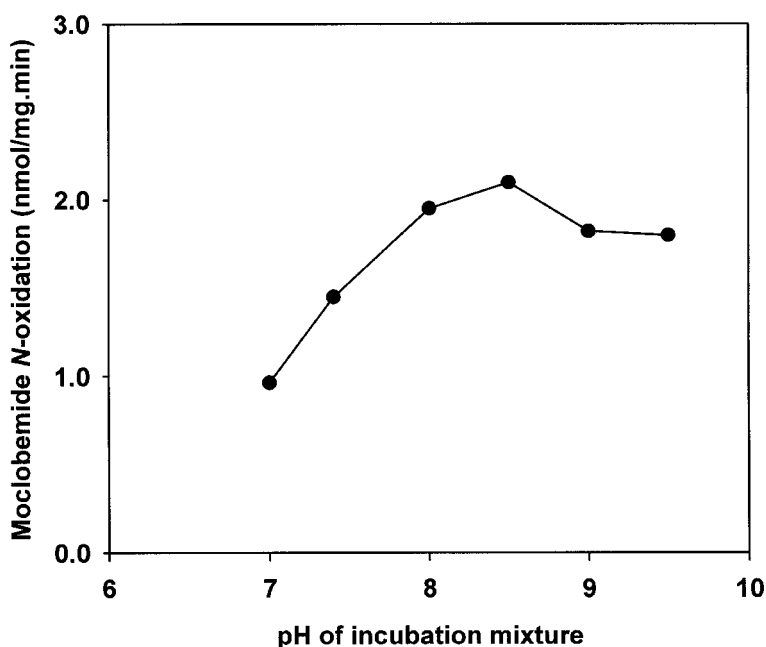


Figure 4. Effect of pH on *N*-oxidation of moclobemide. Moclobemide (1.3 mM) was incubated for 60 min at 37°C with HLM2, an NADPH-generating system and 0.2 M Tris-HCl buffer at increasing pH. Each point represents the average of duplicate measurements.

Discussion

The formation of the *N*-oxide metabolite of moclobemide, Ro12-5637, was NADPH dependent indicating that the reaction is FMO and/or cytochrome P450-mediated. FMOs are inactivated selectively by pre-incubating HLM at 50°C for 1 min (Grothusen *et al.* 1996) in the absence of an NADPH-regenerating system. These heat conditions greatly diminished the formation of Ro12-5637 whereas HLM heat-treatment in the presence of an NADPH-regenerating system protects the enzyme from inactivation. Furthermore, the reaction velocity is maximal at pH 8.5, a characteristic of FMO-mediated reactions (Cashman *et al.* 1993, Brunelle *et al.* 1997), and the rate of Ro12-5637 formation is strongly correlated with the *N*-oxidation of benzydamine, an FMO substrate (Lang *et al.* 1998). Together these observations are consistent with FMOs catalysing the *N*-oxidation of moclobemide in human liver, *in vitro*. Five isoforms of FMO (1–5) have been identified in humans. FMO3 and FMO5 are expressed in the adult human liver and FMO3 is the major isoform (Overby *et al.* 1997). A recent study using recombinant human FMOs showed that FMO3 catalyses benzydamine *N*-oxidation with greater efficiency than FMO5 (FMO3: $K_m = 60 \mu\text{M}$; $V_{\max} = 46 \text{ min}^{-1}$, FMO5: $K_m > 2 \text{ mM}$; $V_{\max} < 1 \text{ min}^{-1}$) (Lang and Rettie 2000), suggesting benzydamine *N*-oxidation is a specific probe reaction for FMO3 activity in HLM. Given the strong relationship between benzydamine and moclobemide *N*-oxidation it is likely that the formation of Ro12-5637 is also mediated by FMO3 in HLM.

Large variability in moclobemide *N*-oxidation activity was observed (26- and 65-fold), however, when the HLM sample with the lowest activity was excluded, the variability (6-fold) was similar to that reported for other FMO mediated

reactions (Overby *et al.* 1997, Tugnait *et al.* 1997, Lang *et al.* 1998). Individuals homozygous for a missense Pro153 to Leu153 mutation in exon 4 of *FMO3* have impaired *N*-oxidation of trimethylamine (Dolphin *et al.* 1997, Zschocke *et al.* 1999). These individuals suffer from 'fish-odour' syndrome because they excrete the fishy smelling trimethylamine in their sweat, urine and breath. A number of other mutations of *FMO3* have been identified, some of which result in amino acid changes that may contribute to interindividual variation in FMO catalytic activity (Cashman *et al.* 2000, Kang *et al.* 2000). The variability observed in the group of human liver microsomal samples tested in this study may be due to interindividual variability in *FMO3* genotypes, a hypothesis which remains to be confirmed.

To the authors' knowledge there is one report of benzydamine *N*-oxidation activities in HLM (Lang *et al.* 1998). Using the same incubation conditions, the benzydamine *N*-oxidation activities observed in the present study are lower than those previously reported (0.15–2.3 versus 2.8–9.5 nmol mg⁻¹ min⁻¹) (Lang *et al.* 1998). These lower activities may reflect different microsomal preparation techniques (Sadeque *et al.* 1993). FMOs are very sensitive to thermal inactivation in the absence of NADPH and labile to mechanical disruption (Cashman 1999). Although our HLM have lower FMO catalytic activities possibly reflecting lower levels of viable FMOs, a high correlation between benzydamine and moclobemide *N*-oxidation was still observed supporting a major role for FMOs in moclobemide *N*-oxidation.

The general P450 inhibitor, *N*-benzylimidazole, did not affect the formation of Ro12-5637 suggesting a lack of P450 involvement in moclobemide *N*-oxidation. Enzyme correlation experiments, however, indicate minor roles for CYP2C19 and CYP2E1 in catalysis of *N*-oxidation at low and high moclobemide concentrations, respectively. However, these observations are not supported by the chemical inhibition results. Experiments using heterologously expressed P450 enzymes would assist in elucidating the specific P450 isoforms involved in catalysing the reaction and to what extent they contribute to the formation of Ro12-5637.

In conclusion, the *N*-oxidation of moclobemide of human liver microsomes highly correlates with the flavin-containing monooxygenase mediated *N*-oxidation of *N*-benzydamine, indicating that moclobemide *N*-oxidation is predominantly catalysed by FMOs. The role of this group of enzymes was confirmed by the observation that Ro12-5637 formation is maximal at pH 8.5 and that heat pretreatment of the human liver microsomes at 50°C for 1 min in the absence of NADPH abolishes the formation of Ro12-5637. Enzyme correlation and chemical inhibition experiments suggest only minor roles for cytochrome P450s in catalysing the reaction.

Acknowledgements

J. H. was funded by a Dora Lush (Biomedical) Scholarship awarded by the National Health and Medical Research Council of Australia.

References

- BALDOCK, G. A., BRODIE, R. R., CHASSEAUD, L. F. and TAYLOR, T., 1990, Determination of benzydamine and its *N*-oxide in biological fluids by high-performance liquid chromatography. *Journal of Chromatography*, **529**, 113–123.

- BOURRIE, M., MEUNIER, V., BERGER, Y. and FABRE, G., 1996, Cytochrome P450 isoform inhibitors as a tool for the investigation of metabolic reactions catalyzed by human liver microsomes. *Journal of Pharmacology and Experimental Therapeutics*, **277**, 321–332.
- BRUNELLE, A., BI, Y. A., LIN, J., RUSSELL, B., LUY, L., BERKMAN, C. and CASHMAN, J., 1997, Characterization of two human flavin-containing monooxygenase (form 3) enzymes expressed in *Escherichia coli* as maltose binding protein fusions. *Drug Metabolism and Disposition*, **25**, 1001–1007.
- CASHMAN, J., 1999, In T. F. Woolf (ed.), *Handbook of Drug Metabolism* (New York: Dekker), pp. 477–505.
- CASHMAN, J. R., AKERMAN, B. R., FORREST, S. M. and TREACY, E. P., 2000, Population-specific polymorphisms of the human FMO3 gene: significance for detoxication. *Drug Metabolism and Disposition*, **28**, 169–173.
- CASHMAN, J. R., YANG, Z., YANG, L. and WRIGHTON, S. A., 1993, Stereo- and regioselective *N*- and *S*-oxidation of tertiary amines and sulfides in the presence of adult human liver microsomes. *Drug Metabolism and Disposition*, **21**, 492–501.
- CHANG, T. K., GONZALEZ, F. J. and WAXMAN, D. J., 1994, Evaluation of triacetyloleandomycin, alpha-naphthoflavone and diethylthiocarbamate as selective chemical probes for inhibition of human cytochromes P450. *Archives of Biochemistry and Biophysics*, **311**, 437–442.
- CHIBA, K., MANABE, K., KOBAYASHI, K., TAKAYAMA, Y., TANI, M. and ISHIZAKI, T., 1993, Development and preliminary application of a simple assay of *S*-mephenytoin 4-hydroxylase activity in human liver microsomes. *European Journal of Clinical Pharmacology*, **44**, 559–562.
- DA PRADA, M., KETTLER, R., KELLER, H. H., BURKARD, W. P., MUGGLI-MANIGLIO, D. and HAEFELY, W. E., 1989, Neurochemical profile of moclobemide, a short-acting and reversible inhibitor of monoamine oxidase type A. *Journal of Pharmacology and Experimental Therapeutics*, **248**, 400–414.
- DOLPHIN, C. T., JANMOHAMED, A., SMITH, R. L., SHEPHARD, E. A. and PHILLIPS, I. R., 1997, Missense mutation in flavin-containing mono-oxygenase 3 gene, FMO3, underlies fish-odour syndrome. *Nature Genetics*, **17**, 491–494.
- GESCHKE, R., KORNER, J. and EGGERS, H., 1987, Determination of the new monoamine oxidase inhibitor moclobemide and three of its metabolites in biological fluids by high-performance liquid chromatography. *Journal of Chromatography*, **420**, 111–120.
- GRAM, L. F., GUENTERT, T. W., GRANGE, S., VISTISEN, K. and BROSEN, K., 1995, Moclobemide, a substrate of CYP2C19 and an inhibitor of CYP2C19, CYP2D6, and CYP1A2: a panel study. *Clinical Pharmacology and Therapeutics*, **57**, 670–677.
- GROTHUSEN, A., HARDT, J., BRAUTIGAM, L., LANG, D. and BOCKER, R., 1996, A convenient method to discriminate between cytochrome P450 enzymes and flavin-containing monooxygenases in human liver microsomes. *Archives of Toxicology*, **71**, 64–71.
- JAUCH, R., GRIESSER, E., OESTERHELT, G., ARNOLD, W., MEISTER, W., ZIEGLER, W. H. and GUENTERT, T. W., 1990, Biotransformation of moclobemide in humans. *Acta Psychiatrica Scandinavica, Supplementum*, **360**, 87–90.
- KANG, J. H., CHUNG, W. G., LEE, K. H., PARK, C. S., KANG, J. S., SHIN, I. C., ROH, H. K., DONG, M. S., BAEK, H. M. and CHA, Y. N., 2000, Phenotypes of flavin-containing monooxygenase activity determined by ranitidine *N*-oxidation are positively correlated with genotypes of linked FMO3 gene mutations in a Korean population. *Pharmacogenetics*, **10**, 67–78.
- KERRY, N. L., SOMOGYI, A. A., MIKUS, G. and BOCHNER, F., 1993, Primary and secondary oxidative metabolism of dextromethorphan. *In vitro* studies with female Sprague-Dawley and Dark Agouti rat liver microsomes. *Biochemical Pharmacology*, **45**, 833–839.
- LANG, D. H. and RETTIE, A. E., 2000, *In vitro* evaluation of potential *in vivo* probes for human flavin-containing monooxygenase (FMO): metabolism of benzydamine and caffeine by FMO and P450 isoforms. *British Journal of Clinical Pharmacology*, **50**, 311–314.
- LANG, D. H., YEUNG, C. K., PETER, R. M., IBARRA, C., GASSER, R., ITAGAKI, K., PHILPOT, R. M. and RETTIE, A. E., 1998, Isoform specificity of trimethylamine *N*-oxygenation by human flavin-containing monooxygenase (FMO) and P450 enzymes: selective catalysis by FMO3. *Biochemical Pharmacology*, **56**, 1005–1012.
- LEMOINE, A., JOHANN, M. and CRESTEIL, T., 1990, Evidence for the presence of distinct flavin-containing monooxygenases in human tissues. *Archives of Biochemistry and Biophysics*, **276**, 336–342.
- MAYERSOHN, M. and GUENTERT, T. W., 1995, Clinical pharmacokinetics of the monoamine oxidase-A inhibitor moclobemide. *Clinical Pharmacokinetics*, **29**, 292–332.
- NEWTON, D. J., WANG, R. W. and LU, A. Y., 1995, Cytochrome P450 inhibitors. Evaluation of specificities in the *in vitro* metabolism of therapeutic agents by human liver microsomes. *Drug Metabolism and Disposition*, **23**, 154–158.
- ONO, S., HATANAKA, T., HOTTA, H., SATOH, T., GONZALEZ, F. J. and TSUTSUI, M., 1996, Specificity of substrate and inhibitor probes for cytochrome P450s: evaluation of *in vitro* metabolism using cDNA-expressed human P450s and human liver microsomes. *Xenobiotica*, **26**, 681–693.

- OVERBY, L. H., CARVER, G. C. and PHILPOT, R. M., 1997, Quantitation and kinetic properties of hepatic microsomal and recombinant flavin-containing monooxygenases 3 and 5 from humans. *Chemico-Biological Interactions*, **106**, 29–45.
- RIEUTORD, A., STUPANS, I., SHENFIELD, G. M. and GROSS, A. S., 1995, Glucalazide hydroxylation by rat liver microsomes. *Xenobiotica*, **25**, 1345–1354.
- RING, B. J., CATLOW, J., LINDSAY, T. J., GILLESPIE, T., ROSKOS, L. K., CERIMELE, B. J., SWANSON, S. P., HAMMAN, M. A. and WRIGHTON, S. A., 1996, Identification of the human cytochromes P450 responsible for the *in vitro* formation of the major oxidative metabolites of the antipsychotic agent olanzapine. *Journal of Pharmacology and Experimental Therapeutics*, **276**, 658–666.
- SADEQUE, A. J., THUMMEL, K. E. and RETTIE, A. E., 1993, Purification of macaque liver flavin-containing monooxygenase: a form of the enzyme related immunochemically to an isozyme expressed selectively in adult human liver. *Biochimica et Biophysica Acta*, **1162**, 127–134.
- SUTTON, D., BUTLER, A. M., NADIN, L. and MURRAY, M., 1997, Role of CYP3A4 in human hepatic diltiazem N-demethylation: inhibition of CYP3A4 activity by oxidized diltiazem metabolites. *Journal of Pharmacology and Experimental Therapeutics*, **282**, 294–300.
- TUGNAIT, M., HAWES, E. M., MCKAY, G., RETTIE, A. E., HAINING, R. L. and MIDHA, K. K., 1997, N-oxygenation of clozapine by flavin-containing monooxygenase. *Drug Metabolism and Disposition*, **25**, 524–527.
- ZSCHOCKE, J., KOHLMUELLER, D., QUAK, E., MEISSNER, T., HOFFMANN, G. F. and MAYATEPEK, E., 1999, Mild trimethylaminuria caused by common variants in FMO3 gene. *Lancet*, **354**, 834–835.



Suppression of CYP3A2 mRNA expression in the warfarin-resistant roof rat, *Rattus rattus*: possible involvement of cytochrome P450 in the warfarin resistance mechanism

S. SUGANO^{†*}, T. KOBAYASHI[§], T. TANIKAWA[¶],
Y. KAWAKAMI[§], H. KOJIMA[‡], K. NAKAMURA[‡], A. UCHIDA[§],
N. MORISHIMA[†] and Y. TAMAI^{‡**}

[†] Department of Biochemistry, School of Nursing, and [‡] Department of Biochemistry, School of Medicine, Kitasato University, Sagamihara, Kanagawa 228-0829, Japan
[§] Laboratory of Medical Zoology, Azabu University, Sagamihara, Kanagawa 229-8501, Japan

[¶] Technical Research Laboratory, Ikari Disinfection Co., Chiba 260-0844, Japan

Received 11 December 2000

1. The continual use of warfarin as a rodenticide has caused the development of populations of warfarin-resistant roof rat. To study the biochemical mechanism of warfarin resistance, the mRNA expression levels of the major P450 forms in the warfarin-resistant and -susceptible roof rat liver following exposure to warfarin were quantified by competitive RT-PCR.

2. The constitutive levels of CYP2C11 and CYP3A2 mRNAs in the warfarin-resistant and -susceptible roof rat were extremely low compared with those in the SD rat. In response to warfarin administration, the CYP3A2 mRNA level in the warfarin-susceptible rat increased to about 3-fold of that before the treatment, whereas in the warfarin-resistant roof rat, CYP3A2 mRNA remained at a low level.

3. The present results suggest the possibility that reduced synthesis of CYP3A2 mRNA is involved in the warfarin-resistant mechanism in the roof rat.

Introduction

Warfarin, an antagonist of vitamin K, extensively inhibits the activity of vitamin K epoxide reductase in the endoplasmic reticulum of hepatocytes (Suttie 1985, Cain *et al.* 1997), whereby the production of vitamin K-dependent plasma coagulation factors decreases (Thompson 1977), and the anticoagulant effect is manifested. Warfarin has been used widely as a rodenticide due to its lethal effect of haemorrhaging. The continual use of it, however, has led to the appearance and infestations of rodents that have acquired warfarin resistance (Boyle 1960, Jackson *et al.* 1988). Because of the importance of rodent pest control, many studies have been performed to elucidate the warfarin-resistance mechanism using warfarin-resistant rat strains developed from the warfarin-resistant wild rat (*Rattus norvegicus*) trapped in Scotland, Wales and Chicago, USA the pharmacokinetic and pharmacodynamic properties of the warfarin-resistant and -susceptible rat strains having mainly been compared. Pharmacodynamic studies have shown that vitamin K epoxide reductase is altered in warfarin-resistant strains. Though the level of

* Author for correspondence; e-mail: sugano@kitasato-u.ac.jp

** Present address; University of Human Arts and Sciences, Iwatsuki, Saitama 339-8539, Japan

reductase activity differs among the rat strains, production of an altered form of vitamin K epoxide reductase appears to be responsible for the inherited component of warfarin resistance in *R. norvegicus* (Hilderbrandt and Suttie 1982, MacNicol 1985, Thijssen 1987, Misenheimer and Suttie 1990, Cain *et al.* 1998).

Warfarin is regioselectively metabolized by multiple rat hepatic cytochrome P450 (CYP) isozymes to various hydroxylated derivatives: *R*-warfarin is metabolized by CYP1A1 to 6- and 8-hydroxywarfarin, by CYP2B1 to 4'-hydroxywarfarin, by CYP2C6 to 7-hydroxywarfarin, by CYP2C11 to 4'- and 6-hydroxywarfarin, and by CYP3A2 to 10-hydroxywarfarin (Guengerich *et al.* 1982). Since 4'-, 6-, 7- and 8-hydroxywarfarins are excreted in the urine or faeces as pharmacologically inactive metabolites, these hydroxylation reactions catalysed by P450s are assumed to be linked to the detoxification mechanism (Ikeda *et al.* 1968). The results of pharmacokinetic studies reported to date are considered not to be sufficient to account for the warfarin resistance mechanism, because no correlation between resistance and the absorption of warfarin, the rate of its metabolism or its half-life in plasma has been found (Pool *et al.* 1968, Townsend *et al.* 1975, Fasco and Cashin 1980). As to urinary or faecal metabolites, no difference between the warfarin-resistant and -susceptible rat has been detected (Hermodson *et al.* 1969). On the other hand, the possibility cannot be excluded that in the warfarin-susceptible rat active or toxic metabolites are produced by P450-catalysed reaction.

The warfarin-resistant roof rat, *Rattus rattus*, was first found in Liverpool in 1973, and later in Europe and the USA (Greaves *et al.* 1973, Jackson and Ashton 1979). In Japan, a warfarin-resistant colony of roof rat has infested the Shinjuku area of Tokyo in recent years and caused serious hygiene problems, accompanying environmental changes which are favourable for the wild roof rat to thrive, such as the increase in high-rise buildings and the use of air-conditioning equipment during the winter. It was reported that the Shinjuku colony of roof rat was highly resistant to the haemorrhaging effect of bait containing 0.025% warfarin: the active ingredient intake and median lethal days were 3703.8 mg kg⁻¹ body weight and 145 days, respectively, these being about 36 and 20 times those of the warfarin-susceptible roof rat, an Amami-Oshima colony (Tanikawa 1991). This resistance property was also inheritable (Tanikawa 1992).

The various hygiene problems caused by the wild roof rat have forced us to study the mechanism of warfarin resistance. The present study aimed to elucidate the involvement of P450 as to the acquisition of warfarin resistance in the roof rat. Reported here is the expression levels of CYP mRNAs in the liver of warfarin-resistant and -susceptible roof rat in response to warfarin administration.

Materials and methods

Animals and warfarin administration

Male SD rat, *Rattus norvegicus*, aged 7 weeks, were obtained from Japan SLC, Inc. Wild roof rat, *Rattus rattus*, were trapped in the Shinjuku area of Tokyo, it being an infestation area of the warfarin-resistant roof rat. Warfarin-resistant roof rats were identified on the basis of their survival after exposure to 0.025% (w/w) warfarin (Aldrich Chemical Company, Inc., MI, USA) in a 28-day feeding trial (Tanikawa 1991). They were fed further a warfarin-free standard diet for 30 days before use. Warfarin-susceptible roof rat, originally trapped on the Ogasawara islands, 800 km south of Tokyo in the Pacific Ocean, were maintained on a standard diet in our laboratory. Healthy male rats weighing about 110 g were used in the study. Fifteen warfarin-resistant roof rats were divided into four groups, one control group (no warfarin administration) and three warfarin-administered groups, 3, 29 and 89 days

respectively. Eight warfarin-susceptible rats were divided into two groups, one control group (no warfarin administration) and one warfarin-administered group, 3 days. The 89 days warfarin-administered groups comprised of three roof rats and other each group comprised of four roof rats. The control groups were fed a warfarin-free standard diet and the warfarin-administered groups were fed a flour-type diet containing 0.025% (w/w) warfarin. The rats were killed under CO₂ gas, and then their livers were removed, immediately frozen in liquid N₂ and stored at -80°C until use.

RNA extraction and competitive RT-PCR

Total RNA was isolated from rat livers using Isogen (Nippongene Co., Tokyo, Japan) and purified to an A260/A280 ratio > 1.80. All RNAs were quantified by measuring the absorbance at 260 nm and their integrity was confirmed by denaturing agarose gel electrophoresis.

The quantitation of the major CYP mRNAs was carried out by competitive RT-PCR using a rat cytochrome P450 competitive RT-PCR set and a Takara RNA LA PCRTM Kit (AMV), v.1.1 (Takara Shuzo Co., Kyoto, Japan), according to the manufacturer's instructions with a slight modification. The reaction was performed with a Perkin-Elmer 2400 thermal cycler (Perkin-Elmer, Norwalk, CT, USA). The mRNA levels of each CYP in rat livers were compared using competitor RNA dilution C (4×10^7 copies/reaction) with 25 ng of total RNA, and 1 pmol sense and antisense primers for rat CYP1A1, CYP1A2, CYP2B1/2, CYP2C11, CYP2E1, CYP3A1, CYP3A2, CYP4A1 and cyclophilin, respectively. The levels of CYP1A1 and CYP3A2 mRNAs were quantified using 4-fold serial dilutions of the competitor RNA: A (2.5×10^6 copies/reaction), B (10^7), C (4×10^7), and D (1.6×10^8). Cyclophilin, as a housekeeping gene, which was used to determine the constitutive level of gene transcription and to control variations in RNA recovery from the liver samples, was similarly co-amplified and quantified.

Quantitation of CYP mRNAs

The amplified products were separated on a 2.5% agarose gel, stained with ethidium bromide, and then quantified using an FAL-2000 Fluoroimage Analyzer with the Mac BAS and Image Reader software program (Fuji Photo Film, Tokyo, Japan). The CYP mRNA level was determined by calculating the ratio of the fluorescence intensity of the CYP mRNA to that of the competitor RNA, and the data for each rat liver sample were normalized by quantitating the amounts of cyclophilin cDNA products.

Results

Analysis of warfarin-resistant roof rat liver CYP mRNA expression

We first attempted to analyse the expression of major CYP mRNAs in the wild warfarin-resistant roof rat liver by competitive RT-PCR, using competitor RNA C with total RNA and primers originating from SD rat CYP forms. As shown in figure 1A, in the SD rat, two bands derived from CYP mRNA and competitor RNA of the expected sizes were clearly detected for each CYP form except for CYP1A1. Since two bands corresponding to each CYP form in the SD rat of identical sizes were also obtained for the warfarin-resistant roof rat, except in the cases of CYP1A1 and 3A2 (figure 1B), the primers for SD rat liver CYP forms were found to be useful for amplifying CYP cDNAs of roof rat liver. The expression of CYP1A1 mRNA was not detected under these conditions in either the SD or roof rat, this finding being in agreement with a previous report that CYP1A1 mRNA was expressed only following exposure to a P450 inducer (Morris and Davila 1996).

We compared the level of each P450 mRNA in the SD rat with that in the warfarin-resistant roof rat as the cyclophilin mRNA level was almost equal in the two rat strains. The constitutive levels of CYP1A2, CYP2B1/2, CYP2E1, CYP3A1 and CYP4A1 mRNAs in the warfarin-resistant roof rat apparently corresponded to those in the SD rat. It is known that CYP2C11 and CYP3A2 mRNAs are constitutively expressed in the liver of the sexually mature male rat but not in the female rat (Zaphiropoulos *et al.* 1988) and are transcriptionally down-regulated by stress hormones (Morgan *et al.* 1998). The levels of these latter two P450s were

A SD rat

B Warfarin-resistant roof rat

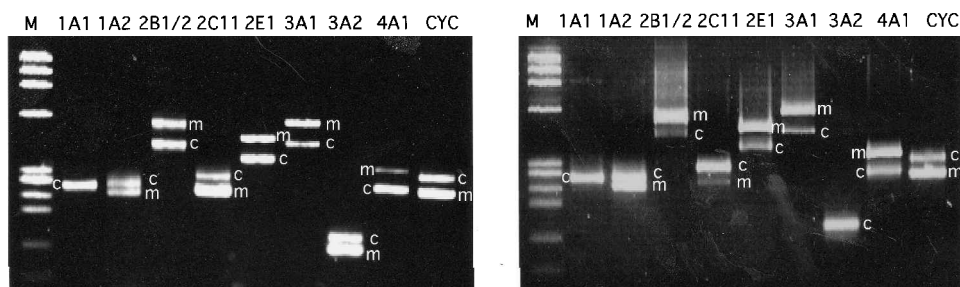


Figure 1. Analysis of the CYP mRNA expression in the liver of the SD and wild warfarin-resistant roof rat by competitive RT-PCR. Total RNA was isolated from the liver of the SD (A) and warfarin-resistant roof (B) rat. Competitive RT-PCR was performed using competitor RNA C (4×10^7 copies/reaction) with 25 ng total RNA, and the primers for the rat CYPs, CYP1A1, 1A2, 2B1/2, 2C11, 2E1, 3A1, 3A2 and 4A1, and cyclophilin (CYC). An agarose gel stained with ethidium bromide shows the cDNA fragments derived from CYP and CYC mRNAs (m), and competitor RNA (c). M, DNA molecular weight standard (ϕ X174-HaeIII digest).

very low in the warfarin-resistant roof rat, even in the adult male, in contrast to the finding for the SD rat. The low expression levels of CYP2C11 and CYP3A2 mRNAs were also found in the female warfarin-resistant roof rat (data not shown), suggesting that CYP2C11 and CYP3A2 mRNA expression is constitutively at a low level and/or is affected by stress in the wild warfarin-resistant roof rat.

Effect of warfarin administration on CYP mRNA expression in the warfarin-resistant and -susceptible roof rat

To determine whether P450 is involved in the acquisition of warfarin resistance by the roof rat, we examined the effects of warfarin administration on the levels of CYP mRNA expression in the warfarin-resistant and -susceptible roof rat. Since it was previously observed that the warfarin-susceptible roof rat died of haemorrhaging on > 4 day exposure to warfarin, both warfarin-resistant and -susceptible roof rat was administered warfarin for 3 days. To reconfirm the warfarin resistance, the warfarin-resistant roof rat was further administered warfarin for 29 and 89 days. Typical results are shown in figure 2. No distinct difference due to warfarin administration was observed in the expression levels of CYP1A2, CYP2B1/2, CYP2C11, CYP2E1, CYP3A1 and CYP4A1 mRNAs from the warfarin-resistant and -susceptible roof rat, showing that the expression of these mRNAs was apparently unaffected by warfarin treatment. However, two findings should be noted. First, the CYP1A1 mRNA was induced to a detectable level in the warfarin-resistant roof rat exposed to warfarin for 3 days (figure 2D), and then became undetectable on longer exposure to warfarin (figure 2E and F). Second, the constitutive CYP3A2 mRNA level in the warfarin-resistant roof rat was very low, and the level was unaltered following exposure to warfarin for 3, 29 and 89 days (figure 2B and D–F), whereas in the warfarin-susceptible roof rat CYP3A2 mRNA expression was markedly increased on exposure to warfarin (figure 2A and C). The present results clearly show that warfarin administration affected the expression

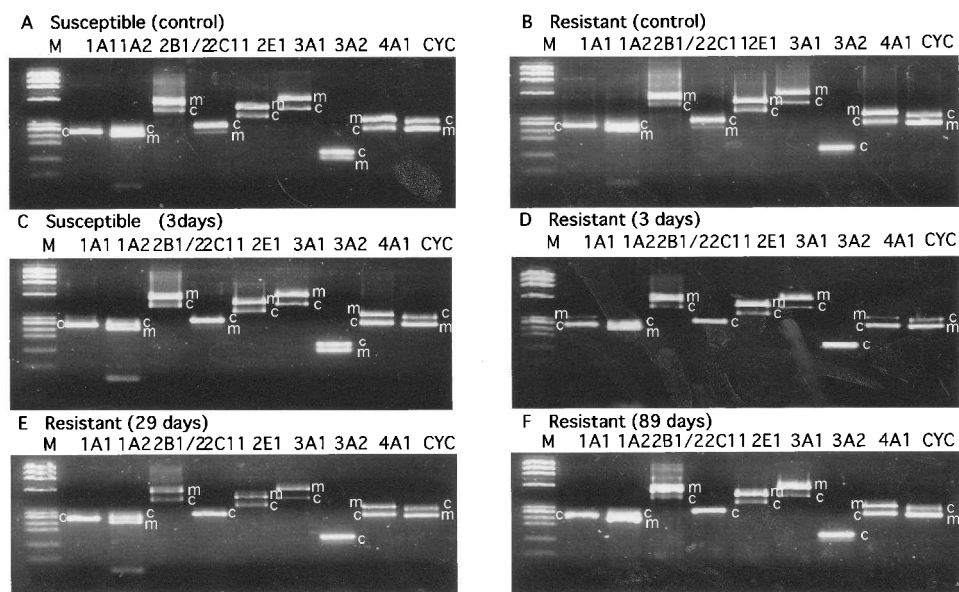


Figure 2. Effects of warfarin administration on CYP mRNA expression in the warfarin-resistant and -susceptible roof rat. Control warfarin-susceptible (A) and -resistant (B) roof rats were fed a specific diet. The warfarin-susceptible roof rat was fed the same diet but containing 0.025% (w/w) warfarin for 3 days (C), and the warfarin-resistant roof rat was fed the same diet for 3 (D), 29 (E) and 89 days (F). Total RNA was isolated from the warfarin-resistant and -susceptible roof rat liver, and competitive RT-PCR was performed as described in the legend to figure 1. An ethidium bromide-stained agarose gel shows the cDNA fragments derived from CYP and CYC mRNAs (m), and competitor RNA (c). A small band (about 50 bp) in the CYP1A2 tracks may be caused by the primer dimer formation. M, DNA molecular weight standard (ϕ X174-HaeIII digest).

level of CYP1A1 mRNA in the warfarin-resistant roof rat and that of CYP3A2 mRNA in warfarin-susceptible roof rat.

Increase of CYP1A1 mRNA expression in both the warfarin-resistant and -susceptible roof rat exposed to warfarin for 3 days

To clarify the above findings, we carried out quantitation of the CYP1A1 and CYP3A2 mRNAs by competitive RT-PCR. Cyclophilin was co-amplified for the quantitation and the ratio to competitor RNA C was about 1:1 and did not vary between the control and warfarin-treated roof rat liver (figure 3A and B). Constitutive CYP1A1 mRNA expression was not detected in the warfarin-susceptible roof rat, whereas that in the warfarin-resistant roof rat was detected with a small amount of competitor RNA (figure 3A). On warfarin exposure for 3 days, CYP1A1 mRNA was distinctly detectable with a small amount of competitor RNA in both the warfarin-susceptible and -resistant roof rat (figure 3B). However, the CYP1A1 mRNA level in both the warfarin-resistant and -susceptible roof rat exposed to warfarin for 3 days was about 10^5 copies ng^{-1} total RNA. In the warfarin-resistant roof rat, a decrease to the constitutive level was observed following exposure to warfarin for 29 days (figure 2E). These results suggest

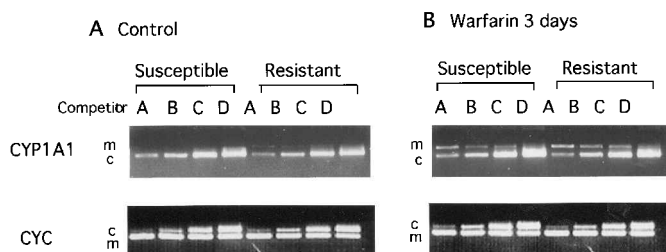


Figure 3. Quantitation of CYP1A1 mRNA expression in the warfarin-resistant and -susceptible roof rat exposed to warfarin. Competitive RT-PCR was performed as described in the legend to figure 2 using competitor RNA A (2.5×10^6 copies/reaction), B (1×10^7), C (4×10^7), and D (1.6×10^8) with 25 ng total RNA, and the primers for rat CYP1A1 and CYC. An ethidium bromide-stained agarose gel shows the cDNA fragments derived from CYP1A1 mRNA (m, 332 bp) and its competitor RNA (c, 264 bp), and CYC mRNA (m, 266 bp) and its competitor RNA (c, 320 bp). (A) CYP1A1 and CYC cDNA products in the control warfarin-resistant and -susceptible roof rat. (B) CYP1A1 and CYC cDNA products in the warfarin-resistant and -susceptible roof rat exposed to warfarin for 3 days.

that warfarin, on exposure, can activate the expression of CYP1A1 mRNA in the warfarin-resistant and -susceptible roof rat.

Increase of CYP3A2 mRNA expression in the warfarin-susceptible roof rat exposed to warfarin for 3 days

Figure 4 shows the CYP3A2 mRNA levels quantified by competitive RT-PCR. The constitutive level of CYP3A2 mRNA in the warfarin-resistant roof rat was about 8×10^4 copies ng^{-1} total RNA, and showed little change following exposure to warfarin for 3, 29 and 89 days, whereas in the warfarin-susceptible roof rat it was about 4×10^5 copies ng^{-1} total RNA and increased 3-fold on exposure to warfarin for 3 days. These findings clearly showed that CYP3A2 mRNA was constitutively expressed at a lower level in the warfarin-resistant roof rat than in the warfarin-susceptible one, and following further exposure to warfarin, the level increased markedly in the warfarin-susceptible one, but did not increase in the warfarin-resistant one.

Discussion

We quantified the levels of mRNA expression of the major CYP forms in the warfarin-resistant and -susceptible roof rat liver following exposure to warfarin using competitive RT-PCR. This method was highly specific and sensitive for detecting the expression of CYP mRNAs in the roof rat liver. By the use of rat treated with PB as a P450 inducer, it was also observed that this method is applicable to the assessment of the induction and down-regulation of the P450 enzyme, which in general is regulated primarily at the mRNA level (data not shown), the findings corresponding to those obtained with the semiquantitative RT-PCR method (Morris and Davila 1996).

In the current study, it was shown that warfarin induced changes in liver CYP1A1 and CYP3A2 mRNA expression in the warfarin-resistant and -susceptible roof rat. Since many studies have reported that rat CYP1A1 catalyses the 6-

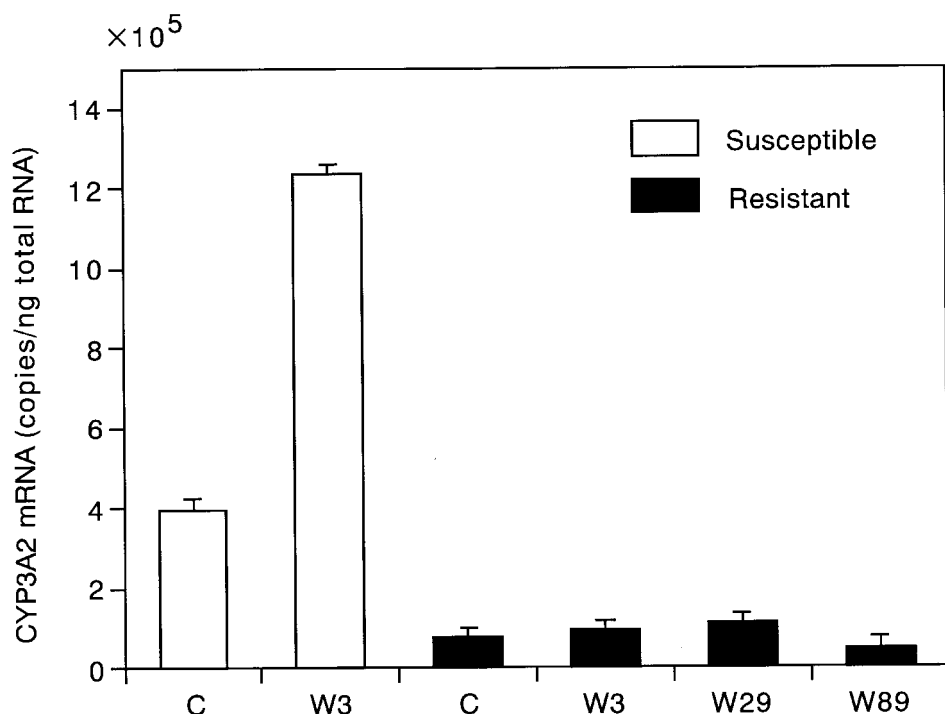


Figure 4. Quantitation of CYP3A2 mRNA expression in the warfarin-resistant and -susceptible roof rat exposed to warfarin. The experiments were carried out as described in the legend to figure 3 except that the primer for rat CYP3A2 was used in place of that for CYP1A1. The CYP1A1 mRNA level normalized to CYC was expressed as the number of copies of mRNA ng⁻¹ total RNA. Each bar represents the mean SD of at least three independent experiments.

and 8-hydroxylation of warfarin, it is assumed that the increased P4501A1 enhances warfarin metabolism at an initial stage of warfarin exposure and, as a consequence, leads to a decrease in its toxicity. However, the higher constitutive level of CYP1A1 mRNA in the warfarin-resistant roof rat compared with the warfarin-susceptible one (figure 3A) may be attributed to the prior warfarin exposure carried out to confirm the warfarin resistance. A similar increase of CYP1A1 mRNA level in both the warfarin-resistant and -susceptible roof rat exposed to warfarin for 3 days cannot be linked to the warfarin resistance mechanism at present.

With regard to CYP3A2 mRNA expression, we have demonstrated a distinct difference in warfarin-induced changes between the warfarin-resistant and -susceptible roof rat: CYP3A2 mRNA expression is suppressed in the warfarin-resistant roof rat, whereas it is induced in the warfarin-susceptible one in response to warfarin administration. In addition, it was observed that the constitutive level of CYP3A2 mRNA in both the warfarin-resistant and -susceptible roof rat is considerably lower than that in the SD rat, which is susceptible to warfarin. Only the warfarin-susceptible roof rat exhibited induction of CYP3A2 mRNA accumulation on warfarin administration, and the level became comparable with the constitutive expression level in the SD rat (figures 1A and 2C). It has been established that CYP3A2 selectively catalyses the 10-hydroxylation of warfarin,

the reaction serving as a probe for the CYP3A family (Guengerich *et al.* 1982). Thus, it is suspected that the increase in the CYP3A2 mRNA causes an increase in the P4503A2 protein, and the enzyme may generate 10-hydroxywarfarin, which has a toxic, but not a detoxification effect and resulting in lethality. The toxicity and/or anticoagulant activity of 10-hydroxywarfarin, however, has not been elucidated to date as far as is known. It has only been reported that dehydrowarfarin has no anticoagulant activity (Fasco *et al.* 1978).

CYP3A2 is closely related to CYP3A1 (90% amino acids similarity), and both forms can be induced by DEX by transcriptional activation (Telhada *et al.* 1992). We have recently found in preliminary experiments that treatment with warfarin and DEX has a lethal effect on the warfarin-resistant roof rat, indicating that the warfarin-resistant roof rat became sensitive to warfarin, probably through an increase in the CYP3A mRNA. Taking the results together, we propose the possibility that CYP3A is involved in the warfarin resistance mechanism in the roof rat. Using this hypothesis, it may be possible to develop an effective rodenticide against the warfarin-resistant rat and an assay method for detecting resistant populations. A study to clarify the involvement of P450 in the acquisition of warfarin resistance in the roof rat, including the treatment of the warfarin-resistant roof rat with DEX, is in progress. We have demonstrated that the expression levels of CYP2C11 and CYP3A2 mRNAs in the wild roof rat are very low compared with those in the SD rat. The warfarin-resistant roof rat may be the first example of a mammal that has acquired a survival capability by regulating CYP expression in response to environmental toxins.

Previously, it has been clearly demonstrated that the vitamin K epoxide reductase is altered in some warfarin-resistant rat strains of *R. norvegicus* and that this alteration that results in altered warfarin pharmacodynamics is responsible in part for warfarin resistance (Hilderbrandt and Suttie 1982, MacNicol 1985, Thijssen 1987, Misenheimer and Suttie 1990, Cain *et al.* 1998). The present data do not exclude that alteration in vitamin K epoxide reductase structure/expression may also be involved in the warfarin resistance mechanism in the roof rat (*R. rattus*) as well as in *R. norvegicus*.

In conclusion, we report here that there is a difference of CYP3A2 mRNA expression in response to warfarin administration between the warfarin-resistant and -susceptible roof rat, the findings suggesting the possibility that decreased metabolism of warfarin by CYP3A2 may be involved in the warfarin resistance mechanism.

Acknowledgement

The authors thank Dr M. Katagiri, Osaka Kyoiku University Division of Natural Science, for valuable discussions.

References

- BOYLE, C. M., 1960, Case of apparent resistance of *Rattus norvegicus* Berkenhout to anticoagulant poisons. *Nature*, **188**, 517.
- CAIN, D., HUTSON, S. M. and WALLIN, R., 1997, Assembly of the warfarin-sensitive vitamin K 2,3-epoxide reductase enzyme complex in the endoplasmic reticulum membrane. *Journal of Biological Chemistry*, **272**, 29068–29075.

- CAIN, D., HUTSON, S. M. and WALLIN, R., 1998, Warfarin resistance is associated with a protein component of the vitamin K 2,3-epoxide reductase enzyme complex in rat liver. *Thrombosis and Haemostasis*, **80**, 128–133.
- FASCO, M. J. and CASHIN, M. J., 1980, Effects of induction on *R*- and *S*-warfarin and metabolite concentrations in rat plasma. *Toxicology and Applied Pharmacology*, **56**, 101–109.
- FASCO, M. J., DYMERSKI, P. P., WOS, J. D. and KAMINSKY, L. S., 1978, A new warfarin metabolite: Structure and function. *Journal of Medical Chemistry*, **21**, 1054–1059.
- GREAVES, J. H., RENISON, B. D. and REDFEN, R., 1973, Warfarin resistance in the ship rat in Liverpool. *International Pest Control*, **15**, 17.
- GUENGERICH, F. P., DANNAN, G. A., WRIGHT, S. T., MARTIN, M. V. and KAMINSKY, L. S., 1982, Purification and characterization of liver microsomal cytochromes P-450: Electrophoretic, spectral, catalytic, and immunochemical properties and inducibility of eight isozymes isolated from rat treated with phenobarbital or beta-naphthoflavone. *Biochemistry*, **21**, 6019–6030.
- HERMODSON, M. A., SUTTIE, J. W. and LINK, K. P., 1969, Warfarin metabolism and vitamin K requirement in the warfarin-resistant rat. *American Journal of Physiology*, **217**, 1316–1319.
- HILDERBRANDT, E. F. and SUTTIE, J. W., 1982, Mechanism of coumarin action: sensitivity of vitamin K metabolizing enzymes of normal and warfarin-resistant rat liver. *Biochemistry*, **21**, 2406–2411.
- IKEDA, M., CONNEY, A. H. and BURNS, J. J., 1968, Stimulatory effect of phenobarbital and insecticides on warfarin metabolism in the rat. *Journal of Pharmacology and Experimental Therapy*, **162**, 338–343.
- JACKSON, W. B. and ASHTON, A. D., 1979, Present distribution of anticoagulant resistance in the United States. In *Proceedings of the 8th Steenbock Symposium*, Madison, WI, pp. 1–6.
- JACKSON, W. B., ASHTON, A. D. and DELVENTHAL, K., 1988, Overview of anticoagulant rodenticide usage and resistance. In J. W. Suttie (ed.), *Current Advances in Vitamin K Research* (New York: Elsevier), pp. 381–388.
- MACNICOLL, A. D., 1985, A comparison of warfarin resistance and liver microsomal vitamin K epoxide reductase activity in rat. *Biochimica et Biophysica Acta*, **840**, 13–20.
- MISENHEIMER, T. M. and SUTTIE, J. W., 1990, Warfarin resistance in a Chicago strain of rat. *Biochemical Pharmacology*, **40**, 2079–2084.
- MORGAN, E. T., SEWER, M. B., IBER, H., GONZALEZ, F. J., LEE, Y. H., TUKEY, R. H., OKINO, S., VU, T., CHEN, Y. H., SIDHU, J. S. and OMIECINSKI, C. J., 1998, Physiological and pathophysiological regulation of cytochrome P450. *Drug Metabolism and Disposition*, **26**, 1232–1240.
- MORRIS, D. L. and DAVILA, J. C., 1996, Analysis of rat cytochrome P450 isoenzyme expression using semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR). *Biochemical Pharmacology*, **52**, 781–792.
- POOL, J. G., O'REILLY, R. A., SCHNEIDERMAN, L. J. and ALEXANDER, M., 1968, Warfarin resistance in the rat. *American Journal of Physiology*, **215**, 627–631.
- SUTTIE, J. W., 1985, Vitamin K-dependent carboxylase. *Annual Reviews in Biochemistry*, **54**, 459–477.
- TANIKAWA, T., 1991, Difference in consumption of warfarin baits and lethal days between resistant and susceptible colonies of the roof rat, *Rattus rattus* in Japan. *Eiseidoubutsu*, **42**, 99–102 [in Japanese].
- TANIKAWA, T., 1992, Studies on genetic analysis of warfarin resistance in roof rat, *Rattus rattus*, in Japan by crossing resistant and susceptible colonies. *Eiseidoubutsu*, **42**, 323–329 [in Japanese].
- TELHADA, M. B., PEREIRA, T. M. and LECHNER, M. C., 1992, Effect of dexamethasone and phenobarbital on run-on transcription rate and CYP3A mRNA concentration in rat liver: Changes during development. *Archives in Biochemistry and Biophysics*, **298**, 715–725.
- THIJSEN, H. H. W., 1987, Warfarin resistance: vitamin K epoxide reductase of Scottish resistance genes is not irreversibly blocked by warfarin. *Biochemical Pharmacology*, **36**, 2753–2757.
- THOMPSON, A. R., 1977, Factor IX antigen by radioimmunoassay: abnormal factor IX protein in patients on warfarin therapy and with hemophilia B. *Journal of Clinical Investigation*, **59**, 900–910.
- TOWNSEND, M. G., ODAM, E. M. and PAGE, J. M. J., 1975, Studies of the microsomal drug metabolism system in warfarin-resistant and -susceptible rat. *Biochemical Pharmacology*, **24**, 729–735.
- ZAPHIROPOULOS, P. G., MODE, A., STROM, A., MOLLER, C., FERNANDES, C. and GUSTAFSSON, J.-A., 1988, cDNA cloning, sequence, and regulation of a major female-specific and growth hormone-inducible rat liver cytochrome P-450 active in 15 β -hydroxylation of steroid sulfates. *Proceedings of the National Academy of Sciences, USA*, **85**, 4214–4217.

Development of diltiazem deacetylase and demethylase activities during ontogeny in rabbit

L. J. FRAILE[†], M. A. BREGANTE^{†*}, M. A. GARCIA[‡] and C. SOLANS[†]

Departments of [†]Pharmacology and Physiology, and [‡]Analytical Chemistry, Faculty of Veterinary Science, University of Zaragoza, E-50013 Zaragoza, Spain

Received 11 October 2000

1. Diltiazem (DTZ) undergoes extensive metabolism in hepatic and extrahepatic tissues. Deacetyldiltiazem (M1) and *N*-desmethyldiltiazem (MA) are two of the main basic metabolites of DTZ that retain pharmacological activity. The development of DTZ deacetylase and demethylase activities through ontogeny has not been addressed. In order to address this issue, *in vitro* studies have been carried out using the blood and several tissues of rabbit as enzyme sources. In addition, *in vivo* studies using a pharmacokinetic approach were carried out to support the *in vitro* findings.

2. DTZ was incubated with homogenates of selected tissues and in whole blood and DTZ, and its metabolites were assayed by HPLC. In addition, a pharmacokinetic study after intraperitoneal administration of DTZ in the 1-, 8-, 16-, 30-day-old and adult rabbit were also carried out.

3. DTZ deacetylase activity was detected whatever the age and tissue examined (including blood). Except in gut homogenates, this activity was shown to be higher at earlier postnatal ages. DTZ demethylase activity was only detected in the liver and gut homogenates and in whole blood. This activity increases from the 1- to 30-day-old rabbit (except for blood), after which it decreases slightly to reach the adult level.

4. *In vivo* experiments showed a close pharmacokinetic profile throughout ontogeny (except for the 30-day-old rabbit) after DTZ intraperitoneal administration.

5. Extrahepatic metabolism may play a more significant role in the overall metabolism and pharmacokinetics of DTZ at earlier stages of development.

6. Finally, *in vivo* studies suggest that age does not seem to modify DTZ disposition and, for this reason, dosage may not have to be taken into account as a function of age.

Introduction

A consistent body of evidence indicates that as animals mature the ability to metabolize xenobiotics usually increases, reaching the value observed in the adult in a variable period, depending on the particular enzyme and the species involved (Short 1984). It is well known that the liver is the primary site of metabolism. As a result, studies on the development of drug-metabolizing enzymes have mainly focused on this organ. However, other sites including the gastrointestinal tract, the lung, the brain, etc. have been identified as potential sites of metabolism (Krishna and Klotz 1994). Depending on the drug itself, the enzyme involved and the route of administration, these extrahepatic organs may contribute significantly to the overall elimination of a drug (Krishna and Klotz 1994).

Diltiazem (DTZ), a calcium channel blocker widely prescribed for the treatment of cardiovascular disorders, has been shown to be extensively metabolized by

* Author for correspondence; e-mail: Bregante@posta.unizar.es

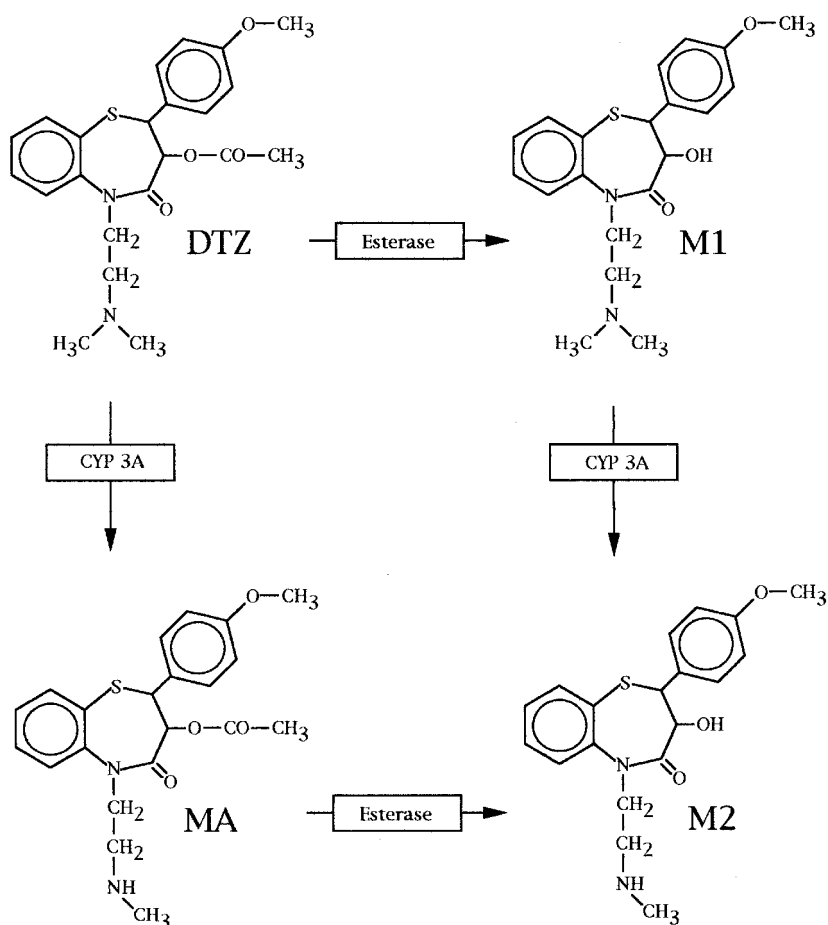


Figure 1. Structural formulae of DTZ and of its active metabolites deacetyldiltiazem (M1), *N*-demethyldiltiazem (MA) and *N*-demethyldeacetyldiltiazem (M2). The enzyme responsible for each metabolic step is indicated in a box.

at least five distinct pathways including deacetylation, *N*-demethylation, *O*-demethylation, ring hydroxylation and acid formation in hepatic and extrahepatic tissues in several animal species (Homsy *et al.* 1995, Fraile *et al.* 1996). These various reactions lead to the formation of at least 20 distinct metabolites (Sugawara *et al.* 1988), some of which – deacetyldiltiazem (M1), *N*-demethyldiltiazem (MA) and *N*-demethyldeacetyldiltiazem (M2) – retain potent pharmacological activity as antihypertensives and coronary vasodilators (Yabana *et al.* 1985). M1 and MA have been recognized as primary metabolites of DTZ, whereas M2 is the product of a secondary reaction (figure 1). Moreover, M1 and MA seem to be the most important basic metabolites in mammals (Yeung *et al.* 1990, 1998).

Fraile *et al.* (1996) reported that DTZ deacetylation takes place in several tissues of rabbit including the liver, lung, brain, gut and blood. In addition, evidence has been presented that hepatic CYP3A is the major enzyme involved in *N*-demethylation of DTZ in rabbit and humans (Pichard *et al.* 1990). This enzyme has been reported to be present in several organs (Murray *et al.* 1988), which may

also contribute to the overall metabolism of the parent drug. These findings make DTZ an interesting tool in order to study the development (throughout the perinatal period) of several enzymes involved in the hepatic and extrahepatic metabolism of the drugs that may share the same metabolic routes of degradation in the organism.

The current study was designed to provide new insights into the diversity of drug metabolism and disposition throughout the perinatal period. To this end, the formation of M1 and MA in different rabbit tissue homogenates (liver, lung, brain, gut) and in whole blood has been examined as a function of age. The tissues were selected because of the previously described occurrence of DTZ deacetylase and/or demethylase activity (Pichard *et al.* 1990, Fraile *et al.* 1996). Experiments were conducted in both the presence and absence of a NADPH-regenerating system in order to exclude the formation of MA and the degradation of M1 to M2 (figure 1). In addition, *in vivo* studies were carried out using a pharmacokinetic approach to support the *in vitro* findings. Rabbit was chosen as the animal model since the disposition in the species is similar to that in humans (Yeung *et al.* 1990, 1998).

Materials and methods

Reagents

Diltiazem, imipramine, glucose 6-phosphate, glucose 6-phosphate dehydrogenase, NAD⁺, and NADP⁺ were purchased from Sigma Chemical Company (St Louis, MO, USA). Deacetyldiltiazem (M1) and demethyldiltiazem (MA) were a gift from Dr R. Latini, Mario Negri Institute, Milan. Other chemicals were obtained from commercial sources and were of reagent or HPLC grade.

Animals

1-, 8-, 16-, 30-day-old and adult (> 1 year) New Zealand White female rabbits were used throughout the study. Animals were obtained from 15 pregnant New Zealand White rabbits (body weights 4.5–5.0 kg), which were acclimatized in our laboratory 15 days prior to delivery.

All the experiments described here were approved by the Institutional Animal Care and Use Committee of the University of Zaragoza and the research also adhered to the principles of Laboratory Animal Care (NIH publication #85-23, revised 1985).

Homogenate preparation

1- ($n = 12$), 8- ($n = 12$), 16- ($n = 4$), 30-day-old ($n = 4$) and adult ($n = 4$) rabbits were used. Pups were obtained from four litters of at least nine members, and those coming from the same litter were assigned to each age group. Immediately before tissue collection, arterial blood samples (5 ml) were drawn from all animals for *in vitro* studies. Heparin (10 μ l 150 IU ml⁻¹ solution) had been previously added to the tubes to prevent blood clotting. To obtain a suitable volume of neonatal blood, samples from three littermates were pooled and the pool used as a single specimen ($n = 4$). Thereafter, the rabbits were anaesthetized by intraperitoneal administration of sodium pentobarbital (50 mg kg⁻¹), the abdomen was opened and the portal vein cannulated. Ice-cold isotonic saline (0.9% NaCl) was perfused through the cannula at a flow rate of 3–5 ml min⁻¹ g⁻¹ liver. In order to minimize the blood content of the organs, the animals were bled via the abdominal cava vein. When the colour of the liver became light tan (3–5 min), the liver, small intestine (jejunum), lung and brain were excized. Faeces remaining in the small intestine were removed by ice-cold saline perfusion. Precisely weighed tissue samples (~3 g) were minced and homogenized with ice-cold 1.15% (w/v) KCl–0.01 M phosphate buffer (pH 7.4) in a Potter-Elvehjem to yield 20% (w/v) homogenates. Each homogenate was centrifuged first at 1000g for 10 min, and then at 9000g for 20 min at 4°C. The supernatant was collected and stored frozen at –80°C until used. Previous experiments (data not shown) have demonstrated that this procedure to store the supernatant does not significantly modify the DTZ deacetylase and demethylase activities for, at least, three months for each organ and age studied. The protein content of the supernatant was determined by the method described by Lowry *et al.* (1951), using bovine serum albumin as standard.

Determination of DTZ demethylase and deacetylase activity in homogenates and blood

Previous experiments (data not shown) have demonstrated that both assays were linear with regard to incubation time and protein concentration under the conditions described below. Furthermore, pH 7.4 was considered optimal for the enzymatic activities studied and that 1 mM DTZ was a saturating concentration for both activities. These experiments to optimize the enzyme assays were carried out as described by Leboeuf and Grech-Belanger (1987).

Demethylation of DTZ in the tissue homogenates was assessed in the presence of an NADPH-regenerating system. In a final volume of 2 ml, a typical reaction mixture contained 1 mg protein, 1 mM DTZ as substrate and an NADPH-regenerating system composed of NAD⁺ (26 mM), NADP⁺ (2 mM), glucose 6-phosphate (53 mM) and glucose 6-phosphate dehydrogenase (4 units). Because of the different protein contents of the homogenates (lower at the earlier ages), the required volume of 1.15% (w/v) KCl-0.01 M phosphate buffer (pH 7.4) was added to the mixture to yield a final volume of 2 ml. Incubations were conducted in glass vials in an oscillating water bath at 37°C for 10 min in air. At the end of this period, an aliquot of 50 µl was withdrawn from the incubation mixture and placed in a tube containing 20 ml cold (-20°C) extraction medium (see HPLC assay), and further assayed for MA and M1 concentrations. Vials containing the same composition, but without the NADPH-regenerating system, were used to assess the DTZ deacetylase activity.

In order to measure enzyme activities in blood, the incubation mixtures consisted of 2 ml whole blood containing DTZ (1 mM). At the end of the incubation (3 min), an aliquot of 100 µl was withdrawn from the incubation mixture and immediately haemolyzed by the addition of 100 µl cold water and then placed in 20 ml cold (-20°C) extraction medium. The samples were assayed for M1 and MA concentration, as described above.

The chemical breakdown of DTZ, M1 and MA was assessed in vials containing the same composition, but inactivated by heat (5 min at 75°C). DTZ and its metabolites (M1 and MA) were spiked to the incubation mixture, yielding a concentration of 1.0, 0.1 and 0.01 mM, respectively. Serial samples were drawn at 0, 10, 30 and 60 min and assayed by HPLC. The amount of M1 or MA formed enzymatically was then calculated by subtracting the amount formed by chemical breakdown from that formed during incubations of DTZ with the enzyme source. DTZ deacetylase or demethylase activity in homogenates or whole blood was expressed as nmol mg⁻¹ protein min⁻¹ or as nmol ml⁻¹ blood min⁻¹, respectively.

In addition, two separate studies on the stability of M1 and MA in whole blood and homogenates were carried out for each organ and age studied. MA was spiked in the incubation mixtures, yielding a final concentration close to that observed in the main experiments in the presence of the NADPH-regenerating system or in whole blood. Aliquots of 50 µl were sampled from the incubation medium at 0, 3, 5 and 10 min and assayed for MA concentrations. Similarly, the stability of M1 was checked, but in the absence of NADPH.

Determination of the blood component responsible for the DTZ deacetylase and demethylase activities

DTZ deacetylase and demethylase activity was also assessed in plasma, whole blood and washed blood cells. To this end, arterial blood samples (10 ml) from four additional 30-day-old rabbits were withdrawn and placed into heparinized tubes. The haematocrit was also measured. An aliquot (2 ml) was immediately separated and maintained at 5°C. Plasma was obtained from the remaining sample by centrifuging at 900g for 10 min and maintained at 5°C. Red blood cells were washed three times with 2 vols isotonic saline. After centrifuging at 900g for 10 min, the upper layer was removed and discarded. The resulting packed red blood cells were diluted to the initial haematocrit by the addition of isotonic saline. DTZ was spiked in each specimen (whole blood, plasma and resuspended red blood cells) to yield a concentration of 1 mM. M1 and MA concentration was assayed in aliquots of washed blood cells and whole blood (100 µl), and in plasma (1 ml), after 3 min of incubation.

Pharmacokinetic study

Five adult rabbits were cannulated in the central ear artery for blood sampling. Thereafter, they were given a single intraperitoneal (i.p.) dose of 5 mg kg⁻¹ DTZ in isotonic saline. The route of administration and dose were chosen because this way may be used for all ages studied and because several authors have used the dose for carrying out pharmacokinetic studies in rabbit (Yeung *et al.* 1990, 1998). Blood samples (1 ml) were drawn from the cannula at 30, 60, 120, 180, 240, 360 and 480 min after drug administration. In addition, 21 rabbits of either 1, 8, 16 or 30 days of age were also used. Each animal was given i.p. 5 mg kg⁻¹ DTZ in isotonic saline. In this case, arterial blood samples (0.5 ml) were obtained by cardiac puncture, under light ether anaesthesia, at the same times as above (three animals were used for each kinetic time). The blood samples were immediately haemolyzed by the addition of an equal volume of cold water.

HPLC assay of DTZ and its metabolites

DTZ and its metabolites were assayed in the blood and tissue homogenates, following hexane/isobutanol (98:2) extraction, using a previously published HPLC method (Yeung *et al.* 1989). Quantitation was performed using a calibration curve of spiked blank blood or tissue homogenates over the range of concentrations assayed. The method was shown to be linear at concentrations between 10 and 5000, 10 and 1000, and 10 and 500 ng ml⁻¹ DTZ, M1 and MA, respectively. The detection limit was 5, 5 and 10 ng ml⁻¹ for DTZ, M1 and MA, respectively. Recovery was determined by the analysis of blood and homogenate standards containing 50, 200 and 500 ng of either drug ml⁻¹, respectively. Each concentration was assayed in quintuplicate and mean recovery was determined to be > 85% for each drug. For DTZ, M1 and MA, the inter- and intra-assay precisions, determined by the injection of control standards, were in all cases < 10 and 7%, respectively.

Data analysis

The DTZ blood concentration versus time data of the adult rabbit were analysed individually. However, because of the sampling protocol, data from 1-, 8-, 16- and 30-day-old rabbits were analysed as a single curve. Equation (1) was used to describe the time-course of DTZ concentration in rabbits of different ages:

$$C = \sum_{i=1}^n A_i * e^{-\lambda_i t}, \quad (1)$$

where A_i and λ_i are the compartmental kinetic variables obtained by non-linear least squares regression analysis using a weighting factor of $1/(\text{concentration})^2$. The half-life in the postdistributive phase was calculated as $t_{1/2\lambda_n} = \ln 2/\lambda_n$. Body clearance was calculated as $CL = \text{dose}/AUC_{0-8}$, where AUC_{0-8} is the area under the DTZ blood concentration versus time curve calculated by the trapezoidal rule to the last data point (AUC_{0-t}) and extrapolated to infinity by dividing C_{last} for the slope of the terminal phase. The volume of distribution under the area was calculated as $V_{d(\text{area})} = \text{dose}/(AUC_{0-8} * \lambda_n)$. The M1 and MA blood concentration versus time data were analysed using non-compartmental methods.

Statistics

All data are presented as mean $\pm$ SD, unless otherwise indicated. Differences between ages were assessed using ANOVA. If a difference was detected, Duncan's multiple range test was used for multiple comparisons. All other comparisons were performed using an unpaired *t*-test with equal variance or parallelism test. $p < 0.05$ was chosen for the acceptance or rejection of the null hypothesis.

Results*In vitro studies*

DTZ chemical breakdown was < 2% of the initial amount after 60 min of incubation in all the tissues and ages examined, indicating that most of the M1 and MA formed under our experimental conditions was due to enzymatic formation. In addition, we could not observe any appreciable chemical degradation of either M1 or MA in our experimental conditions (data not shown).

The greatest protein concentration in homogenates (table 1) was measured in the liver, followed by the intestine, lung and brain, whatever the age examined.

Table 1. Total protein concentration in the 9000g supernatant of several tissue homogenates from rabbit at different postnatal ages.

	Postnatal age (days)				
	1	8	16	30	Adult
Liver	65.4 $\pm$ 9.2 ^a	72.7 $\pm$ 17.0	82.2 $\pm$ 6.1	88.3 $\pm$ 7.0	93.0 $\pm$ 16.0
Lung	42.8 $\pm$ 5.8 ^a	46.1 $\pm$ 5.2	48.3 $\pm$ 9.0	47.3 $\pm$ 6.0	57.5 $\pm$ 9.2
Intestine	50.0 $\pm$ 7.5 ^a	54.2 $\pm$ 9.0	58.5 $\pm$ 8.6	60.1 $\pm$ 8.0	70.1 $\pm$ 12.7
Brain	16.5 $\pm$ 2.1 ^a	19.5 $\pm$ 2.8	23.0 $\pm$ 1.2	22.2 $\pm$ 6.0	23.7 $\pm$ 3.6

Data are presented as mg g⁻¹ tissue and are the mean ($\pm$ SD) of four experiments.

^a Significantly different from the adult.

The protein content tended to increase with postnatal age in all tissues examined. However, significant differences in the protein content of 1-day-old rabbit homogenates as compared with the adult were only detected.

When DTZ was incubated in the absence of NADPH, we could only detect the formation of M1 (figure 2); however, when DTZ was incubated in the presence of a NADPH-regenerating system, the formation of both M1 and MA was evident. In this latter case, and depending on the tissue and age studied, the amount of M1 formed was 20–30% less than that observed when NADPH was not included in the incubation medium.

DTZ deacetylase activity was detected in all the tissue homogenates and in whole blood whatever the age examined (figure 3). Significant differences were observed when comparing the activities observed in the liver, lung and brain homogenates and in the blood of the 1- and 8-day-old rabbit with those of the adult. Thereafter, the differences were not significant. However, the gut followed a different pattern. In this tissue, DTZ deacetylase activity remained unchanged throughout the period examined. When expressed as mg^{-1} protein, the DTZ deacetylase activity was in the following rank order: brain > lung > liver = gut in

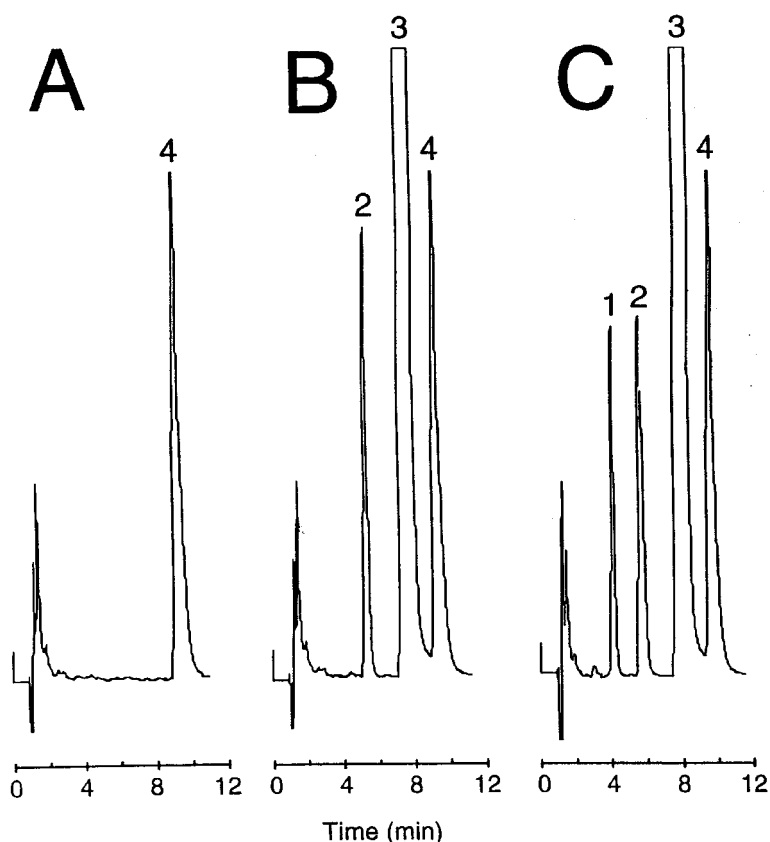


Figure 2. HPLC analysis of diltiazem metabolism without DTZ (A) and the *in vitro* conversion of DTZ to the metabolites M1 and MA in the absence (B) or presence (C) of an NADPH-regenerating system by the 9000g supernatant of the 30-day-old rabbit lung homogenates. 1, demethyldiltiazem; 2, deacetyldiltiazem; 3, diltiazem; 4, imipramine (internal standard).

rabbit < 16 days of age, but brain > lung > gut > liver in the older animal. This enzymatic activity showed the same tendency when data were expressed as g^{-1} wet tissue. Although an important age-dependent DTZ deacetylase activity was observed in the blood, it was not included in the comparison between organs because of the unequal composition of the incubation medium ($\text{nmol min}^{-1} \text{ml}^{-1}$ blood versus $\text{nmol min}^{-1} \text{mg}^{-1}$ protein).

DTZ demethylase activity was only detected in the liver and gut homogenates and in whole blood (figure 4). Liver and gut homogenates from the 1-, 8- and 16-day-old rabbit displayed $\sim 25\%$ of the activity observed in the adult ($p < 0.05$). In these tissues, the maximal activity was detected in the 30-day-old rabbit and was significant higher than that observed in the adult. This enzymatic activity showed the same tendency when data were expressed as g^{-1} wet tissue. However, the DTZ demethylase activity observed in the blood was unchanged in all ages studied. As mentioned above, the activity determined in the blood could not be compared with that described in tissue homogenates because of the unequal composition of the incubation medium.

No differences were found when comparing the DTZ deacetylase and demethylase activity observed in washed blood cells (3.52 ± 0.62 and $5.04 \pm 1.88 \text{ nmol ml}^{-1} \text{ washed blood cells min}^{-1}$, respectively) with that observed in whole blood (3.82 ± 0.84 and $5.72 \pm 1.75 \text{ nmol min}^{-1} \text{ ml}^{-1}$, respectively). However, highly significant differences were observed when the comparison was made with that found in the plasma (1.46 ± 0.09 and $0.05 \pm 0.02 \text{ nmol ml}^{-1} \text{ plasma min}^{-1}$, respectively).

In vivo studies

Whatever the age examined, the best fit for DTZ blood concentration versus time profile after i.p. administration of 5 mg kg^{-1} of this drug was obtained for $n = 1$ as determined by the Akaike's criterion as described by Yamaoka *et al.* (1978) ($r > 0.99$; figure 5). With the exception of that displayed by the 30-day-old rabbit, the DTZ blood concentration versus time profile showed no pronounced changes throughout the period studied. In fact, no significant differences were found in most of the pharmacokinetic parameters described in the present study (C_n , λ_n , $V_{\text{d(area)}}$, AUC, CL) among the 1-, 8-, 16-day-old and adult rabbit (table 2). However, most of the pharmacokinetic parameters associated with DTZ elimination in the 30-day-old rabbit were significantly greater than those observed at other ages.

The mean blood concentration versus time profiles of M1 and MA following the intraperitoneal administration of DTZ are also shown in figure 5. As seen, the M1 concentrations in the 1-, 8- and 16-day-old rabbit were higher than those observed in the adult. However, the M1 concentration observed in the 30-day-old rabbit was significant lower than that observed in the adult. Moreover, the maximal concentration attained (C_{max}), AUC and the AUCM1/AUCDTZ ratio (table 2) in the younger animal (1, 8 and 16 days old) were significantly greater than that observed in the adult, whereas C_{max} and AUC in the 30-day-old rabbit were significantly lower than in the adult. Surprisingly, the MA blood concentrations are similar in all the ages examined. However, the 30-day-old rabbit again showed a different concentration versus time profile, with the slope of the terminal phase being four times greater than that observed in the adult.

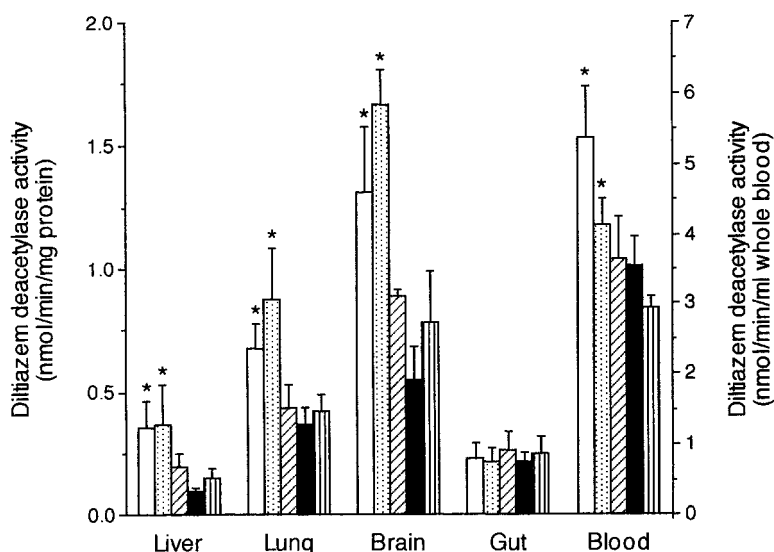


Figure 3. Diltiazem deacetylase activity measured in the 9000g supernatant of several tissue homogenates and in whole blood from rabbit at different postnatal ages. Data are the mean ($\pm$ SD) of four experiments. Incubation conditions are described in the Materials and methods. $\square$, 1-; $\dots$, 8-; /// , 16-; $\blacksquare$, 3-day-old; ||| , adult rabbit. *Significantly different from the adult rabbit.

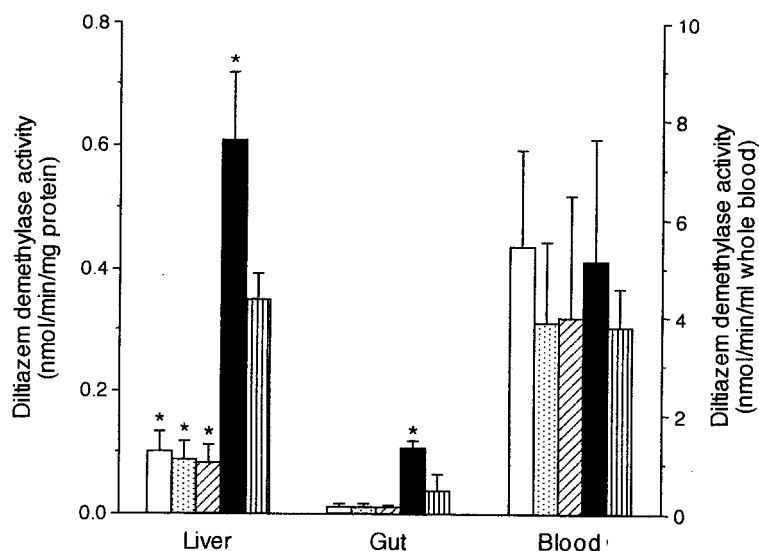


Figure 4. Diltiazem demethylase activity measured in the 9000g supernatant of several tissue homogenates and in whole blood from rabbit at different postnatal ages. Data are the mean ($\pm$ SD) of four experiments. Incubation conditions are described in the Materials and methods. $\square$, 1-; $\dots$, 8-; /// , 16-; $\blacksquare$, 30-day-old; ||| , adult rabbit. The same experiments were performed by using brain and lung homogenates as enzyme sources, but MA concentrations were below the limit of quantitation of the assay (10 ng/ml^{-1}). *Significantly different from the adult rabbit.

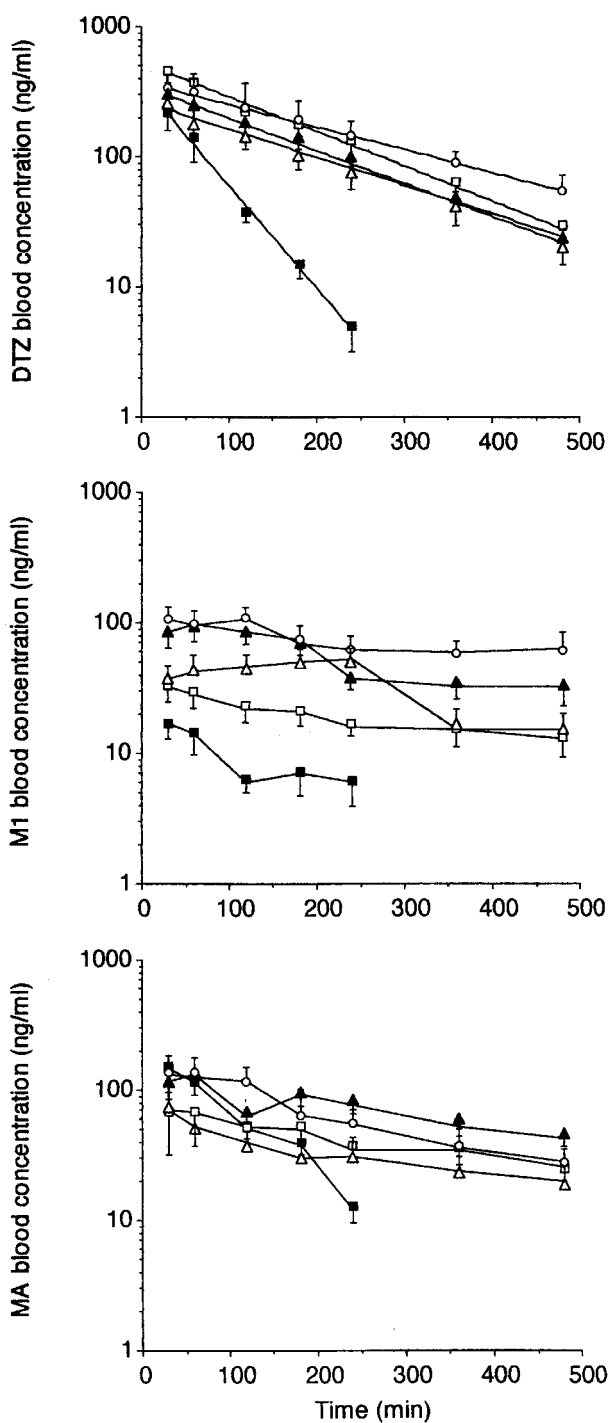


Figure 5. Pharmacokinetic profile of diltiazem and its metabolites in neonate and the adult rabbit. Concentrations of DTZ (top), M1 (central) and MA (bottom) in blood after a single intraperitoneal administration of 5 mg kg^{-1} DTZ in rabbit at different postnatal ages. Data are the mean ($\pm$ SD) of four experiments. ○, 10; ▲, 8-; △, 16-; ■, 30-day-old; □, adult rabbit.

Table 2. Pharmacokinetic parameters of diltiazem and its metabolites, deacetyldiltiazem and N-demetyldiltiazem, determined following a single 5 mg kg⁻¹ i.p. dost to the rabbit.

	Postnatal age (days)				
	1	8	16	30	Adult ^a
Diltiazem					
C _n (ng ml ⁻¹)	375	396	387	362	531 ± 155
λ _n (min ⁻¹)	0.0040	0.00612	0.00507	0.01804 ^c	0.00610 ± 0.00110
t _{1/2λ_n} (min)	173.2	113.0	136.6	38 ^c	113.7 ^b
V _{d(area)} (liters kg ⁻¹)	12.7	12.0	12.4	12.1	9.5 ± 2.6
AUC _{0-∞} (ng min ml ⁻¹)	98491	68152	78280	23002 ^c	97069 ± 41370
CL (ml min ⁻¹ kg ⁻¹)	50.8	73.4	81.6	217 ^c	59.9 ± 26.2
Deacetyldiltiazem					
C _{max} (ng ml ⁻¹)	108 ^c	98 ^c	52 ^c	17 ^c	33 ± 8
λ _n (min ⁻¹)	0.0012	0.0030	0.0052 ^c	0.0050 ^c	0.0018 ± 0.0010
AUC _{0-∞} (ng min ml ⁻¹)	36195 ^c	26925 ^c	16215 ^c	2355 ^c	9435 ± 1838
AUC _{M1} /AUC _{DTZ}	0.3675 ^c	0.3950 ^c	0.2646 ^c	0.1024	0.1131 ± 0.0520
N-Demethyldiltiazem					
C _{max} (ng ml ⁻¹)	132	133	71	148 ^c	85 ± 40
λ _n (min ⁻¹)	0.0037	0.0022	0.0026	0.0114 ^c	0.0031 ± 0.0011
AUC _{0-∞} (ng min ml ⁻¹)	33435 ^c	36615 ^c	16740	17535	21593 ± 4467
AUC _{MA} /AUC _{DTZ}	0.3395	0.5375	0.2732	0.7623 ^c	0.2689 ± 0.1473

^a Each value is the mean ± SD of four animals.

^b Harmonic mean.

^c Significantly different from adults.

C_n is the coefficient and λ_n is the exponent derived by non-linear least-squares regression fit of concentratoin data to equation (1). These terms are related to rate constants for the specific one-compartment pharmacokinetic model and were used to derive the following pharmacolinetic values: t_{1/2λ_n}, elimination half-life; V_{d(area)}, apparent volume of distribution calculated by area method; AUC, area under the blood concentration–time curve; CL, body clearance; C_{max}, maximal experimental blood concentration.

Discussion

Most studies on DTZ demethylase and/or deacetylase activity have been carried out using the microsomal fraction of several tissues, especially the liver. However, evidence has been presented showing that the deacetylation of DTZ is catalysed by microsomal and/or cytosolic enzymes (Leboeuf and Grech-Belanger 1987). Thus, we have selected the 9000g supernatant fraction from the homogenates of selected organs because the microsomal and cytosolic components are included, making the preliminary discrimination between microsomal or cytosolic activities unnecessary.

It is known that DTZ demethylation is catalysed by the NADPH-dependent CYP3A in rabbit and humans (Pichard *et al.* 1990). In addition, it has been demonstrated that DTZ deacetylation is catalysed by a specific esterase, without co-factors being required (Leboeuf and Grech-Belanger 1987). Furthermore, in the presence of NADPH, M1 could be the substrate of a second reaction yielding the secondary metabolite M2 (figure 1). Thus, two separate experiments were designed: the first, including NADPH in the incubation medium, for the determination of the DTZ demethylase, and the second, without NADPH, for the determination of the DTZ deacetylase activity. Obviously, a certain amount of M1 was formed in the first protocol (figure 2). This amount was less than that formed under the conditions used to measure the DTZ deacetylase activity, probably because a fraction was additionally metabolized (Homsy *et al.* 1995). In this sense,

the second set of experiments is designed not to underestimate M1 formation by avoiding its possible degradation.

Under these conditions, DTZ deacetylase activity in the supernatant of 9000g homogenates of the liver, lung, brain, gut and whole blood could be observed at all the ages examined. Except in gut homogenates, DTZ deacetylase activity decreased after the early postnatal period until reaching the value observed in the adult. This finding may be considered as unusual but agrees with previous data published by our group (Fraile *et al.* 1997). In general, the activity of most drug-metabolizing enzymes appears to increase with postnatal age, until reaching the value observed in the adult (Short *et al.* 1972, Morselli 1989, Nouws 1992). However, there are exceptions to this general rule and several authors have observed the activity of several enzymes responsible for the biotransformation of drugs increasing or decreasing during ontogenesis (Ding *et al.* 1991, Gregus and Klaasen 1992, Borlakoglu *et al.* 1993). In fact, a particular isoform may be in a majority at one age but in a minority at another. The different expression of several esterase isoenzymes throughout the postnatal period may explain why the observed activity is actually higher at earlier stages of development than when the animal reaches adulthood. Nevertheless, other factors could contribute to explain these findings. The dietary changes that accompany birth and weaning may be important determinants of enzymatic activity in the liver and other tissues (Hall and Widdowson 1979) and the shift from intra- to extra-uterine life and from high fat breast milk to a solid matter diet at weaning are likely to engender changes in a variety of metabolic enzymes.

Leboeuf and Grech-Belanger (1987) measured DTZ deacetylase activity in rat under experimental conditions directly comparable with ours, reporting a remarkable DTZ deacetylase activity in the liver and blood of the adult rat. However, the activity observed in other organs (small intestine, brain, kidney, heart) was negligible. Esterases constitute a complex group of enzymes that exist in a variety of molecular forms within the tissues or cells of a single organism, with broad and overlapping chemical characteristics (McCracken *et al.* 1993). This family of enzymes has been described as having an almost even and widespread distribution throughout organs. Such complexity permits changes in its expression between different ages, organs and species.

We have observed that the liver, gut and blood can transform DTZ into MA. Pichard *et al.* (1990) demonstrated that the CYP3A subfamily is the major enzyme family involved in the *N*-demethylation of DTZ in rabbit and humans. The tissue distribution pattern of the different isoforms belonging to this subfamily has been repeatedly studied in animals and humans (Murray *et al.* 1988). For example, it has been reported that the small intestine contained the second highest specific content of CYP3A after the liver (Krishna and Klotz 1994). Our findings agree with the literature on the distribution pattern of CYP3A throughout the different organs examined. Surprisingly, we have also observed a remarkable DTZ demethylase activity in the red blood cells of rabbit. Erythrocytes are specialized cells in which the amount of smooth endoplasmic reticulum may be considered as negligible, suggesting that the reaction which metabolizes DTZ into MA in the blood may be catalysed by a non-microsomal enzyme.

It is now conventional knowledge that, at birth, many functions are developed at a rate lower than that predicted on a size basis (Nouws 1992, Schwark 1992). In general, this rule holds in processes involving drug elimination. In fact, it is widely

accepted that during development, microsomal mixed-function oxidase activity increases over several weeks depending on the particular enzyme and species, after which it plateaus or decreases slightly to reach the adult level (Schwark 1992). Our findings on DTZ demethylase activity throughout the rabbit postnatal period are in agreement with this assertion. However, the demethylase activity found in the blood remained unchanged throughout the period examined, reinforcing the hypothesis that, in the blood, this reaction was catalysed by a non-microsomal enzyme.

Although DTZ is extensively metabolized by several routes, deacetylation and *N*-demethylation seem to be the most important pathways in order to generate basic metabolites in mammals (Yeung *et al.* 1990). Moreover, higher blood concentrations of the metabolites after an intravenous (i.v.) dose of DTZ compared with oral (p.o.) doses have been reported, suggesting that extrahepatic metabolism of DTZ may be taking place in the rabbit (Yeung *et al.* 1991). Our results support this hypothesis as many organs could metabolize DTZ to M1 and/or MA. These reactions, occurring in several tissues, may lead to higher concentrations of the metabolites after an i.v. dose, since more parent drug is available systemically for biotransformation.

To confirm further the *in vitro* results, DTZ was administered i.p. to a set of rabbits at the same ages as examined in the *in vitro* experiments. In order to minimize the number of animals used in the study, the sampling protocol was varied among the adult rabbit and the rest of the ages studied (central ear artery versus intracardiac sampling). Previous experiments (data not shown) indicated that the pharmacokinetic profile of DTZ and its metabolites was unchanged by the sampling protocol. This result was expected because there is no organ between the left ventricle and central ear artery that could metabolize and/or eliminate DTZ.

The DTZ pharmacokinetic profile seemed to remain unchanged throughout the rabbit postnatal period when compared with the adult except for 30-day-old animals where a marked increase in DTZ elimination characteristics appeared to develop. These surprising results are not in accord with most of the reports describing drug disposition during ontogeny (Shoaf *et al.* 1987, Kawalek and El Said 1990a, b, Yoo *et al.* 1994). In fact, it is generally admitted that the pre- and postnatal immaturity of the organs involved in the metabolism and elimination of drugs (e.g. liver and kidney) imply a lower capacity to eliminate xenobiotics from the body. This affirmation is almost absolute for xenobiotics with predominant renal excretion. However, other authors have described some exceptions for drugs widely metabolized before renal excretion (Gregus and Klaasen 1992). For example, Miller *et al.* (1976) observed that the pharmacokinetics of acetaminophen did not change throughout the ontogeny in humans, because one of the main biotransformation reactions of this compound (sulphation) has a precocious development that offsets the immaturity of the other biotransformation pathways (glucuronidation). From the DTZ pharmacokinetic profile described in the current study, it may be inferred that some of the reactions which metabolize DTZ were, at least in part, developed in young animals. This assertion is supported by the *in vitro* findings on DTZ deacetylase and demethylase activity in several tissues and in the blood. On the other hand, other organs not investigated in the present study, e.g. the kidney, may have contributed to explain the lack of difference between the young and adult rabbit concerning DTZ clearance.

From the *in vivo* studies, one is tempted to conclude that age does not substantially influence DTZ elimination and that dosage should not be adjusted for a paediatric population. Nevertheless, caution must always be used in extrapolating findings between different species even if the disposition in rabbit is close to that in humans (Yeung *et al.* 1990, 1998).

Acknowledgements

The work was supported by the Spanish DGICYT (PB92-0350); L. J. F. was supported by the Diputacion General de Aragón (BCB-17/93). The authors thank Dr R. Latini and the Istituto di Ricerche Farmacologiche 'Mario Negri' for providing the diltiazem metabolites.

References

- BORLAKOGLU, J. T., SCOTT, A., HENDERSON, C. J. and WOLF, C. R., 1993, Expression of P450 isoenzymes during rat liver organogenesis. *Internal Journal of Biochemistry*, **13**, 1659–1668.
- DING, X., PERNECKY, S. J. and COON, M. J., 1991, Purification and characterization of cytochrome P450 2E2 from hepatic microsomes of neonatal rabbits. *Archives of Biochemistry and Biophysics*, **291**, 270–276.
- FRAILE, L. J., ARAMAYONA, J. J., BREGANTE, M. A., GARCIA, M. A. and ABADIA, A. R., 1996, Deacetylation of diltiazem by several rabbit tissues. *Pharmaceutical Research*, **13**, 1874–1879.
- FRAILE, L. J., ARAMAYONA, J. J., GARCIA, M. A. and BREGANTE, M. A., 1997, Enhanced diltiazem deacetylase activity in pre-term and full-term rabbits compared with adult rabbits. *Biology of the Neonate*, **72**, 51–61.
- GREGUS, Z. and KLAASEN, C. D., 1992, Hepatic disposition of xenobiotics during prenatal and early postnatal development. In R. A. Polin and W. W. Fox (eds), *Fetal and Neonatal Physiology* (Philadelphia: W. B. Saunders), pp. 1103–1123.
- HALL, R. A. and WIDDOWSON, E. M., 1979, Response of the organs of rabbits to feeding during the first days after birth. *Biology of the Neonate*, **35**, 131–139.
- HOMSY, W., LEFEBVRE, M., CAILLE, G. and DU SOUICH, P., 1995, Metabolism of diltiazem in hepatic and extrahepatic tissues of rabbits: *in vitro* studies. *Pharmaceutical Research*, **12**, 609–614.
- KAWALEK, J. C. and EL SAID, K. D., 1990a, Maturational development of drug-metabolizing enzymes in sheep. *American Journal of Veterinary Research*, **51**, 1736–1741.
- KAWALEK, J. C. and EL SAID, K. D., 1990b, Maturational development of drug-metabolizing enzymes in dogs. *American Journal of Veterinary Research*, **51**, 1742–1745.
- KRISHNA, D. R. and KLOTZ, U., 1994, Extrahepatic metabolism of drugs in humans. *Clinical Pharmacokinetics*, **26**, 144–160.
- LEBOEUF, E. and GRECHBELANGER, O., 1987, Deacetylation of diltiazem by rat liver. *Drug Metabolism and Disposition*, **15**, 122–126.
- LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. and RANDALL, R. J., 1951, Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry*, **193**, 265–275.
- MCCRACKEN, N. W., BLAIN, P. G. and WILLIAMS, F. M., 1993, Nature and role of xenobiotic metabolizing esterases in rat liver, lung, skin and blood. *Biochemical Pharmacology*, **45**, 31–36.
- MILLER, R. P., ROBERTS, R. J. and FISCHER, L. J., 1976, Acetaminophen elimination kinetics in neonates, children and adults. *Clinical of Pharmacological Therapy*, **19**, 284–294.
- MORSELLI, P. L., 1989, Clinical pharmacology of the perinatal period and early infancy. *Clinical Pharmacokinetics*, **17**, 13–28.
- MURRAY, G. I., BARNES, T. S., SEWELL, H. F., EWEN, S. W. B. and MELVIN, W. T., 1988, The immunocytochemical localisation and distribution of cytochrome P450 in normal hepatic and extrahepatic tissues with a monoclonal antibody to human cytochrome P450. *British Journal of Clinical Pharmacology*, **25**, 465–475.
- NOUWS, J. F. M., 1992, Pharmacokinetics in immature animals: a review. *Journal of Animal Science*, **70**, 3627–3634.
- PICHARD, L., GILLET, G., FABRE, I., DALETBELUCHE, I., BONFILS, C., THENOT, J. P. and MAUREL, P., 1990, Identification of the rabbit and human cytochromes P-450IIB and P-450IIC as the major enzymes involved in the N-demethylation of diltiazem. *Drug Metabolism and Disposition*, **18**, 711–719.
- SCHWARK, W. S., 1992, Factors that affect drug disposition in food-producing animals during maturation. *Journal of Animal Science*, **70**, 3635–3645.

- SHOAF, S. E., SCHWARK, W. S. and GUARD, C. L., 1987, The development of hepatic drug-metabolizing enzyme activity in the neonatal calf and its effect on drug disposition. *Drug Metabolism and Disposition*, **15**, 677–681.
- SHORT, C. R., 1984, Drug disposition in neonatal animals. *Journal of American Veterinary Medical Association*, **184**, 1161–1162.
- SHORT, C. R., MAINES, M. D. and WESTFALL, B. A., 1972, Postnatal development of drug-metabolizing enzyme activity in liver and extrahepatic tissues of swine. *Biology of the Neonate*, **21**, 54–68.
- SUGAWARA, Y., NAKAMURA, S., USUKI, S., ITO, Y., SUZUKI, T., OHASHI, M. and HARIGAYA, S., 1988, Metabolism of diltiazem. II. Metabolic profile in rat, dog and man. *Journal of Pharmacobiodynamics*, **11**, 224–233.
- YABANA, H., NAGAO, T. and SATO, M., 1985, Cardiovascular effects of the metabolites of diltiazem in dogs. *Journal of Cardiovascular Pharmacology*, **7**, 152–157.
- YAMAOKA, K., NAKAGAWA, T. and UNO, T., 1978, Statistical moments in pharmacokinetics. *Journal of Pharmacokinetics and Biopharmacy*, **6**, 547–558.
- YEUNG, P. K. F., FENG, J. Z. and BUCKLEY, S. J., 1998, Pharmacokinetics and hypotensive effect of diltiazem in rabbits: comparison of diltiazem with its major metabolites. *Journal of Pharmacy and Pharmacology*, **50**, 1247–1253.
- YEUNG, P. K. F., MONTAGUE, T. J., TSUI, B. and MCGREGOR, C., 1989, High-performance liquid chromatographic assay of diltiazem and six of its metabolites in plasma: application to a pharmacokinetic study in healthy volunteers. *Journal of Pharmaceutical Science*, **78**, 592–597.
- YEUNG, P. K. F., MOSHER, S. J., QUILLIAM, M. A. and MONTAGUE, T. J., 1990, Species comparison of pharmacokinetics and metabolism of diltiazem in humans, dogs, rabbits and rats. *Drug Metabolism and Disposition*, **18**, 1055–1059.
- YEUNG, P. K. F., MOSHER, S. J. and POLLAK, P. T., 1991, Pharmacokinetics and metabolism of diltiazem in rabbits after a single intravenous or single oral administration. *European Journal of Drug Metabolism and Pharmacokinetics*, **16**, 69–74.
- YOO, S. D., BURGIO, D. E. and MCNAMARA, P. J., 1994, Phenobarbital disposition in adult and neonatal rabbits. *Pharmaceutical Research*, **11**, 1204–1206.

Metabolism of the analgesic drug, tramadol hydrochloride, in rat and dog

W. N. WU^{†*}, L. A. MCKOWN[†], A. D. GAUTHIER[‡], W. J. JONES[‡] and R. B. RAFFA[§]

Divisions of [†]Preclinical Development and [‡]Drug Discovery, The R. W. Johnson Pharmaceutical Research Institute, Spring House, PA 19477, USA

Received 15 January 2001.

1. Metabolism of the analgesic agent, tramadol hydrochloride, was investigated after a single oral administration of ¹⁴C-tramadol to four rats (50 mg kg⁻¹) and two dogs (20 mg kg⁻¹).

2. Recovery of total radioactivity in rat and dog urine samples over 24 h was 73 and 65% of the radioactive dose, respectively.

3. Unchanged tramadol and a total of 24 metabolites, consisting of 16 Phase I metabolites and eight conjugates (seven glucuronides, one sulphate), were isolated and tentatively identified, which accounted for > 52% of the dose in urine of both species.

4. Of the metabolites, five (M1-5) were previously identified.

5. The metabolites were formed via the following six metabolic pathways: *O*-demethylation, *N*-demethylation, cyclohexyl oxidation, oxidative *N*-dealkylation, dehydration and conjugation.

6. Pathways 1-3 appear to be major steps, forming seven *O*-desmethyl/*N*-desmethyl and hydroxy-cyclohexyl metabolites in major quantities.

7. Pathways 1-3 in conjunction with pathway 6 produced four glucuronides along with four minor conjugates.

8. In addition, the *in vitro* metabolism of tramadol was conducted using rat hepatic S9 fraction in the presence of an NADPH-generating system. Unchanged tramadol (30% of the sample) plus nine metabolites, M1-7, tramadol-*N*-oxide (M31) and OH-cyclohexyl-M1 (M32), were profiled and tentatively identified based on MS and MS/MS data.

Introduction

Tramadol hydrochloride (ULTRAM[®]), (1 *RS*, 2 *RS*)-2-[(dimethylamino-methyl]-1-(3-methoxyphenyl)-cyclohexanol HCl (figure 1), is an orally active, centrally acting opioid analgesic currently being marketed in the USA and more than 90 other nations. Tramadol is well absorbed and extensively metabolized after oral administration, and its metabolites are excreted primarily in the urine (Lintz *et al.* 1981, Wu *et al.* 1997a-c). The mechanism of action, pharmacokinetics and clinical attributes of tramadol have been published (Flohe' *et al.* 1978, Friderichs *et al.* 1978, Lintz *et al.* 1981, Lintz and Besker 1986, Hennies *et al.* 1988, Hills *et al.* 1992, Raffa *et al.* 1992, 1993, 1995, Lee *et al.* 1993, Abel 1995). The excretion and metabolism of tramadol in animals and humans were also previously investigated, and five metabolites, *O*-desmethyl tramadol (M1), *N*-desmethyl tramadol

* Author for correspondence.

§ Present address: Temple University School of Pharmacy, Philadelphia, PA 19140, USA.

(M2), *N,N*-didesmethyl (M3), *N,N,O*-tridesmethyl tramadol (M4) and *N,O*-didesmethyl tramadol (M5), and three uncharacterized conjugates of M1, M4 and M5 were reported (Lintz *et al.* 1981). The *in vitro* metabolism of tramadol was conducted and identified CYP2D6 and CYP3A4 as the principal cytochrome P450 isoforms catalysing *O*-demethylation and *N*-demethylation of tramadol in human liver microsomes (Paar *et al.* 1992, 1993, Dayer *et al.* 1997). In animal models, M1 is up to six times more potent than tramadol in producing analgesia and 200 times more potent in μ -opioid binding (Raffa *et al.* 1992). Preliminary results from our laboratories on the metabolism of tramadol in the Wistar rat, beagle dog and humans have been reported (Wu *et al.* 1997a–c). The purpose of the present study was: (1) to reconfirm the previously identified metabolites, (2) to characterize the nature of M1, M4 and M5 conjugates, and (3) to identify any other metabolites. This paper describes the excretion, isolation and identification of unchanged tramadol, its 26 metabolites consisting of 18 Phase I metabolites (five known metabolites, 13 new metabolites) and eight conjugates (seven glucuronides, one sulphate) and the proposed metabolic pathways in rat hepatic S9 fraction, and rat and dog urine after oral administration of tramadol.

Materials and methods

Chemicals and materials

^{14}C -tramadol hydrochloride, ($\pm$) *cis*-2[(dimethylamino)- ^{14}C -methyl]-1-(3-methoxyphenyl)-cyclohexanol hydrochloride (figure 1) was supplied by The R. W. Johnson Pharmaceutical Research Institute, Spring House, PA, USA, with a radiochemical purity > 97% (radioTLC and HPLC) (Weaner, unpublished data). The compound was used at a specific activity of $12.7\ \mu\text{Ci mg}^{-1}$ for the rat and dog studies. Tramadol (RWJ-26898), RWJ-41907 (M1), RWJ-41908 (M2), RWJ-41909 (M3), RWJ-41910 (M4), RWJ-41911 (M5) and RWJ-50803 (M6) (purity of each compound was > 97% by HPLC and MS) (figure 1) were synthesized at The R. W. Johnson Pharmaceutical Research Institute. Diazald and *N*-ethyl-*N'*-nitroso guanidine were purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). Glusulase[®] was obtained from Endo Laboratory (Wilmington, DE, USA). Formula-989 (scintillation solution) was purchased from New England Nuclear (Boston, MA, USA). HPLC grade solvents were obtained from Fisher Scientific (Fair Lawn, NJ, USA), and glass-distilled solvents were purchased from Burdick and Jackson Laboratories (Muskegon, MI, USA). The incubation components for hepatic S9 (Tris, potassium chloride, magnesium chloride, NADP^+ , glucose-6-phosphate), were purchased from Sigma Co. (St Louis, MO, USA).

Hepatic S9 fraction

The rat hepatic S9 fraction was generated from a male CrI:CD[®] (SD)IGS BR, VAF/Plus[®] rat (~275 g) at The R.W. Johnson Pharmaceutical Research Institute.

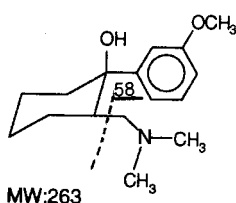
Incubations

Chilled, freshly made components were added to each flask (on ice) in the following order: 1.15% KCl in 0.05 M Tris buffer (pH 7.4), 5 mM MgCl_2 , 5 mM glucose-6-phosphate, 0.5 mM NADP^+ , test substrate (hepatic S9), and tramadol spike, to obtain a final volume of 5 ml and a tramadol concentration of $10\ \mu\text{g ml}^{-1}$. After the addition of the last component, each flask was incubated in a 37°C Dubnoff Metabolic Shaker Incubator (Precision Scientific, Chicago, IL, USA). Samples were removed at 0 and 60 min. A control flask was incubated without rat subcellular fraction to determine drug stability under the incubation conditions.

Animal experiments

^{14}C -tramadol ($20\ \text{mg kg}^{-1}$ dose) and tramadol ($40\ \text{mg kg}^{-1}$ dose) were administered by gavage to each of two male beagle dogs (9–16 kg; Marshall Farms, Rose, NY, USA), respectively, as a single aqueous dose. Four rat (two males, two females; 250–350 g), CrI:CD[®]BR (Charles River Laboratories, Raleigh, NC, USA) were given a single 50 or $150\ \text{mg kg}^{-1}$ of ^{14}C -tramadol and tramadol, respectively (one per sex for each regimen). Urine samples were collected over 24 h, and each collected sample was assayed for total radioactivity by LSC, TLC and HPLC.

Tramadol

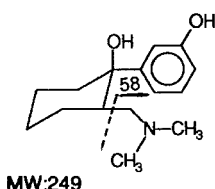

CI and EI-MS and Ionspray-MS/MS data
***m/z* (% relative abundance)**

292([M+C₂H₅]⁺, 3), 264([M+H]⁺, 100), 246
 ([M+H]⁺-H₂O, 4), 217(1), 189(3), 135(1),
 121(1), 114(1)

ionspray-MS/MS

264([M+H]⁺, 8), 246([M+H]⁺-H₂O, 3), 201(4),
 187(2), 145(2), 131(1), 121(3), 107(2), 58(100)

M1

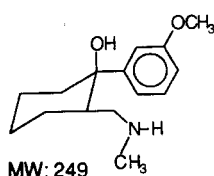


249(M⁺, 8), 231(M⁺-H₂O, 1), 169(2), 152(4),
 121(2), 110(8), 58(100)

ionspray-MS/MS

250([M+H]⁺, 3), 232([M+H]⁺-H₂O, 1), 145
 (1), 58(100)

M2

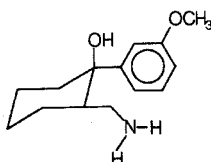


250([M+H]⁺, 100), 232([M+H]⁺-H₂O, 3), 188
 (11), 135(2)

ionspray-MS/MS

250([M+H]⁺, 17), 232([M+H]⁺-H₂O, 7), 201
 (24), 189(93), 175(3), 173(3), 159(11), 147
 (2), 135(8), 121(100), 107(3), 91(7)

M3

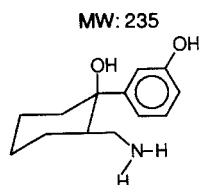


236([M+H]⁺, 100), 218([M+H]⁺-H₂O, 41), 189
 (54), 135(2), 86(2)

ionspray-MS/MS

236([M+H]⁺, 100), 218([M+H]⁺-H₂O, 11),
 204(6), 189(58), 176(11), 145(6), 135(3),
 121(54), 107(2)

M4

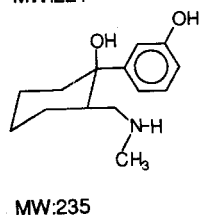


222([M+H]⁺, 100), 204([M+H]⁺-H₂O, 28),
 175(5), 138(48), 121(84), 108(2)

ionspray-MS/MS

222([M+H]⁺, 1), 204([M+H]⁺-H₂O, 4), 187
 (3), 175(50), 162(6), 145(5), 133(3), 121(2),
 107(100), 81(17)

M5



236([M+H]⁺, 100), 218([M+H]⁺-H₂O, 2),
 174(4), 121(10)

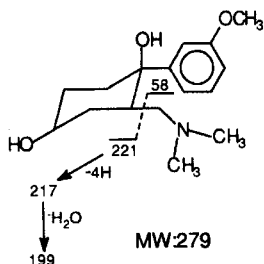
ionspray-MS/MS

236([M+H]⁺, 81), 218([M+H]⁺-H₂O, 25),
 187(17), 175(5), 159(14), 145(35), 133(9),
 121(7), 117(3), 107(100), 93(4)

Figure 1.

Continued

Continued

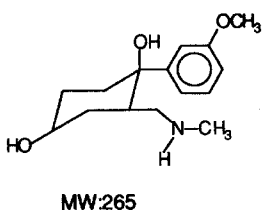
M6

279(M^+ , 11), 261($M^+ - H_2O$, 2), 234(4), 217(2), 186(1), 169(4), 149(2), 135(5), 121(2), 107(4), 58(100)

308($[M + C_2H_5]^+$, 2), 280($[M + H]^+$, 100), 262($[M + H]^+ - H_2O$, 25), 234(2), 187(1), 135(2), 116(3)

ionspray-MS/MS

280($[M + H]^+$, 50), 262($[M + H]^+ - H_2O$, 18), 217(5), 201(2), 199(17), 184(5), 149(1), 135(10), 121(16), 107(2), 91(4), 58(100)

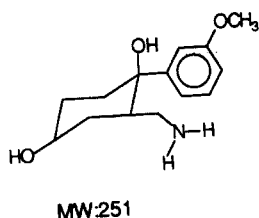
M7

265(M^+ , 3), 247($M^+ - H_2O$, 5), 204(34), 160(26), 150(8), 135(9), 121(2), 107(4), 44(100)

294($[M + C_2H_5]^+$, 2), 266($[M + H]^+$, 100), 248($[M + H]^+ - H_2O$, 30), 236(3), 204(6), 187(3), 135(1), 107(3)

ionspray-MS/MS

266($[M + H]^+$, 100), 248($[M + H]^+ - H_2O$, 5), 187(3), 121(2), 58(7), 44(45)

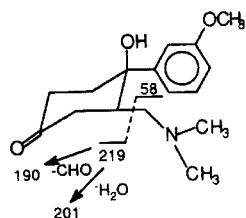
M8

251(M^+ , 11), 234(33), 233($M^+ - H_2O$, 22), 174(44), 149(45), 124(78), 121(56), 106(100)

252($[M + H]^+$, 23), 234($[M + H]^+ - H_2O$, 22), 191(18), 173(34), 147(100), 129(45), 107(92)

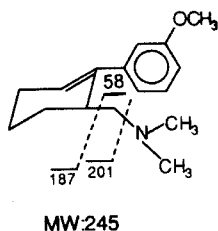
ionspray-MS/MS

252($[M + H]^+$, 100), 234($[M + H]^+ - H_2O$, 4), 216(6), 205(13), 187(32), 121(3), 101(18)

M9

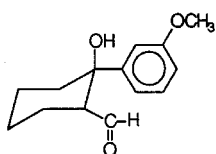
ionspray-MS/MS

278($[M + H]^+$, 17), 261(7), 250(1), 237(38), 219(26), 201(10), 190(100), 121(3), 58(28)

M10

ionspray-MS/MS

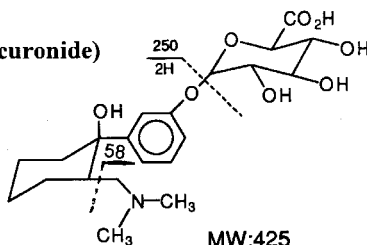
246($[M + H]^+$, 16), 201(25), 187(30), 159(10), 145(8), 135(2), 121(10), 107(2), 72(11), 58(100)

M11

MW:234

ionspray-MS/MS

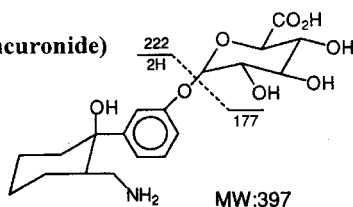
235([M+H]⁺, 2), 217([M+H]⁺-H₂O, 4), 189
([M+H]⁺-H₂O-CO₂, 2), 135(100), 121(7), 107(6)

M13**(M1-glucuronide)**

MW:425

ionspray-MS/MS

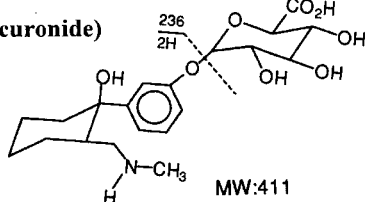
426([M+H]⁺, 25), 408([M+H]⁺-H₂O, 1), 250
([M+H]⁺, 6), 232([M+H]⁺-H₂O, 2), 58(100)

M14**(M4-glucuronide)**

MW:397

ionspray-MS/MS

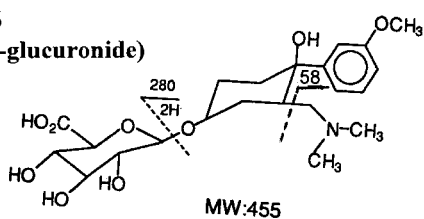
398([M+H]⁺, 100), 380([M+H]⁺-H₂O, 50), 222
([M+H]⁺, 6), 204([M+H]⁺-H₂O, 22), 107(13)

M15**(M5-glucuronide)**

MW:411

ionspray-MS/MS

412([M+H]⁺, 100), 394([M+H]⁺-H₂O, 2), 236
([M+H]⁺, 18), 218([M+H]⁺-H₂O, 3), 117(2)

M16**(M6-glucuronide)**

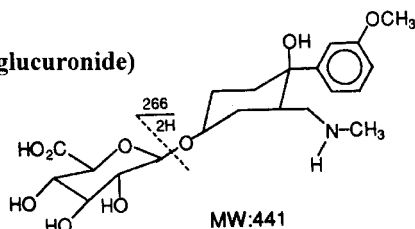
MW:455

ionspray-MS/MS

456([M+H]⁺, 100), 438([M+H]⁺-H₂O, 2), 280
([M+H]⁺, 3), 262([M+H]⁺-H₂O, 1), 131(28),
131(28), 58(32)

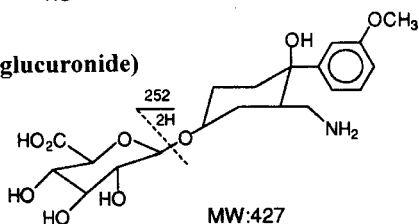
Continued

Continued

M17**(M7-glucuronide)**

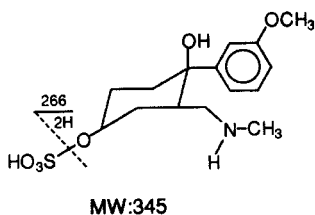
ionspray-MS/MS

442([M+H]⁺, 100), 424([M+H]⁺-H₂O, 10), 266
([M7+H]⁺, 6), 248([M7+H]⁺-H₂O, 6), 187(7),
135(2), 121(3)

M18**(M8-glucuronide)**

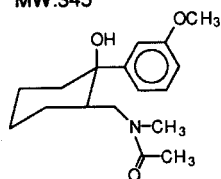
ionspray-MS/MS

428([M+H]⁺, 100), 410([M+H]⁺-H₂O, 5), 252
([M8+H]⁺, 8), 205(14), 187(3)

M24**(M7 sulfate)**

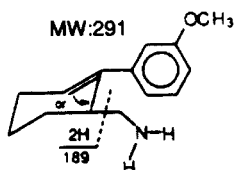
ionspray-MS/MS

346([M+H]⁺, 100), 266([M7+H]⁺, 7), 167(22),
139(24), 121(12), 107(20)

M25

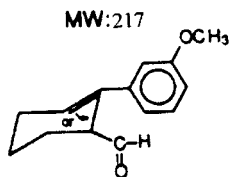
ionspray-MS/MS

292([M+H]⁺, 100), 274([M+H]⁺-H₂O, 14), 251
(9), 231([M+H]⁺-HAc, 38), 201(58), 172(7),
151(5), 135(3), 117(5), 86(55), 60(33)

M26

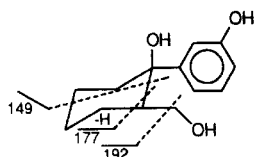
ionspray-MS/MS

218([M+H]⁺, 1), 201([M+H]⁺-NH₃, 2), 189(18),
147(4), 135(2), 121(100, MeO-PhCH₂⁻), 91(5),
81(8)

M27

ionspray-MS/MS

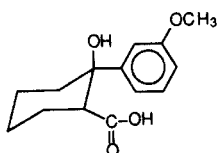
217([M+H]⁺, 4), 199([M+H]⁺-H₂O, 3), 184(199
-Me, 2), 135(80), 121(10), 107(31), 69
(COCHCH₂CH₂⁺, 100)

M28

ionspray-MS/MS

223([M+H]⁺, 51), 205([M+H]⁺-H₂O, 2), 192
(1), 177(100), 163(2), 149(23), 105(5)

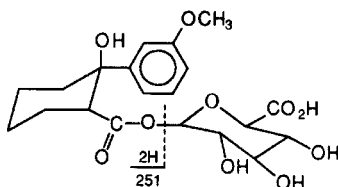
MW:222

M29

ionspray-MS/MS

251([M+H]⁺, 100), 233([M+H]⁺-H₂O, 2), 221
([M+H]⁺-CHO, 1), 190(2), 175(2), 159(3), 121
(1), 117(2), 99(4), 59(13)

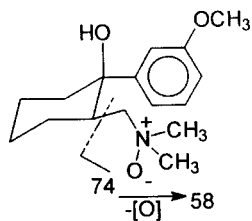
MW:250

M30

ionspray-MS/MS

427([M+H]⁺, 36), 409([M+H]⁺-H₂O, 1), 251
([M29+H]⁺, 6), 233([M29+H]⁺-H₂O, 2), 58(100)

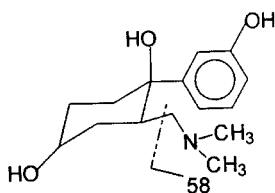
MW:426

M31

ionspray-MS/MS

280([M+H]⁺, 12), 262([M+H]⁺-H₂O, 22), 221(2)
201(11), 191(3), 173(4), 159(6), 135(64), 121(6)
110(18), 107(3), 74(4), 58(100)

MW:279

M32

ionspray-MS/MS

266([M+H]⁺, 25), 248([M+H]⁺-H₂O, 3), 135(5),
121(11), 58(100)

MW:265

Figure 1. Structures and mass spectral data for tramadol and its metabolites.

Analytical equipment

Total radioactivity in each sample was determined using Formula-989TM LSS cocktail in a Packard Tri-Carb[®] 2300 TR Liquid Scintillation Analyzer (Packard Instruments, Downers Grove, IL, USA). Thin-layer radiochemical analyses were performed using 5 × 20 cm silica gel GF plates (250 µm gel thickness, Anatech, Inc., Newark, DE, USA). TLC plates were analysed using a Radiochromatogram Imaging System (BID 100) (Bioscan, Inc., Washington DC, USA). The HPLC used was a gradient liquid chromatograph (Beckman Instrument Co., Fullerton, CA, USA) connected to a radioactive flow detector (RAMONA; IN/US Service, Corp., Fairfield, NJ, USA). LiChrosorb RP-18 Hibar guard and analytical column (10 µm, 25 cm × 4.6 mm i.d.) were used. Electron-impact (EI) and desorption chemical ionization (CI, methane) spectra were obtained in a Hewlett-Packard 5989A MS Engine (Hewlett-Packard Co., Valley Forge, PA, USA), and operating conditions for the spectrometer included: source temperature at 220°C (EI) and 100°C (CI); ionization potential at 70 eV (EI) and 100 eV (CI); multiplier voltage at 1.8 KeV; electrometer setting at 10⁻⁷ A/V; reagent gas source pressure of 1000 µm for methane; mass ranges 18–1000 *m/z* at 1 s decade⁻¹. API-ionspray MS and MS/MS spectra were obtained using a PE Sciex API-III Plus (Perkin-Elmer Sciex, Thornhill, Ontario, Canada), a triple quadrupole mass spectrometer interfaced to a Hitachi HPLC solvent delivery system (L-6200A, Intelligent Pump, Hitachi Co., Tokyo, Japan) via an ionsprayer using nitrogen as a curtain and nebulizing gas and argon as a collision gas for MS/MS and MRM analysis.

Sample preparation

Urine sample pools (0–24 h) were generated as a mixture of equal aliquots of urine from four rats (two per sex) orally dosed with ¹⁴C-tramadol (50 mg kg⁻¹) and tramadol (150 mg kg⁻¹), respectively, and two male dogs each orally dosed with ¹⁴C-tramadol (20 mg kg⁻¹) and tramadol (40 mg kg⁻¹), respectively. The Glusulase[®]-hydrolyzed urine samples were prepared from each species by treating aliquots from the urine pools in the following manner. Each urine sample was adjusted to pH 5.1 with acetic acid and buffered with 1 M sodium acetate solution (10% volume of urine). Glusulase[®] (2% by volume) was added to this mixture and hydrolyzed for 16 h at 37°C. Each hydrolyzed urine was extracted with 1/3 urine volume of Amberlite[®] XAD-2 twice. The combined XAD-2 resin was washed with water and hexane followed by methanol extraction (extraction efficiency 92 and 84% for rat and dog, respectively). Each methanol extract residue was reconstituted in a small volume of methanol and further separated and purified using column chromatography (neutral alumina), TLC and HPLC and further analysed using NMR, CI-MS, EI-MS, and ionspray-MS, MS/MS and MRM. Phenolic metabolites were further derivatized as methyl and ethyl ethers by diazomethane and diazoethane, respectively. For the sample preparation of untreated urine, 25 ml of untreated urine pool (0–24 h) from each species was extracted with Amberlite[®] XAD-2/methanol as described previously. Each methanol extract residue (extraction efficiency 91 and 90% for rat and dog, respectively) was reconstituted in a small volume of methanol and further separated, purified, analysed using TLC, LC/ionspray-MS and MS/MS techniques.

Total radioactivity

All urine samples were assayed for total radioactivity using Formula-989TM LSS cocktail in a Packard Liquid Scintillation Analyzer. Duplicate aliquots of urine (0.1 ml) and purified metabolite fractions in methanol were assayed directly for total radioactivity in 10 ml Formula-989.

Metabolite profiling, isolation, and identification

Sample pools from urine (0–24 h, 73 and 65% of the radioactive dose in rat and dog, respectively), were generated for metabolite isolation and identification. The TLC analyses of the sample pools were performed using the basic solvent system, methylene chloride/methanol/ammonium hydroxide (90/10/1 v/v/v). Each TLC plate was developed to 14 cm above the spot or band origin. Each sample was chromatographed with tramadol and M1-6 and tramadol-*N*-oxide as reference compounds, and visualized by UV light after each plate was air-dried. Each TLC plate was analysed by radiochromatogram imaging. Each HPLC analysis was conducted using a linear gradient program, from 1% methanol (99% water) to 100% methanol in 10 min and 100% methanol in 2 min with both methanol and water containing 0.2% ammonium acetate at a flow rate of 1 ml min⁻¹. Unchanged tramadol and its metabolites were fractionated from the above LC system and analysed using PE Sciex API-ionspray MS, MS/MS and MRM. The mobile phase for this system was acetonitrile/water with 2.5 mM ammonium acetate buffer (pH 4.0, 50/50 v/v) at a flow rate of 0.1 ml min⁻¹.

Data analysis

The Excel for Windows computer program (v.4.0, Microsoft Corp., Redmond, WA, USA) was used to calculate the total percent radioactive dose recovered in the urine pools. An estimate of the relative

Table 1. Metabolism of tramadol in rat and dog, and in rat hepatic S9 fractions.

Analyte	Rat			Analyte	Rat		
	Hepatic S9	Urine (73% dose)	Dog (65% dose)		Hepatic S9	Urine (73% dose)	Dog (65% dose)
Tramadol	30	2	2	M15 (M5 glucuronide)	—	10	7
M1 (RWJ-41907)	27	13	6	M16 (M6 glucuronide)	—	+	8
M2 (RWJ-41908)	30	13	6	M17 (M7 glucuronide)	—	+	7
M3 (RWJ-41909)	+	9	4	M18 (M8 glucuronide)	—	—	+
M4 (RWJ-41910)	+	+	+	M24 (M7 sulphate)	—	—	+
M5 (RWJ-41911)	8	5	6	M25	—	+	+
M6 (RWJ-50803)	+	+	3	M26	—	+	+
M7	+	+	3	M27	—	+	+
M8	—	+	+	M28	—	+	+
M9	—	+	+	M29	—	+	+
M10	—	+	+	M30 (M29 glucuronide)	—	—	+
M11	—	+	+	M31 (tramadol-N-oxide)	+	—	—
M13 (M1 glucuronide)	—	7	+	M32 (OH-M1)	+	—	—
M14 (M4 glucuronide)	—	+	+	Total %	~ 95	~ 90	~ 80

+, < 2%; —, not detected.

percentages of unchanged tramadol and each metabolite in a given sample was made using the integrated chromatogram, and the Sciex API III-Q1 scan MS (TIC, total ion chromatogram).

Results

The 0–24-h rat and dog urine pools contained 73 and 65% of the radioactive dose, respectively. Unchanged tramadol plus 26 metabolites were characterized and tentatively identified from the rat (65% of the dose) and dog (52% of the dose) urine, and rat hepatic S9 incubations (table 1). TLC metabolic profiles in figure 2 were obtained for the rat and dog urine. The *in vitro* metabolism of tramadol was conducted in the rat hepatic S9 fraction, and unchanged tramadol plus nine metabolites were profiled and identified in the 60-min S9 incubate. The structures of tramadol and each metabolite and their MS (figure 1) and NMR (table 2) data are presented. API-Q1 scan MS (TIC) profiles were obtained for untreated and Glusulase[®]-treated urine of both rat, dog (figure 3). The isolation, characterization, and identification of unchanged tramadol and each metabolite are discussed. Representative mass spectra for M1, M6 and each glucuronide are presented in figure 4.

Unchanged tramadol and M1 to M5

Unchanged tramadol (2% of the dose in both species) and known metabolites 1–5 (Lintz *et al.* 1981) were reconfirmed as major metabolic products (table 1) in both untreated and Glusulase[®]-treated urine using TLC, CI-MS, EI-MS, API-ion-spray-MS, MS/MS and MRM, and NMR techniques, and by comparison with synthetic samples. The MS and NMR data are shown in figure 1 and table 2, respectively. In rat hepatic S9 (60-min incubation), unchanged tramadol (30% of the sample) plus M1 and M2 as major metabolites, and M3–5, as minor metabolites were profiled, quantified and tentatively identified (table 1).

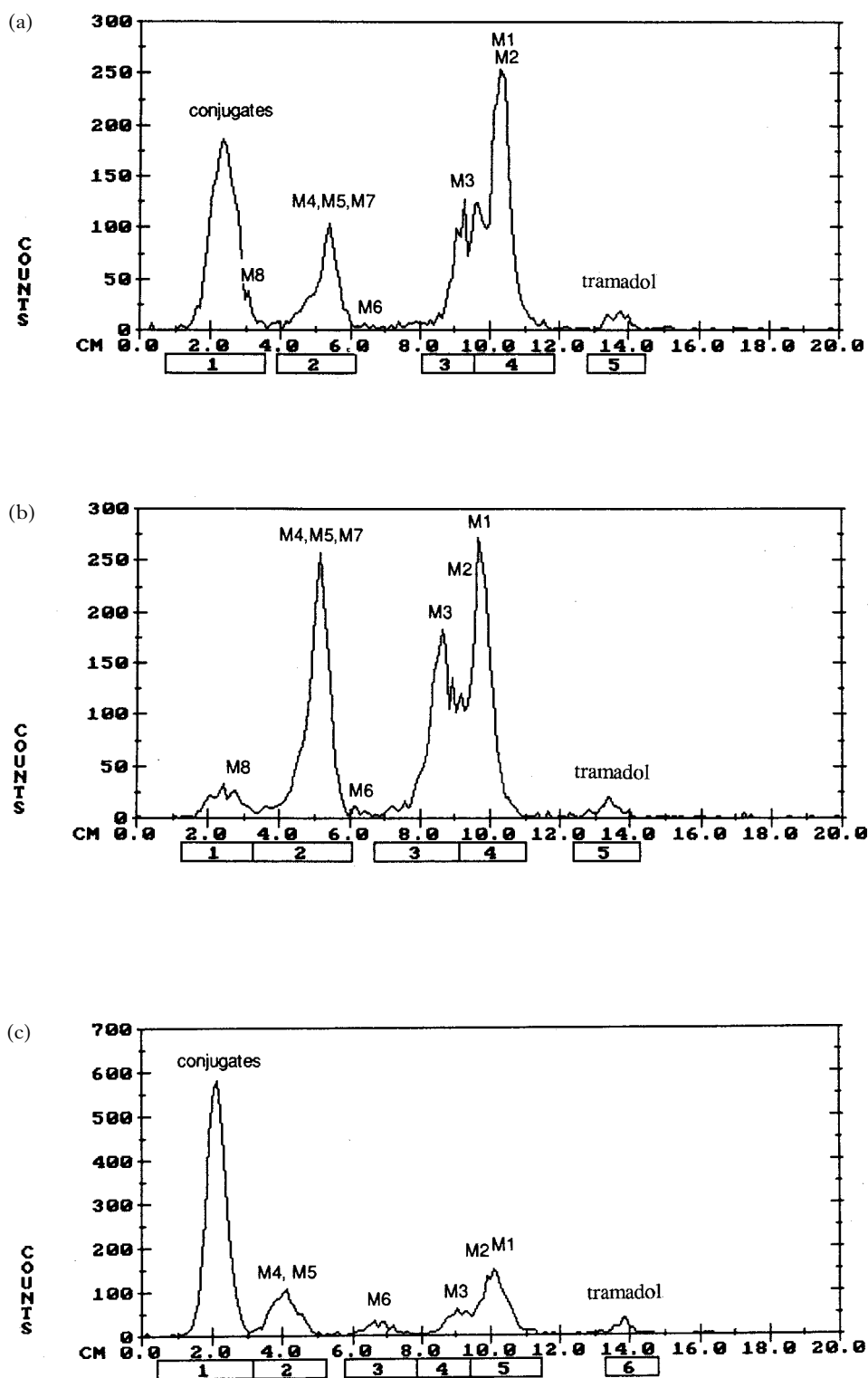


Figure 2.—continued

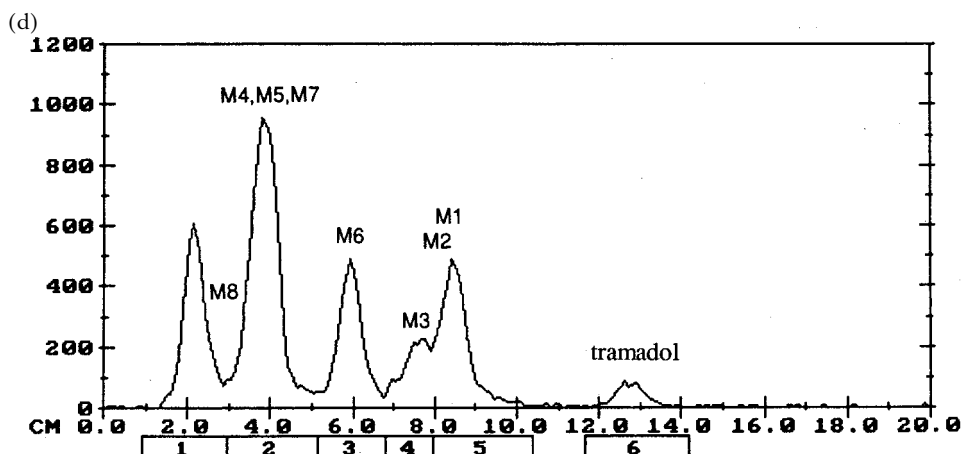


Figure 2. (continued) TLC metabolic profiles of rat and dog urine. (a) Rat untreated urine (0–24 h); (b) rat glucusulase-treated urine (0–24 h); (c) dog untreated urine (0–24 h); (d) dog glucusulase-treated urine (0–24 h).

Table 2. Proton NMR data for tramadol and its metabolites.

Analyte	Aliphatic protons	N-methylene	N-methyl	O-methyl	Aromatic protons
Tramadol	1.40–2.20 (m, 9Hs)	2.45 (d, 2Hs)	2.15 (s, 6Hs)	3.88 (s, 3Hs)	6.80–7.30 (m, 4Hs)
M1	1.35–2.05 (m, 9Hs)	2.40 (d, 2Hs)	2.10 (s, 6Hs)	–	6.67–7.20 (m, 4Hs)
M2	1.45–2.10 (m, 9Hs)	2.35, 2.62 (dd, 2Hs)	2.28 (s, 3Hs)	3.88 (s, 3Hs)	6.80–7.25 (m, 4Hs)
M3	1.40–2.10 (m, 9Hs)	2.50 (d, 2Hs)	–	3.88 (s, 3Hs)	6.75–7.25 (m, 4Hs)
M4	1.48–1.95 (m, 9Hs)	2.52 (d, 2Hs)	–	–	6.72–7.45 (m, 4Hs)
M5	1.40–1.90 (m, 9Hs)	2.40, 2.60 (dd, 2Hs)	2.25 (s, 3Hs)	–	6.72–7.30 (m, 4Hs)
M6	1.65–2.20 (m, 8Hs) 3.80 (m, 1H)	2.45 (d, 2Hs)	2.18 (s, 6Hs)	3.88 (s, 3Hs)	6.80–7.20 (m, 4Hs)
M7	1.70–2.10 (m, 8Hs) 3.80 (m, 1H)	2.50, 2.65 (dd, 2Hs)	2.30 (s, 3Hs)	3.88 (m, 3Hs)	6.80–2.20 (m, 4Hs)
M31	1.40–2.35 (m, 9Hs)	3.15, 3.30 (dd, 2Hs)	3.00, 3.10 (ss, 6Hs)	3.80 (m, 3Hs)	6.73–7.26 (m, 4Hs)

M6–11

M6–9 were not previously identified in rat and dog. EI-MS, CI-MS and Q1 scan MS analysis of M6 revealed a molecular ion and an apparent protonated molecular ion at m/z 279 and 280, respectively (figure 1). MS/MS analysis of protonated molecular ion displayed important daughter ions at m/z 262, 199, 135, 121 and 58 (base peak), together with m/z 280 (figure 1). ^1H NMR (table 2), ^{13}C NMR, and 2D NMR spectra of M6 are obtained. On the basis of MS and NMR data and by comparison with a synthetic sample (RWJ-50803), M6 was identified to be 4-hydroxy-cyclohexyl tramadol. API-ionspray Q1 scan MS and CI-MS analysis of M7, M8 and M9 showed protonated molecular ions at m/z 266, 252 and 278, respectively (figure 1). MS/MS analysis of m/z 266 (protonated M7) exhibited diagnostic daughter ions at m/z 248, 187 and 135, along with protonated parent ion (figure 1). ^1H NMR data of M7 is shown in table 2, which further confirmed the structure of M7. MS/MS analysis of protonated M8 indicated informative daughter ions at m/z 234, 205, 187 and 121 (figure 1). MS/MS analysis of protonated M9 revealed prominent daughter ions at m/z 261, 237, 219, 201, 190,

(a)

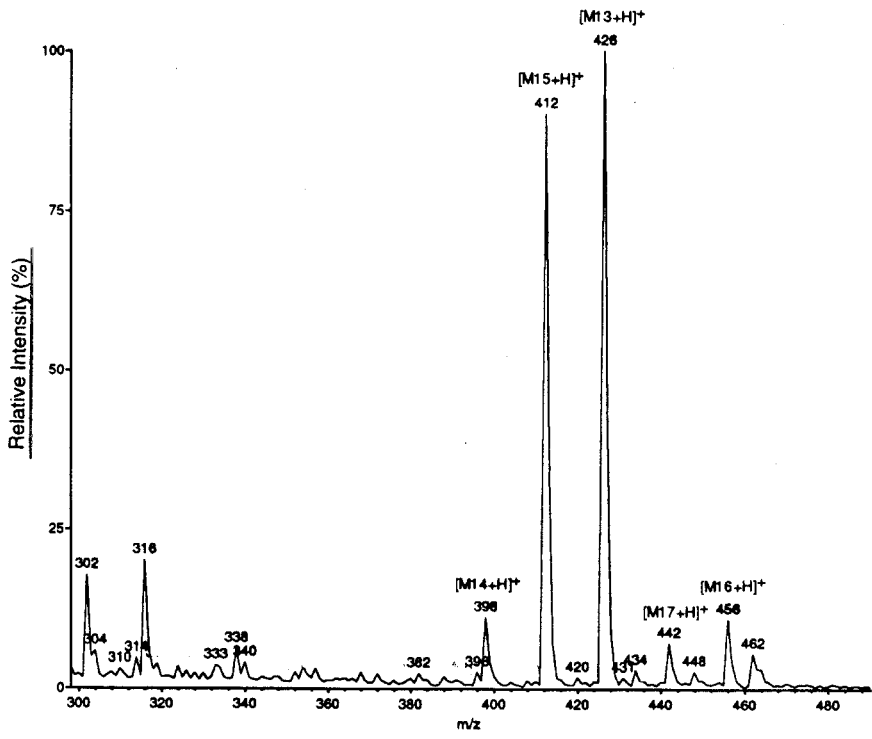
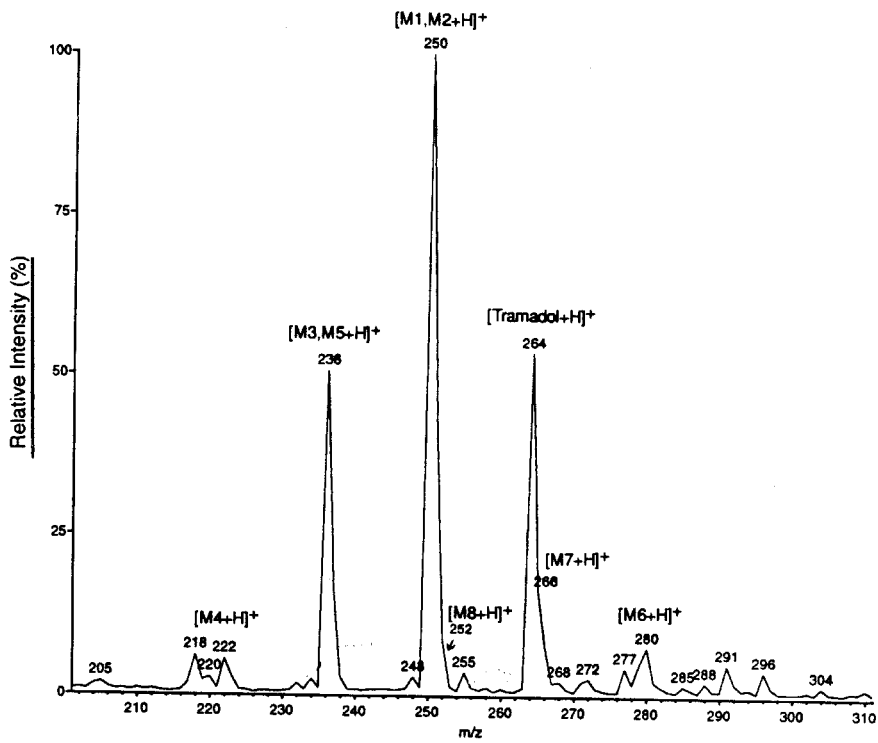


Figure 3.

continued

continued

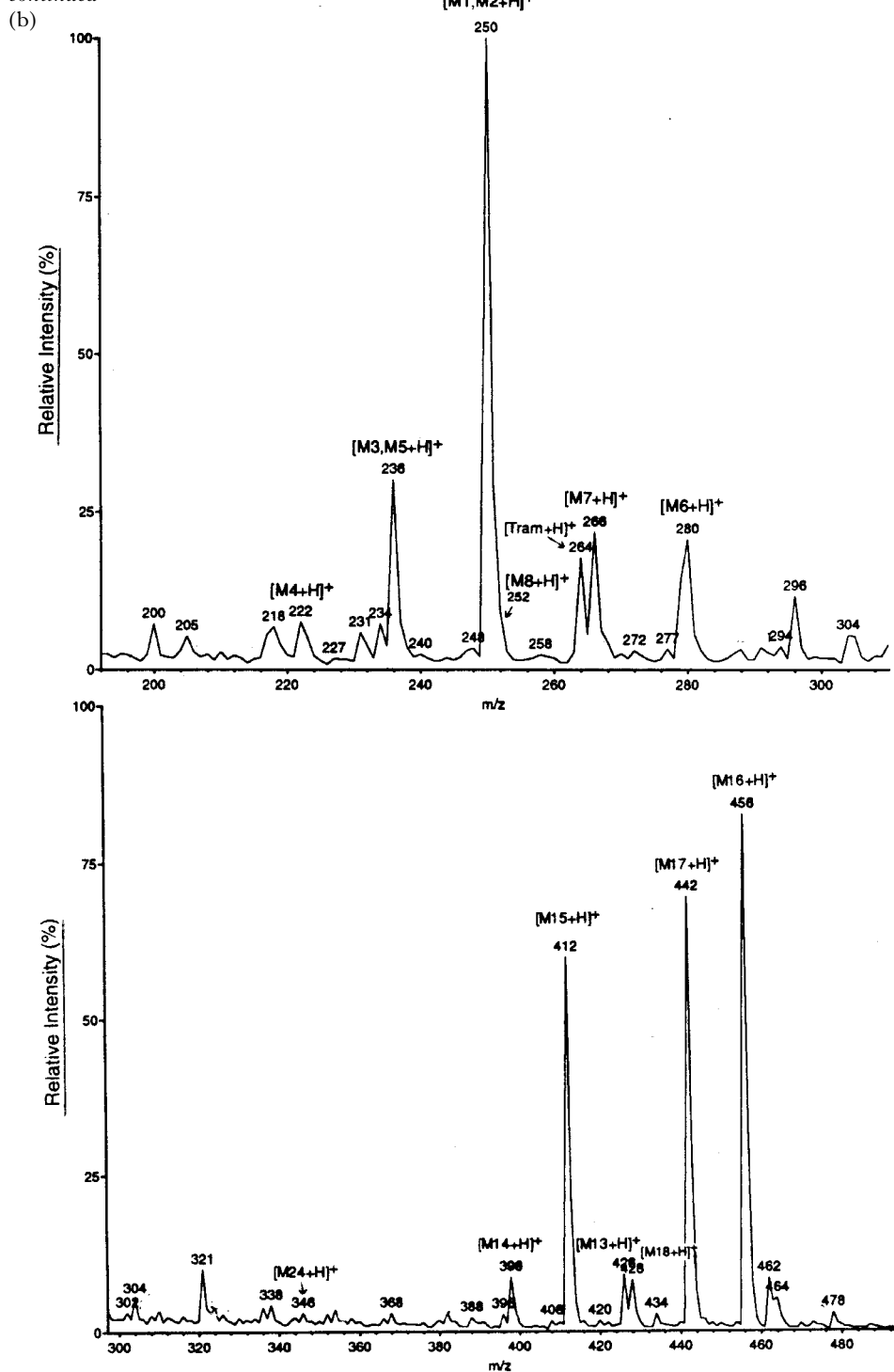


Figure 3. API-Q1 ms of rat and dog tramadol metabolities in untreated urine. (a) rat urine (0-24h); (b) dog urine (0-24h).

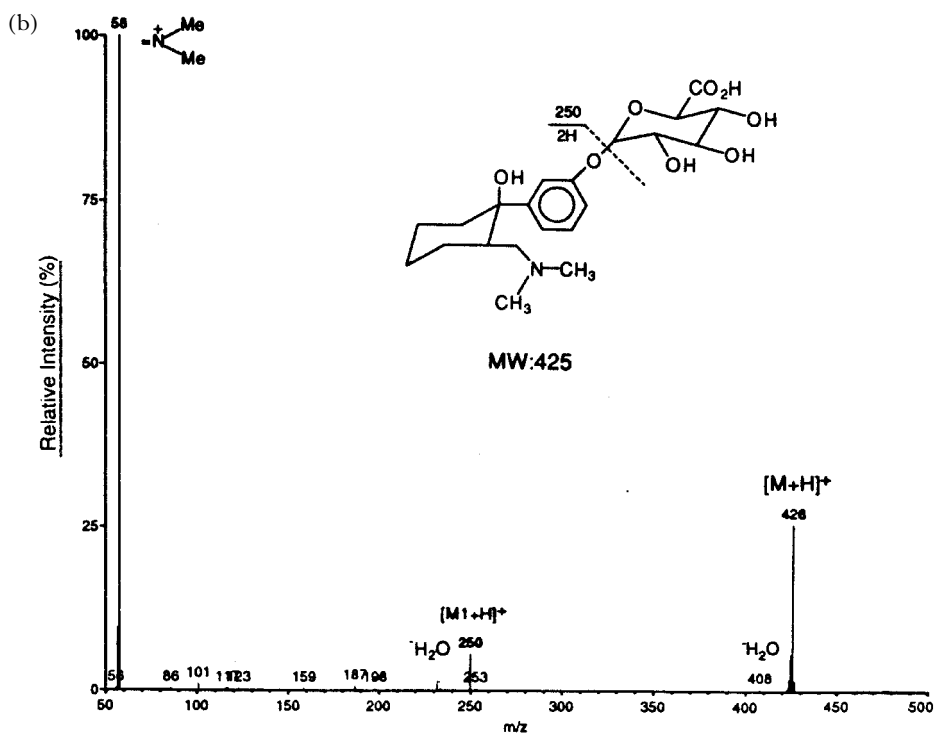
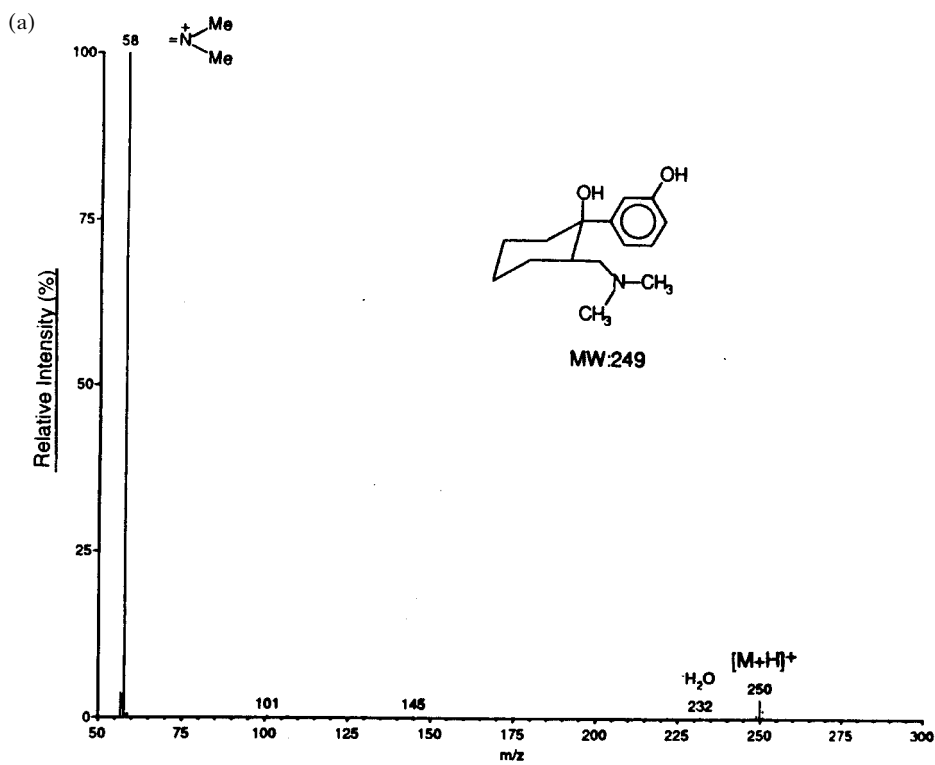


Figure 4.

continued

continued

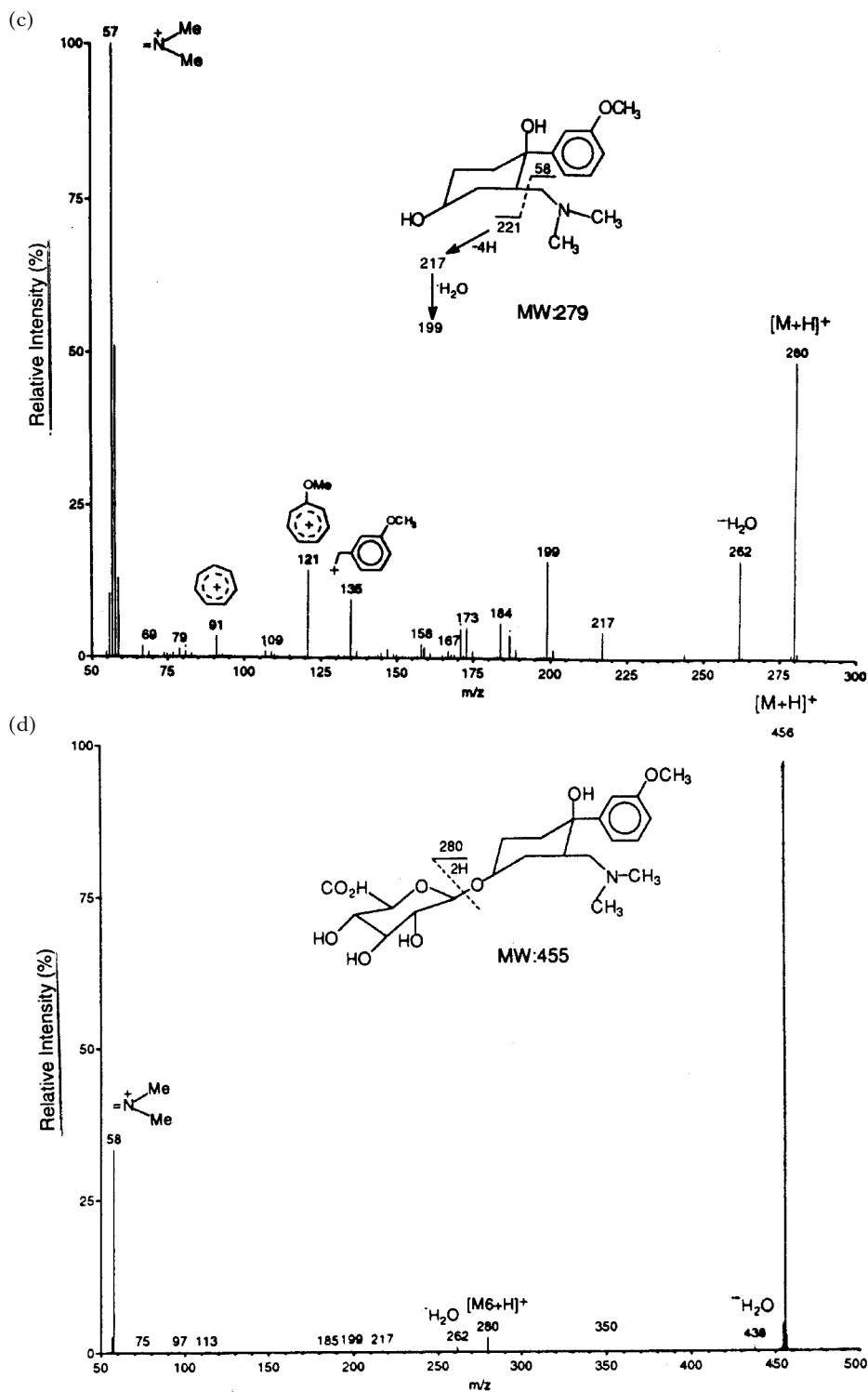


Figure 4. MS/MS spectra of M1, M6 and its glucuronides. (a) M1; (b) M13; (c) M6 (d) M16.

121 and 58 (figure 1). On the basis of MS and NMR data, M7, M8 and M9 were tentatively identified as 4-hydroxy-cyclohexyl-*N*-desmethyl tramadol, 4-hydroxy-cyclohexyl-*N,N*-didesmethyl tramadol, and 4-oxo-cyclohexyl tramadol, respectively. M6 and M7 are present in major quantities in dog, but as minor metabolites in rat and rat hepatic S9 fraction (table 1). M8 and M9 are minor metabolites in both species. M10 and M11 were present in minor quantities (table 1), and not previously identified. MS/MS analysis of protonated M10, m/z 246, displayed important daughter ions at m/z 201, 187, 145, 135, 121, 107, 72 and 58 for the structural assignment of M10, dehydro-tramadol (figure 1). MS/MS analysis of m/z 235 (protonated M11) revealed daughter ions at m/z 217, 189, 135, 121 and 107 (figure 1). M11 was tentatively identified to be an aldehyde, an oxidative *N*-dealkylation product of tramadol.

M13–18

M13–18 were identified as new metabolites. M13 and M15–17 were present in major amounts in both species, and M13–15 were previously reported as M1, M4 and M5 conjugates (Lintz *et al.* 1981) (table 1). M14 and M18 were identified as minor metabolites (table 1). Q1 scan MS analysis of M13–18 provided apparent protonated molecular ions of glucuronides at m/z 426, 398, 412, 456, 442 and 428 for glucuronides of M1, and M4–8, respectively (figure 1). MS/MS analysis of each glucuronide exhibited protonated molecular ion and diagnostic aglycon ion as well as informative daughter ions (figure 1). These six glucuronides of M1, M4–8 were tentatively elucidated on the basis of MS and MS/MS data.

M24–32

M24 was present in minor quantities in dog (table 1). Q1 Scan MS analysis of it gave an apparent protonated molecular ion of sulphate conjugate at m/z 346 (figure 1). MS/MS analysis of m/z 346 indicated a protonated aglycon ion at m/z 266 for protonated M7, along with a protonated sulphate molecular ion (figure 1), which tentatively characterized this metabolite to be a sulphate conjugate of M7. M25–32 were also present in minor quantities in both species (table 1). Ionspray-MS/MS analysis of m/z 292, a protonated molecular ion of M25, revealed informative daughter ions at m/z 274, 231, 201, 135, 86 and 60 (figure 1), which tentatively characterized M25 to be acetyl M2. MS/MS analysis of m/z 218, a protonated molecular ion of M26, provided diagnostic daughter ions at m/z 189, 135 and 121, with m/z 218 (figure 1), which tentatively assigned M26 to be dehydro-M3. MS/MS analysis of m/z 217 (protonated M27) showed daughter ions at m/z 199, 184, 135, 121, 107 and 69 (figure 1), which tentatively postulated M27 to be dehydro-M11. MS/MS analysis of m/z 223, a protonated molecular ion of M28, displayed daughter ions, m/z 205, 192, 177 and 149, accompanied with m/z 233 (figure 1), which tentatively identified M28 to be an alcoholic metabolite. MS/MS analysis of m/z 251, protonated M29, gave daughter ions at m/z 233 and 121, along with m/z 251. The structure of M29 was tentatively proposed to be a carboxylic acid. MS/MS analysis of the protonated M30, exhibited a protonated molecular ion at m/z 427, a diagnostic aglycon ion, m/z 251, as well as informative daughter ions at m/z 409 and 233 (figure 1). The structure of M30 was tentatively characterized to be a glucuronide of M29. M31 was not previously identified and was confirmed as a

The diagram illustrates the metabolic pathways of Tramadol. The central compound is Tramadol, which can be converted into various metabolites (M1 through M32) through different metabolic routes. The metabolites are shown with their chemical structures and their respective elimination half-lives ($t_{1/2}$) in parentheses.

- Tramadol** (central compound)
- M10** (N-methyl-10-hydroxy- Δ^8 -THC derivative, $t_{1/2}$ (g))
- M11** (N-methyl-11-hydroxy- Δ^8 -THC derivative, $t_{1/2}$ (g))
- M9** (N-methyl-9-hydroxy- Δ^8 -THC derivative, $t_{1/2}$ (g))
- M6** (N-methyl-6-hydroxy- Δ^8 -THC derivative, $t_{1/2}$ (g))
- M31** (N-methyl-31-hydroxy- Δ^8 -THC derivative, $t_{1/2}$ (g))
- M7** (N-methyl-7-hydroxy- Δ^8 -THC derivative, $t_{1/2}$ (s))
- M25** (N-methyl-25-hydroxy- Δ^8 -THC derivative, $t_{1/2}$ (g))
- M8** (N-methyl-8-hydroxy- Δ^8 -THC derivative, $t_{1/2}$ (g))
- M3** (N-methyl-3-hydroxy- Δ^8 -THC derivative, $t_{1/2}$ (g))
- M26** (N-methyl-26-hydroxy- Δ^8 -THC derivative, $t_{1/2}$ (g))
- M4** (N-methyl-4-hydroxy- Δ^8 -THC derivative, $t_{1/2}$ (g))
- M5** (N-methyl-5-hydroxy- Δ^8 -THC derivative, $t_{1/2}$ (g))
- M1** (N-methyl-1-hydroxy- Δ^8 -THC derivative, $t_{1/2}$ (g))
- M13** (N-methyl-13-hydroxy- Δ^8 -THC derivative, $t_{1/2}$ (g))
- M15** (N-methyl-15-hydroxy- Δ^8 -THC derivative, $t_{1/2}$ (g))
- M14** (N-methyl-14-hydroxy- Δ^8 -THC derivative, $t_{1/2}$ (g))
- M18** (N-methyl-18-hydroxy- Δ^8 -THC derivative, $t_{1/2}$ (g))
- M24** (N-methyl-24-hydroxy- Δ^8 -THC derivative, $t_{1/2}$ (s))
- M17** (N-methyl-17-hydroxy- Δ^8 -THC derivative, $t_{1/2}$ (g))
- M2** (N-methyl-2-hydroxy- Δ^8 -THC derivative, $t_{1/2}$ (g))
- M27** (N-methyl-27-hydroxy- Δ^8 -THC derivative, $t_{1/2}$ (g))
- M28** (N-methyl-28-hydroxy- Δ^8 -THC derivative, $t_{1/2}$ (g))
- M29** (N-methyl-29-hydroxy- Δ^8 -THC derivative, $t_{1/2}$ (g))
- M32** (N-methyl-32-hydroxy- Δ^8 -THC derivative, $t_{1/2}$ (g))

Figure 5. Proposed metabolic pathways for tramadol in rat and dog. g, Forms glucuronide metabolite; s, forms sulphate metabolite. * ^{14}C label.

58 in MS/MS spectrum, which tentatively assigned M32 to be hydroxy-cyclohexyl-M1 (figure 1).

Discussion

Unchanged tramadol and a total of 26 metabolites consisting of 18 Phase I metabolites (M1–11, M25–29, M31, M32) and eight conjugates (seven glucuronides (M13–18, M30), one sulphate (M24)) were characterized and tentatively identified by means of CI-MS, EI-MS and ionspray-MS and MS/MS, and NMR data in rat and dog urine and rat hepatic S9 fraction. API-ionspray MS and MS/MS revealed apparent protonated molecular ions, and prominent, as well as diagnostic, fragment daughter ions for the structural elucidation of tramadol and its metabolites. Six proposed pathways can explain the formation of these metabolites: *O*-demethylation, *N*-demethylation, cyclohexyl oxidation, oxidative *N*-dealkylation, dehydration and conjugation. The first three and last steps appear to be the major pathways forming seven *O*-desmethyl/*N*-desmethyl metabolites and hydroxycyclohexyl metabolites of tramadol, M1–5 in both species, and M6 and M7 in dog as major Phase I metabolites (table 1). M1 is the only metabolite that has shown to have more analgesic activity than tramadol (Flick *et al.* 1978, Frankus *et al.* 1978, Raffa *et al.* 1992, 1993). The cyclohexyl oxidation of tramadol, forming M6–9 and their subsequent glucuronides, is a newly identified pathway that is only significant in the dog. The first three steps in conjunction with the last step produced four major glucuronides, M13 (M1 glucuronide, rat), M15 (M5 glucuronide, both species), M16 (M6 glucuronide, dog), and M17 (M7 glucuronide, dog), three minor glucuronides, and one sulphate (table 1). The remaining pathways in conjunction with conjugation formed 9 minor metabolites. The proposed metabolic pathways for tramadol in rat and dog are depicted in figure 5. M12 and M19–23 have been found in human urine (Wu *et al.* 1997c).

In summary, tramadol was rapidly and extensively metabolized after oral administration, and renal excretion was the major route of elimination for tramadol and its metabolites. Unchanged tramadol (2% of the dose in both species) and 24 metabolites accounted for > 52% of the radioactive dose were characterized and identified in each rat and dog urine. In the rat hepatic S9 incubations, unchanged tramadol (30% of the sample) plus nine *in vitro* metabolites accounted for > 95% of the sample were tentatively identified.

Acknowledgements

The authors thank Dr L. E. Weaner for purifying ¹⁴C-tramadol, Dr K. L. Sorgi and Miss L. Scott for synthesizing the tramadol metabolites, and Dr R. J. Stubbs for reviewing the manuscript.

References

- ABEL, S. R., 1995, Tramadol: an alternative analgesic to traditional opioids and NSAIDs. *Journal Pharmaceutical Care in Pain and Symptom Control*, **3**, 5–29.
- DAYER, P., DESMEULES, J. and COLLART, L., 1997, Pharmacologie du tramadol. *Drugs*, **53** (suppl. 2), 18–24.
- FLICK, K., FRANKUS, E. and FRIDERICH, E. 1978, Studies on chemical structure and analgesic activity of phenyl substituted aminomethylcyclohexanols. *Arzneim-Forsch/Drug Research*, **28**, 107–113.

- FLOHE', L., AREND, I., COGAL, A., RICHTER, W. and SIMON, W., 1978, Klinische Prüfung der Abhängigkeitsentwicklung nach Langzeitapplikation von Tramadol. *Arzneim-Forsch/Drug Research*, **28**, 213–217.
- FRANKUS, E., FRIDERICHs, E., KIM, S. M. and OSTERLOH, G., 1978, On separation of isomers, structural elucidation and pharmacological characterization of 1-(*m*-methoxyphenyl)-2-(dimethylamino-methyl)-cyclohexan-1-ol. *Arzneim-Forsch/Drug Research*, **28**, 114–121.
- FRIDERICHs, E., FELGENHAUER, F., JONSCHAAP, P. and OSTERLOH, G., 1978, Pharmakologische Untersuchungen zur Analgesie, Abhängigkeits- und Toleranzentwicklung von Tramadol, einem stark wirkenden Analgetikum. *Arzneim-Forsch/Drug Research*, **28**, 122–134.
- HENNIES, H. H., FRIDERICHs, E. and SCHNEIDER, J., 1988, Receptor binding analgesic and antitussive potency of tramadol and other selected opioids. *Arzneim-Forsch/Drug Research*, **38**, 877–880.
- HILLS, J. F., LIAO, S. and NAYAK, R. K., 1992, The effect of food on the bioavailability of tramadol. In *1992 AAPS Eastern Regional Meeting*, abst., 32.
- LEE, R. C., MCTAVISH, D. and SORKIN, E. M., 1993, Tramadol, a preliminary review of its pharmacodynamic and pharmacokinetic properties, and therapeutic potential in acute and chronic pain states. *Drugs*, **46**, 313–340.
- LINTZ, W. and BESKER, R., 1986, Determination of tramadol in human serum by capillary gas chromatography with nitrogen-selective detection. *Journal of Chromatography*, **377**, 213–220.
- LINTZ, W., ERLACIN, S., FRANKUS, E. and URAGG, H., 1981, Metabolismus von Tramadol bei Mensch und Tier. *Arzneim-Forsch/Drug Research*, **31**, 1932–1943.
- PAAR, W. D., FRANKUS, P. and DENGLE, H. J., 1992, The metabolism of tramadol by human liver microsomes. *Clinical Investigation*, **70**, 708–710.
- PAAR, W. D., POCHE, S., GERLOFF, J. and DENGLE, H. J., 1993, *In vivo* investigation of the metabolism of tramadol – further evidence for *O*-demethylation polymorphism. *Naunyn-Schmiedeberg's Archives of Pharmacology*, **347** (suppl. R39), 154.
- RAFFA, R. B., FRIDERICHs, E., REIMANN, W., SHANK, R. P., CODD, E. E. and VAUGHT, J. L., 1992, Opioid and nonopioid components independently contribute to the mechanism of action of tramadol, an 'atypical' opioid analgesic. *Journal of Pharmacology and Experimental Therapeutics*, **260**, 275–285.
- RAFFA, R. B., FRIDERICHs, E., REIMANN, W., SHANK, R. P., CODD, E. E., VAUGHT, J. L., JACOBY, H. I. and SELVE, N., 1993, Complementary and synergistic antinociceptive interaction between the enantiomers of tramadol. *Journal of Pharmacology and Experimental Therapeutics*, **267**, 331–340.
- RAFFA, R. B., NAYAK, R. K. and LIAO, S., 1995, The mechanism(s) of action and pharmacokinetics of tramadol. *Review of Contemporary Pharmacotherapeutics*, **6**, 485–497.
- WU, W. N., DESAI, D., MCKOWN, L. A., GAUTHIER, A. D., JONES, W. J., TAKACS, A. R., SCOTT, L., SORGI, K. L. and RAFFA, R. B., 1997a, Metabolism of Ultram[®] (tramadol) in the dog. In ISSX Proceedings of the 6th European ISSX Meeting, **11**, abst. 160.
- WU, W. N., LIAO, S., MCKOWN, L. A. and TAKACS, A. R., 1997c, Metabolism of Ultram[®] (tramadol) in the human. ISSX Proceedings of the 6th European ISSX Meeting, **11**, abst. 159.
- WU, W. N., MCKOWN, L. A., JONES, W. J., GAUTHIER, A. D. and TAKACS, A. R., 1997b, Metabolism of Ultram[®] (tramadol) in the rat. In ISSX Proceedings of the 8th North American ISSX Meeting, **12**, abst. 107.



Metabolism of [^{14}C]1,2,7,8-tetrachlorodibenzo-*p*-dioxin in a ruminating Holstein calf

H. HAKK*, G. L. LARSEN and V. J. FEIL

US Department of Agriculture, Agricultural Research Service, Biosciences Research Laboratory, PO Box 5674 State University Station, Fargo, ND 58105 USA

Received 8 January 2001

1. [UL-7,8-ring ^{14}C]-1,2,7,8-tetrachlorodibenzo-*p*-dioxin (1278-TCDD) was administered orally to a ruminating Holstein bull calf (43.6 kg; 1.2 mg kg $^{-1}$ body weight). Urine and faeces were collected daily for 96 h, while blood was sampled at multiple time points. Tissues were removed for combustion analysis.

2. Each tissue contained < 0.6% of the dose at 96 h. Tissues with highest levels of 1278-TCDD, as a percentage of administered dose, were the large and small intestine, rumen, liver and carcass.

3. Urinary excretion accounted for 10.6% of the dose, and faecal excretion accounted for 81.6% of the administered dose. The major urinary and faecal metabolites were isolated and characterized by mass spectrometry and ^1H -NMR.

4. Plasma levels of ^{14}C peaked at 24 h, and decreased to near background at 96 h. Detectable plasma levels of 1278-TCDD were observed by 2 h.

5. A hydroxylated metabolite of 1278-TCDD was detected in calf plasma, which has the potential to interfere with thyroid hormone homeostasis.

Introduction

Polychlorinated dibenzo-*p*-dioxins (dioxins) are a family of halogenated aromatic hydrocarbons unintentionally produced by numerous industrial activities. The most toxic dioxin congeners are laterally chlorinated at carbons 2, 3, 7 and 8, and toxic effects of dioxins are observed in laboratory animals at levels of ng kg $^{-1}$ body weight. Some metabolism studies have been reported on the most toxic congener, i.e. 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (2378-TCDD), but very low levels of metabolites were produced making characterization difficult (Poiger *et al.* 1982, Poiger and Buser 1984). In addition, all metabolism studies have been performed in small, non-agriculturally relevant species, i.e. rat, mouse, guinea pig and hamster (Allen *et al.* 1975, Gasiewicz *et al.* 1983, Olson 1986, and Olson *et al.* 1980).

The goal of the present research was to administer a relatively high dose of a non-toxic dioxin congener in order to obtain a large mass from which to isolate metabolites. This would allow the characterization of intact, conjugated metabolites by ^1H -NMR and negative-ion fast atom bombardment mass spectrometry. An additional goal was to obtain dioxin metabolism data on an agriculturally relevant species, and to compare these results with previous reports in rodents.

* Author for correspondence; e-mail: hakkh@fargo.ars.usda.gov

Materials and methods

Chemicals

[UL-7,8-ring ^{14}C]1278-TCDD was purchased from ChemSyn Science Laboratories (Lenexa, KS, USA; > 98% purity). Radiochemical purity was determined by silica TLC (50:50 hexane:methylene chloride) and HPLC (described below). Unlabelled 1278-TCDD was synthesized in-house by accepted methods (> 90% purity by GC; Gray *et al.* 1976), and was used to dilute the radiolabel until the desired dose and specific activity was achieved (1.2 mg kg^{-1} body weight; $7.3\text{ }\mu\text{Ci }\mu\text{mol}^{-1}$, respectively). Hydroxylated TCDDs were synthesized in-house by standard methods (> 90% by GC and ^1H -NMR; Singh and Kumar 1993).

Animal treatment

A 43.6 kg Holstein bull calf received a hay- and grain-supplemented diet for 4 weeks prior to the experiment, and was no longer on milk replacer to assure rumination. The animal was dosed orally with [^{14}C]1278-TCDD (1.2 mCi) in a gelatin capsule containing peanut oil. The calf was held in an elevated stall from the time of dosing to sacrifice (96 h). A urinal was attached to allow the complete separation of urine and faeces. Sample collections of excreta were made every 24 h. Blood samples (15 ml) were collected before dosing and at 1, 2, 4, 8, 24, 32, 48, 56 and 96 h via the jugular vein. The calf was euthanized with 10 ml Sleepaway[®] at 96 h, and the selected tissues were removed and stored at -40°C .

Chromatography

Urine samples were purified on a $2.4 \times 30\text{ cm}$ Porapak-Q column (Waters Associates, Milford, MA, USA) eluted with water and methanol (0–24 h; figure 1). The water Porapak-Q fraction was applied to a $2.4 \times 100\text{ cm}$ Sephadex LH-20 column eluted with water. The LH-20 fractions and methanol Porapak-Q fractions were applied to reversed-phase C18 SepPak solid-phase extraction cartridges (Waters Associates), and eluted with water followed by methanol. SepPak fractions were further fractionated by C18 reversed-phase HPLC (described below).

Lyophilized faecal samples were extracted successively with hexane, ethyl acetate and methanol (0–24 h; figure 2). The extracts were applied to silica gel TLC plates ($5 \times 20\text{ cm}$; $250\text{ }\mu\text{m}$; Analtech, Inc., Newark, DE, USA), and developed in 50:50 hexane:methylene chloride. Radioactivity was detected with a System 200 Imaging Scanner (Bioscan, Inc., Washington, DC, USA), and bands were scraped and eluted with ethyl acetate. The ^{14}C was then applied to C18 SepPak cartridges and eluted successively with hexane, toluene, methylene chloride, acetone and methanol. SepPak fractions were further fractionated by reversed-phase HPLC.

Instrumentation

The ^{14}C content of urine and faecal extracts were quantified by liquid scintillation counting using Ecolite (ICN, Costa Mesa, CA, USA) and a Packard 1900 CA liquid scintillation counter (LSC; Meridan, CT, USA). Radioactivity in faeces and tissues was quantified by combustion of lyophilized aliquots (0.1–0.5 g) using a Packard Model 307 sample oxidizer.

High-performance liquid chromatography analyses (HPLC) were performed on a Gilson System 45NC Gradient Analytical HPLC equipped with a Model 715 system controller software (Gilson Medical Electronics, Middleton, WI, USA). Fractionations were accomplished in reversed-phase mode using a C-18 DeltaPak column ($8 \times 100\text{ mm}$; Waters Associates) with a flow rate of 1.0 ml min^{-1} . A step gradient from 5% acetonitrile in water to 100% acetonitrile over 100 min was utilized. Detection of analytes was made with a variable wavelength UV detector set at 254 nm connected in series with a Packard Flow-One liquid scintillation detector.

Purified material (3–10 μg) from urine or faecal extracts were analysed by ^1H -NMR in CD_3OD or CD_3COCD_3 inside an 1.8-mm capillary tube (30 μl). Spectra were obtained with a Bruker AM400 spectrometer (Billerica, MA, USA) operating at 400 MHz, utilizing tetramethylsilane as the reference (80.00 ppm).

Negative-ion fast atom bombardment mass spectra (–FAB/MS) of urine conjugates were obtained on a Micromass VG AutoSpec mass spectrometer (Beverly, MA, USA) equipped with a caesium gun. The samples were dissolved in a glycerol matrix containing 2.6% Proton-Sponge[®]. The electron-impact mass spectra were obtained on a VG AutoSpec spectrometer equipped with a Hewlett-Packard 5990 gas chromatograph (Wilmington, DE, USA) following derivatization with Regisil[®] (Regis Technologies, Inc., Morton Grove, IL, USA) or diazomethane.

Plasma analysis

Whole blood ($\sim 15\text{ ml}$) was obtained in heparinized syringes at 0, 1, 2, 4, 8, 24, 28, 32, 48, 56 and 96 h. Plasma was obtained by centrifugation at 2900g for 20 min at 4°C . ^{14}C levels in the plasma samples were determined by LSC. Plasma samples were fractionated by Sephadex G-75 and Sephacryl S-200

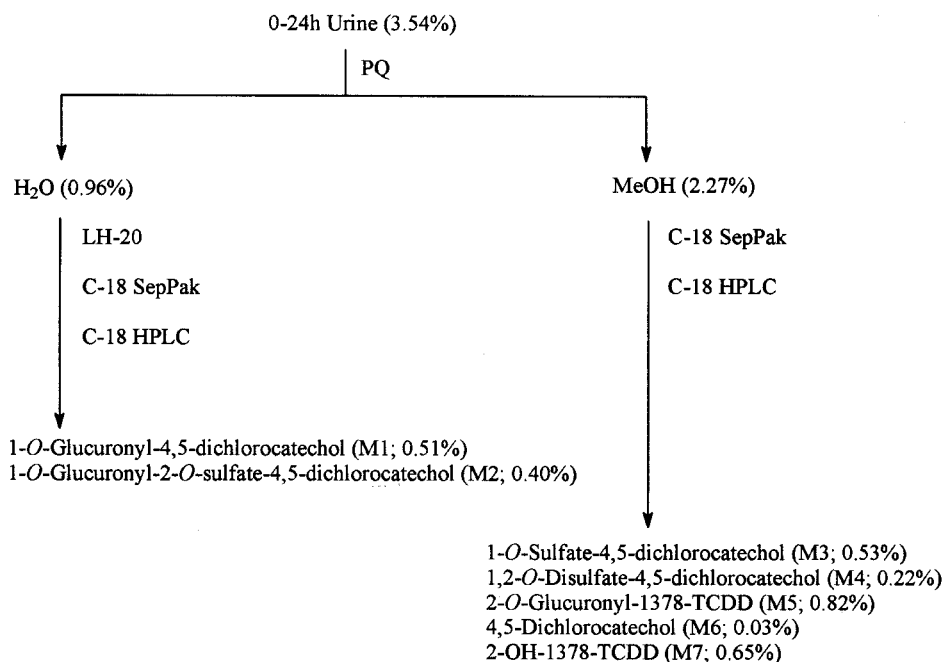


Figure 1. General fractionation method of 0–24-h urine metabolites from a Holstein bull calf orally dosed with ^{14}C -1278-tetrachlorodibenzo-*p*-dioxin in peanut oil administered in a gelatin capsule. Values in parentheses are the per-cent of dose.

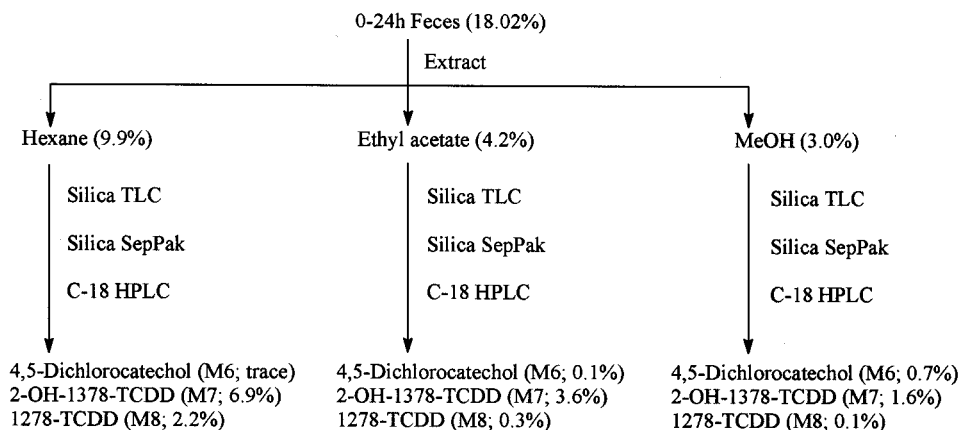


Figure 2. General fractionation method of 0–24-h faeces metabolites from a Holstein bull calf orally dosed with ^{14}C -1278-tetrachlorodibenzo-*p*-dioxin in peanut oil administered in a gelatin capsule. Values in parentheses are the per-cent of dose.

gel filtration (Pharmacia Biotech, Piscataway, NJ, USA), as described (Larsen *et al.* 1990). Isolation and quantification of parent 1278-TCDD and hydroxylated metabolites were performed according to Bergman *et al.* (1994). Briefly, the plasma was diluted with 1 vol. each of water and methanol. Following acidification with 0.5 M H_2SO_4 , the mixture was extracted three times with 1:1 hexane:methyl *t*-butyl ether. The extract was blown to dryness under a stream of nitrogen and redissolved in hexane. Phenolic compounds were partitioned into 1 M KOH in 50% ethanol. The alkaline phase was re-acidified and extracted with hexane:methyl *t*-butyl ether. The organic layer residue was derivatized with diazomethane, purified on neutral alumina and analysed by GC/MS.

Total plasma thyroxine (T4) levels were determined by the use of a AxSYM® Total T4 Fluorescence Polarization Immunoassay (Abbott Laboratories, Abbott Park, IL, USA). The reported values include both the protein-bound and -unbound forms of T4.

Results

Table 1 summarizes the excretion and disposition of [¹⁴C]1278-TCDD orally administered to a bull calf. The majority of the radioactivity (81.6%) was recovered in the faeces, while cumulative urine excretion of ¹⁴C was > 10%. Faecal excretion of 1278-TCDD derived [¹⁴C] peaked at 24–48 h (51.7% of the dose), and levels dropped rapidly at subsequent time points. In the urine, radioactivity was detected in the earliest collection (0–8 h), and peaked at 8–24 h, i.e. 3.3% of dose.

Individual tissues contained < 1% of the administered dose. Calf tissues with ¹⁴C levels > 0.1% of the dose were the small and large intestines, stomachs, liver and carcass (table 1). All other sampled tissues contained well below 0.1% of the administered dose. When the tissue data are expressed on a concentration basis, i.e. ng 1278-TCDD equivalentsg⁻¹ wet tissue (ppb), the large intestine, liver, kidney and small intestine contained the highest concentrations of ¹⁴C, ranging

Table 1. ¹⁴C recovery of [¹⁴C]1,2,7,8-tetrachlorodibenzo-*p*-dioxin in a male Holstein calf following an oral administration of 1.2 mg/kg⁻¹ body weight in a gelatin capsule.

Excreta/tissue	Per-cent of dose	
Urine		
0–8 h	0.3	
8–24 h	3.3	
24–32 h	3.1	
32–48 h	2.0	
48–56 h	0.8	
56–72 h	0.9	
72–80 h	0.1	
80–96 h	0.1	
Subtotal	10.6	
Faeces		
0–24 h	18.0	
24–48 h	51.7	
48–72 h	8.6	
72–96 h	3.3	
Subtotal	81.6	
		Concentration (ppb)
Blood	0.0002	See figure 3
Carcass	0.3	4.3
Fat (perirenal)	–	21.8
Heart	0.003	4.7
Small intestine ^a	0.1	39.5
Large intestine ^a	0.5	222.2
Kidney	0.02	45.3
Liver	0.2	100.2
Lung	0.02	18.8
Muscle (l. dorsi)	–	0.9
Stomachs ^b	0.1	16.3
Subtotal	1.2	
Total recovered	93.4	

^a Includes contents.
^b Rumen, reticulum, omasum and abomasum.

from 40 to 222 ppb (table 1). Perirenal fat, lung and stomachs contained intermediate levels of ^{14}C , whereas the heart, muscle and carcass were exceedingly low in ^{14}C (< 5 ppb). The liver/fat ratio, an indicator of specificity for hepatic binding, was 4.6.

Since the metabolite profiles reported below for 0–24-h urine and faeces were similar to other time collection points, with only the relative amounts of individual components varying, only the fractionation data for the 0–24-h urine and faeces samples were presented. A fractionation scheme was developed to isolate and characterize urine metabolites (figure 1). Urine was pooled daily, applied to a Porapak-Q column (PQ), and eluted with water (PQW) and methanol (PQM). Two metabolites were characterized in the PQW fraction of collected urine. M1 was characterized as 1-*O*-glucuronyl-4,5-dichlorocatechol (0.51% of dose; table 2) by its $-\text{FAB}/\text{MS}$ spectrum, which contained characteristic peaks at m/z 353 (M-H; 2 Cl), and a glucuronide loss at m/z 177 (2 Cl). The ^1H -NMR spectrum of M1 displayed two singlets at 7.28 and 6.97 ppm compatible with an unsymmetrical catechol. M1 was hydrolyzed with β -glucuronidase (*E. coli* recombinant, pH 6.8, no sulphatase activity; Sigma, St Louis, MO, USA), derivatized to the trimethylsilyl ether with Regisil[®] and analysed by GC/MS. The mass spectrum characterized the product as the di-OTMS derivative of 4,5-dichlorocatechol [$\text{M} + 322$ (2 Cl), M-15 (307); M6, table 2].

M2 from the 0–24-h urine PQW fraction (0.40% of the dose) was characterized as the glucuronide ether/sulphate ester diconjugate of 4,5-dichlorocatechol. The ^1H -NMR spectrum of M2 displayed two non-equivalent singlets at 7.56 and 7.37 ppm. These NMR resonances were identical to a glucuronide ether/sulphate ester diconjugate of 4,5-dichlorocatechol found in the urine of male Sprague–Dawley rat after receiving an oral dose of [^{14}C]1278-TCDD (Hakk *et al.* 2001). The $-\text{FAB}/\text{MS}$ was also compatible with this assignment, and contained a two chlorine cluster at m/z 455 representing an M-H sodium adduct, a fragment ion at m/z 375 compatible with a sodium adduct of a sulphate loss, and an ion at m/z 177 (2 Cl) representing a loss of both the glucuronic acid and sulphate moieties. The amount of these highly polar PQW metabolites steadily increased as a percentage of daily urine elimination (27.2% of the 0–24-h urine, to 54.0% of the 72–96-h urine).

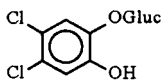
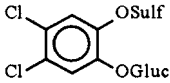
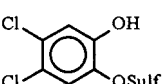
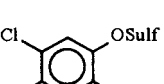
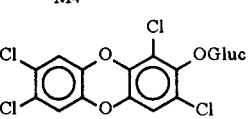
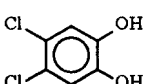
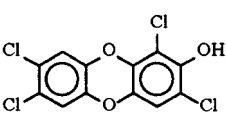
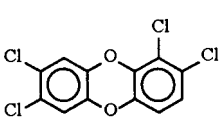
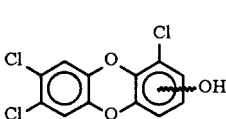
In the 0–24-h urine PQM fractions, three additional metabolites were isolated and characterized. The $-\text{FAB}/\text{MS}$ spectrum of M3 (0.53% of dose) was compatible with an assignment of 1-*O*-sulphate-4,5-dichlorocatechol. A two-chlorine cluster representing a proton loss was evident at m/z 257, and a sulphate loss occurred at m/z 177 (table 2).

M4 (0.22% of dose) was characterized as 1,2-*O*-disulphate-4,5-dichlorocatechol by a characteristic potassium adduct of the parent ion at m/z 375 (M + K-2H, 2 Cl), a sodium adduct of a sulphate loss at m/z 279 (M + Na-2H-80), and loss of both sulphates at m/z 177 in its $-\text{FAB}$ mass spectrum (table 2). A ^1H -NMR spectrum was not obtained for M3 and M4 due to the lack of sample purity.

Finally, M5 (0.82% of dose) was characterized as the ether glucuronide conjugate of a hydroxy TCDD metabolite. The $-\text{FAB}$ mass spectrum displayed a loss of hydrogen at m/z 511 as a four chlorine isotope cluster, a loss of chlorine at m/z 477 (3 Cl) and a glucuronic acid loss at m/z 335.

A ^1H -NMR spectrum of M5 displayed three non-equivalent singlet resonances at 7.20, 7.12 and 6.98 ppm. These chemical shifts were identical with a glucuronide ether of NIH-shifted 2-hydroxy-1378-TCDD found in rat urine and bile

Table 2. Characterization of metabolites of 1278-TCDD in a Holstein bull calf by mass spectrometry and ¹H-NMR. The mass spectra were obtained in either electron impact (EI-GC/MS) mode as the trimethyl silyl ether, the methoxy derivative, or in negative-ion fast atom bombardment (– FAB/MS) mode as intact conjugates. The ¹H-NMR were obtained in CD₃OD, except where otherwise indicated.

Metabolite	Chemical shift (ppm)	Diagnostic MS ions (m/z)
 M1	7.28 (s), 6.97 (s)	M-H + Na ⁺ ,(375); M-H + K (391); M-H (353); M-177 (177; 2 Cl)
 M2	7.56 (s), 7.37 (s)	M-H + Na (455; 2 Cl); M-81 + Na (375; 2 Cl); M-177 (257; 2 Cl); M-80-177 (177; 2 Cl)
 M3	N/A	M-H (257; 2 Cl); M-H-80 (177; 2 Cl)
 M4	N/A	M + K-2H (375; 2 Cl); M + Na-2H-Sulf (279; 2 Cl); M-2(Sulf)-H (177)
 M5	7.20 (s), 7.12 (s), 6.98 (s)	M-H (511; 4 Cl); M-35 (477; 3 Cl); M-177 (335; 4 Cl)
 M6	N/A	M ⁺ 322 (2 Cl); M-15 (307; 2 Cl) as TMS
 M7	7.18 (s), 7.11 (s), 6.91 (s)	M ⁺ 408 (4 Cl); M-15 (393; 4 Cl); M-50 (358; 3 Cl) as TMS
 M8	7.34 (s), 7.26 (s), 7.25 (d), 7.00 (d) in CD ₃ COCD ₃	M ⁺ 320 (4 Cl); M-35 (285; 3 Cl); M-63 (257; 3 Cl)
 M9	N/A	M ⁺ 316 (3 Cl); M-15 (301; 3 Cl); M-43 (273) as OMe

N/A, not acquired.

following an oral dose of 1278-TCDD (Hakk *et al.* 2001). Minor amounts of free 4,5-dichlorocatechol (M6; 0.03% of the dose) and relatively high amounts of 2-hydroxy-1378-TCDD (M7; 0.65% of the dose) were also characterized in the PQM fractions as their TMS ethers, and may have been formed as artefacts from handling (table 2). No parent 1278-TCDD was detected in urine.

The fractionation of 0–24-h faeces yielded three compounds. The major metabolite (M7; 12.1% of dose; table 2) was identified as the NIH-shifted 2-hydroxy-1378-TCDD by comparison of the ^1H -NMR and mass spectral data with the synthesized standard (Petroske *et al.* 1997). The NMR spectrum contained three singlet resonances at 7.18, 7.11 and 6.91 ppm, indicative of a non-*ortho* arrangement for the three protons. GC/MS analysis of the trimethylsilyl ether derivative showed a molecular ion at m/z 408 (4 Cl), a methyl loss at m/z 393 and a chloromethyl rearrangement loss at m/z 358. A second metabolite in 0–24-h faeces (M6; 0.8% of dose; table 2) was characterized as the free 4,5-dichlorocatechol by its HPLC chromatographic retention time and electron-impact mass spectrum. The mass spectrum of the silylated derivative displayed a molecular ion at m/z 322 as a two-chlorine cluster, and also showed a characteristic methyl loss at m/z 307. Parent 1278-TCDD (M8) in 0–24-h faeces accounted for only 2.6% of the administered dose. This low level of parent indicated that 1278-TCDD was readily absorbed from the digestive system of the calf. The ^1H -NMR spectrum of M8 contained a pair of doublets at 7.00 and 7.25 ppm corresponding to the *ortho* protons on carbons 3 and 4, and singlets at 7.25 and 7.34 ppm for the C-6 and C-9 protons, respectively. The ^1H -NMR and mass spectra were identical with that of the starting material. A monohydroxy trichlorodibenzo-*p*-dioxin metabolite (M9) was detected in trace amounts in faeces collected at days 2 and 3 by its electron-impact mass spectrum (table 2), but a stereochemical assignment was not possible due to the limited amount and purity of the sample.

Radioactivity in calf plasma was detectable at 2 h, and peaked by 24 h at 240 ppb (figure 3). By 96 h, nearly background levels of ^{14}C in plasma were observed. Individual plasma samples were fractionated, which resulted in the isolation of a hydroxylated tetrachlorodibenzo-*p*-dioxin metabolite of [^{14}C]1278-TCDD. Most likely, this plasma metabolite was the NIH-shifted, 2-hydroxy-1378-TCDD, which was also observed in the faeces and urine. Support for this stereochemical conclusion came from the mass spectral peak intensities of the molecular ion and the M-15 ion following methylation. Methylated mass spectra of all the possible monohydroxy tetrachlorodibenzo-*p*-dioxin isomers have been published (Petroske *et al.* 1997), and the mass spectrum of synthesized 2-methoxy-1378-TCDD matched that of the unknown plasma metabolite in that the ion ratios of M + to M-15 to M-43 in both samples were $\sim 100:78:47$. The ion ratios of other possible metabolites, i.e. the non-NIH-shifted 3- and 4-OH-TCDD isomers, were 100:31:58 and 100:14:43, respectively. The majority of the total plasma radioactivity was composed of the hydroxy-TCDD, even at the early time points, i.e. 4 h. Only 0–5% of the plasma radioactivity was parent 1278-TCDD by extraction analysis. This indicated that metabolism of 1278-TCDD had commenced soon after absorption from the gastrointestinal tract.

Plasma T4 levels displayed a steady increase from a resting, predose level of 45 ng ml^{-1} to a high of 75 ng ml^{-1} by 56 h (figure 4). By 96 h, T4 levels had decreased to 55 ng ml^{-1} . Plasma spiked with synthesized 2-OH-1378-TCDD did not inter-

fere with the T4 assay in the range from 10 to 1000 nM (3.4–336 ng ml⁻¹; data not shown).

Essentially all of the plasma radioactivity was protein bound at each time point (figure 5). SDS-PAGE and Western immunoblot analysis identified the major ¹⁴C ligand-binding species as serum albumin (data not shown). Previous *in vivo* and *in vitro* studies had shown that hydroxylated polyhalogenated aromatic hydrocarbons can also bind with considerable affinity to transthyretin (TTR), a plasma transport protein for thyroid hormones (Lans *et al.* 1993). However, due to the limit of detection of our electrophoresis analyses, no TTR could be detected on any SDS-PAGE gels run with calf plasma (monomer MW 14 kDa).

Discussion

The data demonstrated that 1278-TCDD was readily excreted from a Holstein bull calf via the urine and faeces (table 1). Therefore, very little 1278-TCDD was retained in the tissues at 96 h. The tissues with the most ¹⁴C were the intestines, stomachs, carcass and liver; however, none exceeded 1% of the dose. Calf tissue ¹⁴C was not extracted and characterized in the present study, but previous results (Rose *et al.* 1976) showed that 2378-TCDD remained in tissues exclusively as parent. The observed tissue disposition and excretion patterns of 1278-TCDD in a ruminating animal resembled those observed in a non-ruminating species (Hakk

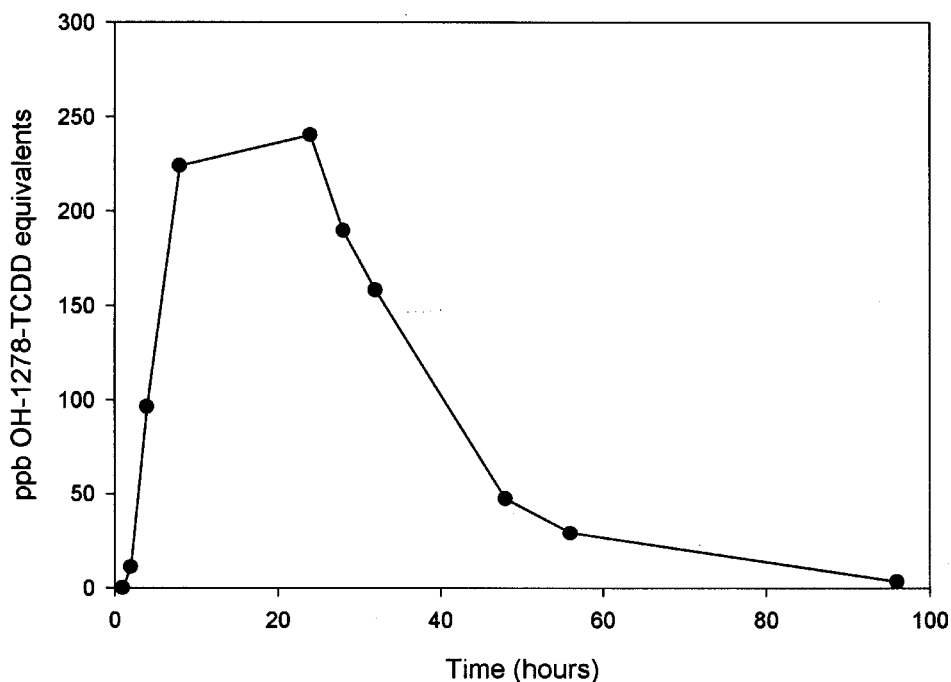


Figure 3. Recovery of ¹⁴C in calf plasma at various times following a single oral administration of [¹⁴C]1278-TCDD in a peanut oil gelatin capsule. Plasma samples (0.1 ml) were counted directly by LSC, then converted to parts per billion (ppb) hydroxy-1278-TCDD (MW 336), the major form of 1278-TCDD in plasma. Density of plasma was assumed to be 0.95. Data are the average of three determinations.

et al. 2001). The male Sprague-Dawley rat given a single oral dose of 1278-TCDD also eliminated the majority of the dose in the faeces by 72 h (80%), and significant amounts in the urine (14%). Total rat tissue disposition of ^{14}C 1278-TCDD was 2.6% at 72 h. Faecal and urinary elimination of ^{14}C 1278-TCDD peaked at 24–48 h in both the rat and calf, an indication that enterohepatic circulation occurred in both mammalian species.

The elimination and tissue disposition patterns were similar to calves given oral doses of other non-toxic dioxin congeners. Nearly 85% of a 2.5 mg kg^{-1} ^{14}C 1478-TCDD dose was excreted in the faeces of a ruminating calf, while only 1% was excreted via the urine (Huwe and Feil 1998). The major tissues for disposition of 1478-TCDD were the large intestine, stomachs and carcass, which accounted for 8% of the administered ^{14}C . It was also demonstrated that nearly 100% and 1% of a 2.1 mg kg^{-1} dose of ^{14}C 1378-TCDD was excreted via the faeces and urine, respectively, in a ruminating calf (Huwe and Feil 1999). The large intestine, stomachs and small intestine were the major sites of tissue disposition.

The liver/fat ratio for 1278-TCDD in calf was determined to be relatively high at 4.6. Values more than unity for the toxic dioxin congeners indicate a specificity for tissue binding, that has been shown to be due to a tight, but non-covalent, association with an inducible monooxygenase cytochrome, i.e. CYP1A2 (Diliberto *et al.* 1997). The present calf data indicated that a hepatic-binding species may also be present in calves to which the non-toxic 1278-TCDD and/or its metabolites can bind. However, due to the more rapid overall metabolism of 1278-TCDD

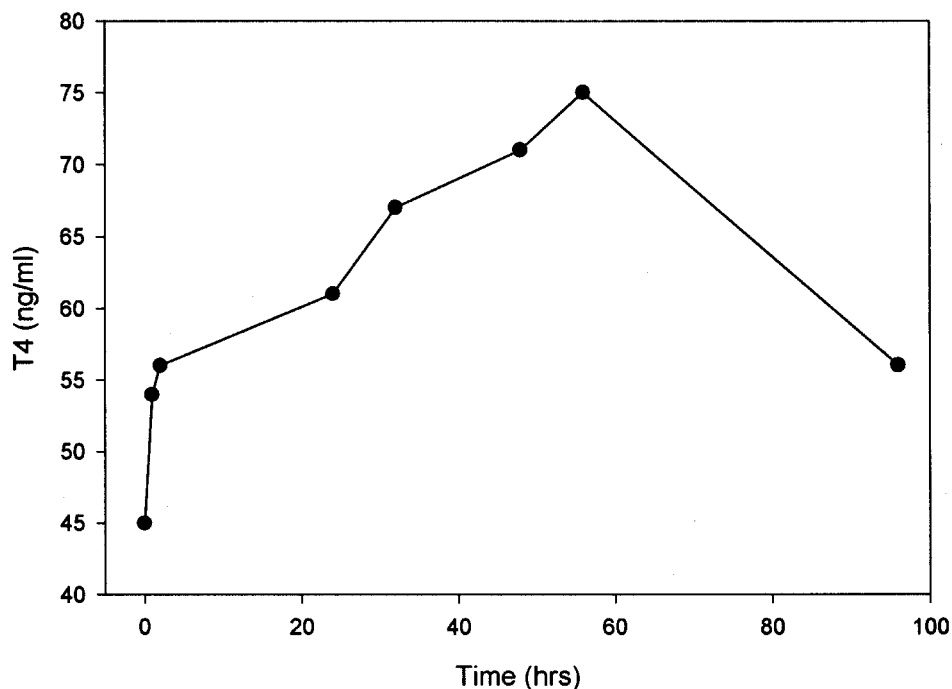


Figure 4. Calf plasma levels of thyroxine (T4) measured at various times following a single oral administration of ^{14}C 1278-TCDD. T4 measurements were obtained by fluorescence polarization immunoassay, and values represent the total of the protein bound and free forms. Data are from a single measurement at each time point of 0.1 ml calf plasma.

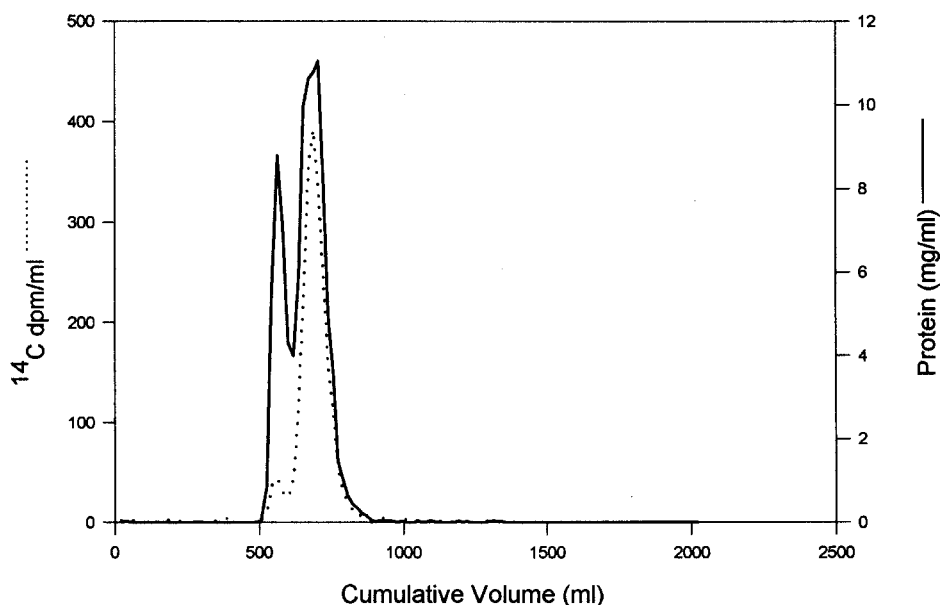


Figure 5. G-75 gel permeation chromatogram of 24–48-h calf plasma after receiving an oral dose of [^{14}C]1278-TCDD. The protein bound region extends from 500 to 800 ml, and the unbound region extends from 1300 to 2000 ml.

compared with 2378-TCDD, total calf liver disposition of 1278-TCDD remained low (0.2% of the administered dose).

In urine, multiple 4,5-dichlorocatechol glucuronide and sulphate conjugates were characterized by their $-\text{FAB/MS}$ or $^1\text{H-NMR}$ spectra (metabolites M1–4; table 2). The glucuronide of 2-OH-1378-TCDD (M5) was present in the urine as a major metabolite. No parent 1278-TCDD was detected in the urine. Therefore, it was concluded that biotransformation of 1278-TCDD played a major role in regulating the excretion of this compound in the urine. In the faeces, the major metabolite was the NIH-shifted 2-OH-1378-TCDD, whereas only minor amounts of 4,5-dichlorocatechol and parent 1278-TCDD were detected. Faecal 2-OH-1378-TCDD and 4,5-dichlorocatechol were probably the result of deconjugation of urinary metabolites, mentioned above, by bacteria located in the rumen of the calf.

The initial event in the metabolism of 1278-TCDD in calf was apparently oxidation at carbons 2 and 3 to form an arene oxide. Opening of the arene oxide with a NIH shift of the chlorine atom on carbon 2 to carbon 3 yielded 2-OH-1378-TCDD (figure 6). No direct hydroxylations of tetrachlorodibenzo-*p*-dioxin were detected by $^1\text{H-NMR}$, i.e. 3-, 4-, 6- or 9-OH-TCDD, all of which were available as synthesized standards. In the calf, the 2-OH-1378-TCDD was efficiently transferred to a microsomal β -glucuronosyl transferase enzyme for conjugation with glucuronic acid. However, the hydroxylated metabolite apparently was not available to cytosolic arylsulphotransferases, because no sulphate ester of 2-OH-1378-TCDD was detected.

Oxidation of an angular carbon of 1278-TCDD to an arene oxide could result in spontaneous or enzymatic cleavage of one of the ether linkages in the dibenzo-*p*-dioxin ring system to form a putative dihydroxy diphenyl ether, which could subsequently decompose to labelled and unlabeled dichlorocatechol (figure 6).

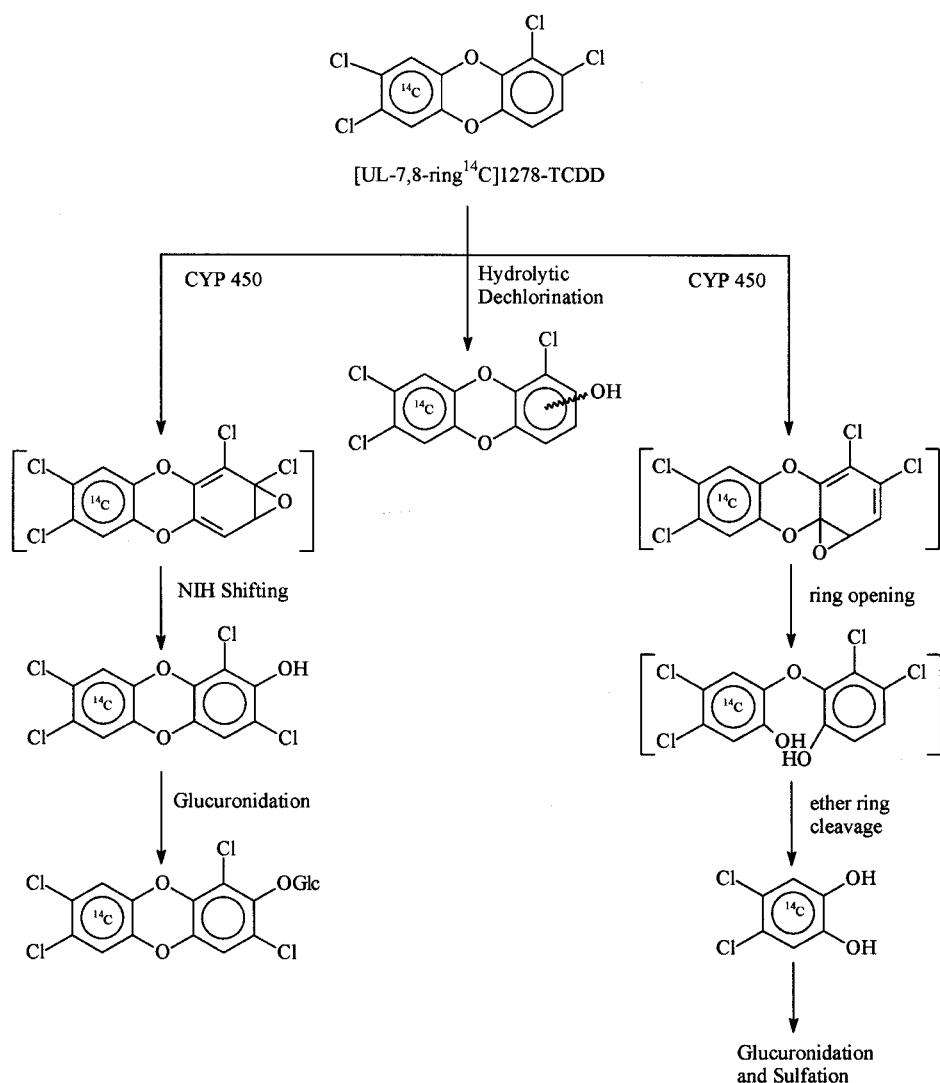


Figure 6. Proposed pathway for the metabolism of 1278-TCDD in a ruminating bull calf.

Hydroxylated diphenyl ethers are considered to be relatively stable species (Poiger *et al.* 1982, Neal *et al.* 1984, Wittich *et al.* 1992); however, no diphenyl ether could be detected in extracts of calf urine or faeces. Therefore, it was concluded that the putative diphenyl ether intermediate was efficiently transferred to a tightly coupled enzymatic activity that resulted in the cleavage of the second ether linkage. The 4,5- and 3,4-dichlorocatechol products were released to serve as substrates for Phase II conjugation. No data were obtained to suggest that a common metabolic pathway for aromatic hydrocarbons, i.e. the glutathione/mercapturic acid pathway, was utilized by the calf for metabolizing 1278-TCDD.

This proposed metabolic pathway is very similar to one proposed previously in the male Sprague-Dawley rat given a single oral dose of 1278-TCDD (Hakk *et al.* 2001). Similar results were obtained when comparing metabolism and disposition data in rat and calf for 1378- and 1478-TCDD (Petroske *et al.* 1997, Huwe *et al.*

1998, Huwe and Feil 1998, Huwe and Feil 1999). These results may suggest a common metabolic pathway utilized by most mammals when exposed to dioxin congeners, and may indicate the suitability of using the smaller laboratory rat to model what occurs in the field with larger production animals following exposure to this family of environmental pollutants.

Particularly interesting were the data that demonstrated a steady elevation in plasma T4 during the 4-day study (figure 4). The highest levels were observed at 56 h. Some reports have demonstrated an increase in total plasma thyroid stimulating hormone (TSH; the pituitary signalling hormone resulting in T4 release) and T4 levels in newborn babies exposed to relatively high levels of dioxins and furans through maternal body burdens (Pluim *et al.* 1992, Koopman-Esseboom *et al.* 1994). However, T4 levels have more often been shown to decrease following administration of dioxins, e.g. the toxic 2378-TCDD in mammals (Lans *et al.* 1990). This decrease in T4 levels primarily occurs by an Ah receptor (AhR)-mediated pathway that increases T4 glucuronidation in the liver, and its subsequent biliary elimination (Beetstra *et al.* 1991). Hydroxylated polyhalogenated aromatic hydrocarbons, which are structurally similar to T4 and T3, can cause a disruption of the thyroid hormone endocrine system by competing with the native hormones for sites on the plasma carrier proteins, i.e. transthyretin (TTR), albumin and thyroxine-binding globulin (TBG). The hydroxylated metabolite of 1278-TCDD identified in calf urine and faeces, and suspected to be the principal form of hydroxylated 1278-TCDD detected in the blood, i.e. 2-OH-1378-TCDD, binds to TTR *in vitro* with a 4.5-fold greater binding affinity than the native T4 (Lans *et al.* 1993). It can be hypothesized that an exchange of T4 for 2-OH-1378-TCDD occurred on the plasma carrier protein TTR. Lower bound plasma T4 levels may have triggered a feedback mechanism to the hypothalamus, which may have lead to a compensatory increase in T4 synthesis and release via the hormone cascade of thyrotropin-releasing hormone and TSH. T4 is the precursor to the more potent thyroid hormone T3, formed enzymatically by iodothyronine de-iodinase. Increasing levels of the precursor T4 may affect the ability of the organism to maintain the proper thyroid hormone homeostasis. Therefore, the data suggests a significant potential for the non-toxic dioxin congener, i.e. 1278-TCDD, to interfere with thyroid hormone homeostasis via hydroxylation to the TTR-competitive binding species, 2-OH-1378-TCDD.

Acknowledgements

The authors gratefully acknowledge the technical assistance of Barbara Magelky and Colleen Pfaff in the significant task of sample preparation, and of Margaret Lorentzsen in producing the mass spectral data. The use of trade, firm or corporation names in this publication is for the information and convenience of the reader. Such use does not constitute an official endorsement or approval by the US Department of Agriculture or the Agricultural Research Service of any product or service to the exclusion of others that may be suitable.

References

- ALLEN, J. R., VANMILLER, J. P. and NORBACK, D. H., 1975, Tissue distribution, excretion, and biological effects of [^{14}C]tetrachlorodibenzo-*p*-dioxin in rats. *Food and Cosmetics Toxicology*, **13**, 501–504.

- BEETSTRA, J. B., VAN ENGELN, J. G. M., KARELS, P., VAN DER HOEK, H. G., DE JONG, M., DOCTER, R., KRENNING, E. P., HEENEMAN, G., BROUWER, A. and VISSER, T. J., 1991, Thyroxine and 3,3',5-triiodothyronine are glucuronidated in rat liver by different uridine diphosphate glucuronyltransferases. *Endocrinology*, **128**, 741–746.
- BERGMAN, Å., KLASSON-WEHLER, E. and KUROKI, H., 1994, Selective retention of hydroxylated PCB metabolites in blood. *Environmental Health Perspectives*, **102**, 464–469.
- DILIBERTO, J. J., BURGIN, D. and BIRNBAUM, L. S., 1997, Role of CYP1A2 in hepatic sequestration of dioxin: studies using CYP1A2 knock-out mice. *Biochemical and Biophysical Research Communications*, **236**, 431–433.
- GASIEWICZ, T. A., GEIGER, L. E., RUCCI, G. and NEAL, R. A., 1983, Distribution, excretion, and metabolism of 2,3,7,8-tetrachloro-*p*-dioxin in C57BL/6J, DBA/2J, and B6D2F1/J mice. *Drug Metabolism and Disposition*, **11**, 397–400.
- GRAY, A. P., CEPÄ, S. P., SOLOMON, I. J. and ANILINE, O., 1976, Synthesis of specific polychlorinated dibenzo-*p*-dioxins. *Journal of Organic Chemistry*, **41**, 2435–2437.
- HAKK, H., LARSEN, G. L. and FEIL, V. J., 2001, Tissue distribution, excretion, and metabolism of 1,2,7,8-tetrachlorodibenzo-*p*-dioxin in the rat. *Chemosphere*, **42**, 975–983.
- HUWE, J. K. and FEIL, V. J., 1998, Major metabolites of 1,4,7,8-tetrachlorodibenzo-*p*-dioxin in a ruminating calf. *Organohalogen Compounds*, **37**, 405–407.
- HUWE, J. K. and FEIL, V. J., 1999, Metabolism of 1,3,7,8-tetrachlorodibenzo-*p*-dioxin in a ruminating calf. *Organohalogen Compounds*, **42**, 147–149.
- HUWE, J. K., FEIL, V. J., LARSEN, G. L. and WIENER, C., 1998, Metabolism and disposition of 1,4,7,8-tetrachlorodibenzo-*p*-dioxin in rats. *Chemosphere*, **37**, 1885–1893.
- KOOPMAN-ESSEBOOM, C., MORSE, D., WEISGLAS-KUPERUS, N., LUTKE-SCHIPHOLT, I., VAN DER PAAUW, C. G., TUINSTRÄ, L. G. M. T., BROUWER, A. and SAUER, P. J. J., 1994, Effects of dioxins and polychlorinated biphenyls on thyroid hormone status of pregnant women and their infants. *Pediatric Research*, **36**, 468–473.
- LANS, M. C., BROUWER, A., KÖPPE, J. G. and VAN DEN BERG, M., 1990, Enzyme induction and alterations in thyroid hormone, vitamin A and K levels by TCDD in neonatal and maternal rats. *Chemosphere*, **20**, 1129–1134.
- LANS, M. C., KLASSON-WEHLER, E., WILLEMSSEN, M., MEUSSEN, E., SAFE, S. and BROUWER, A., 1993, Structure-dependent, competitive interaction of hydroxy-polychlorobiphenyls, -dibenzo-*p*-dioxins and -dibenzofurans with human transthyretin. *Chemical-Biological Interactions*, **88**, 7–21.
- LARSEN, G. L., BERGMAN, Å. and KLASSON-WEHLER, E., 1990, A methylsulphonyl metabolite of a chlorinated biphenyl can serve as a ligand for α 2u-globulin in rat and major urinary protein in mice. *Xenobiotica*, **20**, 1343–1352.
- NEAL, R. A., GASIEWICZ, T. A., GEIGER, L. H., OLSON, J. R. and SAWAHATA, T., 1984, Metabolism of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in mammalian systems. In A. Poland and R. Kimbrough (eds), *Biological Mechanisms of Dioxin Action*. Banbury Report 18 (Cold Spring Harbor: Cold Spring Harbor Laboratory), pp. 49–60.
- OLSON, J. R., 1986, Metabolism and disposition of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in guinea pigs. *Toxicology and Applied Pharmacology*, **85**, 263–273.
- OLSON, J. R., GASIEWICZ, T. A. and NEAL, R. A., 1980, Tissue distribution, excretion and metabolism of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in the Golden Syrian hamster. *Toxicology and Applied Pharmacology*, **56**, 78–85.
- PETROSKE, E., HUWE, J. K., FEIL, V. J. and LARSEN, G. L., 1997, Identification of NIH-shifted metabolites of 1,3,7,8-tetrachlorodibenzo-*p*-dioxin in the rat by NMR comparison with synthesized standards. *Chemosphere*, **34**, 1549–1555.
- PLUIM, H. J., KÖPPE, J. G., OLIE, K., VAN DER SLIKKE, J. W., VULSMA, T., KOK, J. H., VAN TIJN, D. and DE VIJLDER, J. J. M., 1992, Effects of dioxins on thyroid function in newborn babies. *Lancet*, **339**, 1303.
- POIGER, H. and BUSER, H. R., 1984, The metabolism of TCDD in the dog and rat. In A. Poland and R. Kimbrough (eds), *Biological Mechanisms of Dioxin Action*. Banbury Report 18 (Cold Spring Harbor: Cold Spring Harbor Laboratory), pp. 39–47.
- POIGER, H., BUSER, H. R., WEBER, H., ZWEIFEL, U. and SCHLÄTTER, C., 1982, Structure elucidation of mammalian TCDD-metabolites. *Experientia*, **4**, 484–486.
- ROSE, J. Q., RAMSEY, J. C., WENTZLER, T. H., HUMMEL, R. A. and GEHRING, P. J., 1976, The fate of 2,3,7,8-tetrachloro-*p*-dioxin following single and repeated oral doses to the rat. *Toxicology and Applied Pharmacology*, **36**, 209–212.
- SINGH, S. K. and KUMAR, S., 1993, Synthesis of oxygenated derivatives of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. *Journal of Agricultural and Food Chemistry*, **41**, 1511–1516.
- WITTICH, R. M., WILKERS, H., SINNEWELL, V., FRANKE, W. and FORTNAGEL, P., 1992, Metabolism of dibenzo-*p*-dioxins by *Sphingomonas* sp. strain RW1. *Applied Environmental Microbiology*, **March**, 1005–1010.



Comment

The long, hard road: drug metabolism in the lifetime of the DMDG

D. A. SMITH

Department of Drug Metabolism, Pfizer Global Research and Development, Ramsgate Road, Sandwich, Kent, CT13 9NJ, UK

Received 22 January 2001

Introduction

Special issues of *Xenobiotica* marked the 10th and 20th anniversaries of the DMDG. Within these, Drs David Case (1981) and Angus Bell and Mike Tarbit (1992) reviewed the state of the art of the science of drug metabolism and pharmacokinetics at those times as applied within the pharmaceutical industry. Now the DMDG reaches the landmark of its 30th anniversary, which is again marked by this special issue of the journal. This review attempts to take a broad overview of the state of our science and the notable advances that have occurred over the past 10 years. As with the previous anniversary issues, the papers presented here are intended to provide a wider perspective on the impact of the advances within the science as applied to industrial drug metabolism.

Move from drug development to embrace discovery

Drug metabolism in the pharmaceutical industry started around three decades ago, largely to investigate the metabolism of new drug candidates in animal species used for toxicology testing and to compare these findings with humans. Much of the work used radiotracers and much of the philosophy reflected other areas of industry, particularly agrochemicals. Rationalizations of species differences in disposition were limited. Although cytochrome P450 was being widely studied, the idea of a superfamily of isoforms was around a decade or more away. Pharmacokinetics was mainly a theoretical subject, which was only sporadically applied in the drug-discovery process. Sensitive assays for detection of the drug in man were limited to gas chromatography. The advent of HPLC, in particular reverse-phase HPLC and UV and fluorescent detectors, began a revolution in assay methodology that allowed the philosophy of pharmacokinetics to be evaluated for all drug candidates.

Listed below is a list of selected major events in drug metabolism that have shaped a change within the pharmaceutical industry. The dates are approximate in some cases but indicate the pace at which this change has occurred. With a process

* Author for correspondence; e-mail: dennis_a_smith@sandwich.pfizer.com

of discovery and development of a new drug molecule that lasts a decade or more, new drug introductions, almost by definition, are affected by changing viewpoints, concerns and scientific advances during this period:

- 1947 publication of R. Tecwyn Williams, *Detoxification Mechanisms*.
- 1964 cytochrome P450 identified.
- 1968 Drug metabolism departments formed in industry.
- 1971 DMDG formed.
- 1977 polymorphism of debrisoquine oxidation discovered.
- 1980 commercialization of HPLC allows pharmacokinetics to be applied routinely to drug development.
- 1984 polymorphism of mephenytoin oxidation discovered.
wide application of toxicokinetics proposed.
- 1986 human nifedipine oxidase (CYP3A4) and debrisoquine oxidase purified. First evidence/proof of substrate selectivity and multiple forms.
- 1990 genotype test for CYP2D6 (debrisoquine oxidase).
MDR *p*-glycoproteins demonstrated in normal human tissue including the gut.
- 1992 putative substract-structure relationships for P450s proposed.
observations of drug interactions and QT interval: changes in the pharmacokinetics and electrocardiograph pharmacodynamics of terfenadine reported after concomitant administration of erythromycin.
- 1993–94 availability of human liver to industry in research quantities.
- 1994 rationalization of a serious drug interaction with discrete P450s; oxidation of the antihistaminic drug terfenadine in human liver microsomes: roles of CYP3A4 established as a major enzyme responsible for terfenadine metabolism.
- 1996 widespread recognition of the role of antitransport processes in oral bioavailability determination.
- 1998 pregnane X-receptor activated by CYP3A4 inducing agents.

Accompanying this process of change has been a natural evolution of the various departments around the industry. In many cases their actual line allegiance has changed to facilitate new directions. Drug metabolism departments that were initially aligned with toxicology groups have become more closely associated with the drug discovery organization and in particular medicinal chemistry.

Prentis *et al.* (1988) reported for the seven UK-owned pharmaceutical companies that 'by the end of 1985, 49 new chemical entities had been marketed and 198 discontinued from development. The main reason for termination was inappropriate pharmacokinetics (39%)'. This attrition statistic is borne out by more modern analyses that indicate that pharmacokinetics are a major factor in drug development failure (DiMasi 1995). Inappropriate pharmacokinetics can include low bioavailability, variable bioavailability, duration of action too short due to high elimination rate, the presence of active metabolites or drug interaction profiles which impact too heavily on clinical usage. The consequences of such shortfalls can be seen as the impossibility of achieving a reliable pharmacodynamic action with a reasonable dosage regimen in the target patient population. For instance, when variable bioavailability or the presence of active metabolites leads to wide variation in the clinical response, or when short duration renders it

impossible to provide the patient with a convenient dosage regimen that encourages compliance and 24-h drug protection. It should be stressed that drugs may have pharmacokinetic flaws and still be successful. However, there are clearly more examples of drugs that are of limited use because of these flaws. It is perhaps not surprising that drug metabolism and pharmacokinetic technology originally applied within development and deployed for descriptive purposes is now being applied to drug discovery and used in a predictive manner.

The prediction of pharmacokinetics and metabolism for human medicinals cannot rely on animals and there is an increased reliance on humanized or human reagents and *in vitro* approaches. This trend has narrowed substantially the gap between industrial and academic drug metabolism research. Much of the early progress for discovery input was during the period that medicinal chemistry was aimed at structure–activity relationship (SAR)-led single compound synthesis, so called ‘rational synthesis’.

Defining drug-like molecules

New techniques in the last decade or less have rapidly changed the drug discovery environment. High throughput screening (HTS) technologies, originally developed to find the chemical lead in the above process, have been followed by high-speed chemical synthesis. The use of these in combination can accelerate lead identification by rapid screening of very large compound libraries. Moreover, the technology is also available to enhance the traditional labour and time-intensive process of turning chemical leads into development candidates by ‘rational synthesis’ using high-speed chemistry (combinatorial) in which a single template is amenable to derivitization to many analogues in a single process. However, if pharmacokinetic properties are not incorporated into design, then the synthesized compounds will be more lipophilic and larger, whether selected by random screening (HTS) as lead material, or as part of an automated high-speed SAR-led programme against potency. This is for two reasons:

- Lipophilicity is a key property in affinity for a drug target, almost invariably increasing lipophilicity increases potency against a target. Figure 1 shows this trend for β -adrenoceptor antagonists.

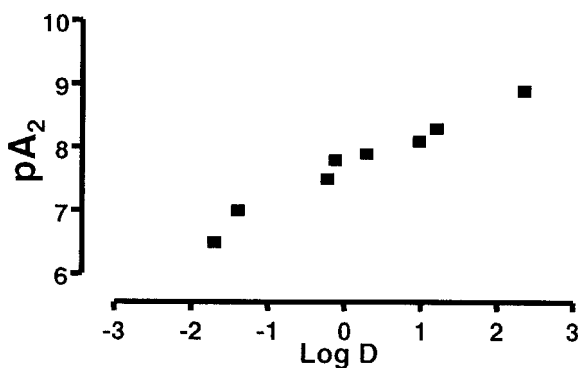


Figure 1. Relationship between lipophilicity ($\log D$) and potency (pA_2) for a series of β -adrenoceptor antagonists.

- Traditional targets such as aminergic 7-*trans*-membrane spanner receptors (7-TMs) have small molecules as agonists, histamine, adrenaline, serotonin, etc., which make excellent leads for agonists and antagonists. The resultant drugs are usually more lipophilic than the lead but still small and compact structures. Non-aminergic 7-TMs, such as endothelin, etc., have much larger molecules as agonists and it is likely that more complex molecules will be needed as agonist and antagonist drugs.

Compounds will also incorporate a significant number of H-bond donating and accepting groups, both to ensure polar interactions with the target and due to the synthetic routes available to large library high-speed synthesis. These compounds will be thus highly potent ligands but not promising as lead or development candidate material.

When the requirements for an oral drug are examined they can be summarized by:

- Aqueous solubility sufficient to allow rapid dissolution.
- Rapid transfer across or through the membranes of the gastrointestinal tract.
- Low clearance/metabolic stability.

The physicochemical and structural features that govern the above criteria are:

- Low aqueous solubility is the result of lipophilic substituents or high intramolecular crystal lattice binding energy.
- Transfer across the membranes of the gastrointestinal tract requires the molecules to be lipophilic and able to shed their sphere. Thus, lipophilic substituents are required in combination with a low number of H-bonding functions. Alternatively, compounds may cross the gastrointestinal tract by the paracellular (aqueous pore) route. The requirements for paracellular absorption is for molecules of small molecular size.
- High intrinsic clearance for compounds is a product of lipophilicity and structure (metabolism) or high molecular weight, lipophilicity and structure (hepatic uptake/biliary clearance).

Based on marketed drugs, Lipinski *et al.* (1997) have published rules which suggest design should exclude:

- molecular weight over 500;
- more than 5 H-bond donors; and
- more than 10 H-bond acceptors.

These are factors that limit membrane transfer:

- $\text{Log } P > 5$.

Highly lipophilic compounds show poor dissolution and therefore poor or erratic absorption.

Although aimed principally at obtaining good oral absorption, the same rules could be applied to ensuring reasonable stability to the clearance processes as outlined above.

Library or any synthesis programmes broadly incorporating these rules will move the emphasis from potent ligands to promising development candidates.

New technology and drug metabolism

The LC/MS/MS instruments have revolutionized bioanalytical procedures in the industrial drug metabolism department. Generic assay methodology with minute run times utilizing the exquisite selectivity and sensitivity of the mass spectrometer as a detector allow huge sample throughput and make sample analysis no longer rate limiting (Cole *et al.* 1998). Actually, carrying out the *in vitro* or *in vivo* procedure becomes a rate-limiting step.

Rapid *in vitro* screening technologies have therefore been developed for absorption using CaCO₂ cells, metabolic stability using recombinant enzymes, liver microsomes or even hepatocytes, protein binding, inhibition of human cytochrome P450s and even drug toxicity (Smith and van de Waterbeemd 1999). Such screens can make use of much of the existing HTS configurations of 96-well microtitre plates and robotics and can be termed high throughput. Screening can also be conducted on mixtures to look for the most stable compound in a series to metabolism (e.g. hydrolysis). Semi-rapid technologies have also been attempted to evaluate whole animal pharmacokinetics. Again, such screens could not be run in parallel to HTS systems but can process compounds rapidly. These techniques rely on administration of mixtures of compounds to a single or few animals and rely on the selectivity of LC/MS/MS instruments to analyse the resultant plasma samples (Beudry *et al.* 1998). Many of these techniques rely on the mixture acting as a control, with accurate pharmacokinetic parameters on the 'optimum' compound or compounds being determined in a separate experiment.

Analysing and interpreting the data now becomes the rate-limiting step and here the dilemma begins. Data are trivial, can be moved electronically and incorporated or disseminated in seconds, knowledge can also be incorporated and disseminated, but the process takes time. Most (all?) successful drug discovery projects will usually succeed or fail due to the wisdom of individuals. Wisdom is the slowest to transfer and the slowest to gain. The danger faced is that as a discipline it stops at the data stage and we believe that the answer to problems is more data. The technology is equally applicable to 'screening' of huge numbers of compounds looking for the ideal combination of properties or performing more complex experiments in a time and people effective way.

Impact of cardiotoxicity and drug interactions on the role of drug metabolism

A growing number of drugs are associated with QT prolongation and its concomitant risks of arrhythmia and sudden death. These drugs have been shown to block the 'rapid' cardiac delayed rectifier potassium current (IKr) or cloned channels encoded by the human ether-a-go-go-related gene (HERG; the gene believed to encode native IKr). IKr plays an important role in ventricular AP repolarization, and its inhibition results in prolongation of both the AP and QT interval of the ECG (Roy *et al.* 1996). The drugs that produce QT prolongation via interaction with this channel include anti-arrhythmics, such as quinidine, non-sedating antihistamines, such as terfenadine, gastric motility agents, such as cisapride, and psychiatric drugs, such as haloperidol. These drugs are basic in nature due to a substituted aliphatic amine function and are of positive lipophilicity. They often have high degrees of first-pass metabolism due to their physicochemistry and structure by intestinal and hepatic CYP3A4. Some of

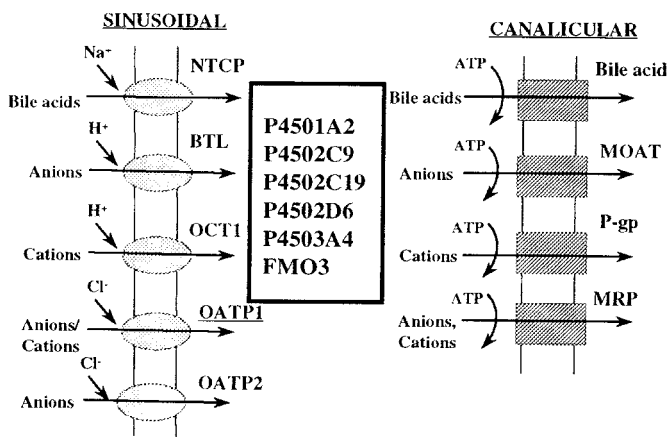


Figure 2. Key drug transporters and metabolizing enzymes in liver. Many enzymes and some transporters are omitted for clarity.

these compounds have proved perfectly safe in clinical use until potent CYP3A4 inhibitors have been co-administered, leading to a marked elevation in drug concentration, QT prolongation, and resultant arrhythmia and sudden death.

Recognition that drug transporters interplay with metabolizing enzymes in determining the pharmacokinetics of a compound

P-glycoprotein (Pgp), which is coded by human MDR1 (multidrug resistance) gene, is an energy-dependent efflux pump that exports its substrates out of the cell. Human Pgp is present in the kidney, liver, small and large intestine, and brain. It acts in concert with cellular influx transporters to provide urinary excretion mechanisms in the kidney, biliary excretion mechanisms in the liver, and importantly an absorption barrier within the gut. In addition, Pgp acts at the blood–brain barrier to limit the accumulation of drugs in the brain. The inhibition of the transporting function of Pgp present in the gut can cause clinically significant drug interactions due to reductions in first-pass gut extraction. Many CYP3A4 substrates are substrates of Pgp and similarly potent CYP3A4 inhibitors such as ketoconazole are inhibitors of Pgp. This makes a complex jigsaw of possible interactions with the structural features of the IKr channel ligands (basicity and lipophilicity showing overlap with the substrate requirements for Pgp and CYP3A4). Thus, guinidine for instance is an IKr blocker, a CYP3A4 substrate and a Pgp inhibitor and substrate. Pgp is an efflux transporter moving drugs from the interior of the cell or the cell membrane to the surrounding media. A battery of influx transporters also exists to move drugs into the interior of cells. These may also be involved in drug interactions. The complex interplay of these transporters is shown for the liver in figure 2.

New demands on drug metabolism: the complete description of ADME and its pharmacodynamic (both beneficial and adverse) consequences—the move to small patient subgroups at risk

To the relationship of structural features and potential life-threatening drug interactions needs to be added the fact that the primary metabolites of many

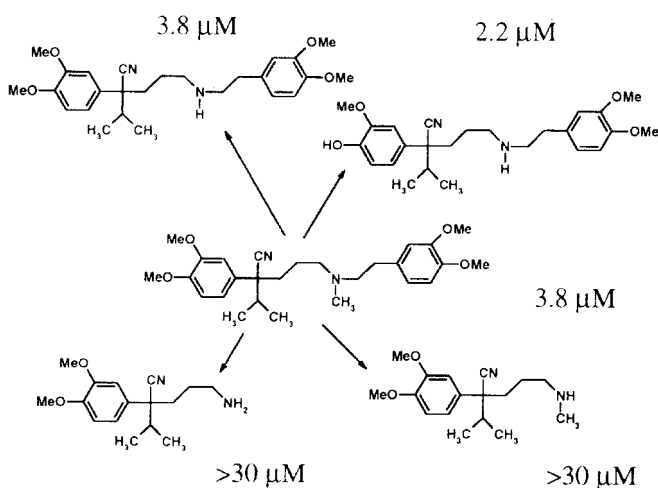


Figure 3. Circulating metabolites of verapamil (Carter *et al.* 1996). The pharmacodynamics of this compound on the cardiovascular system are usually related to its activity against Ca^{++} channels. However, the compound is also active against the IKr channel. The figure shows the potency against this channel for the metabolites (Waldegger *et al.* 1999) and indicates that ADME considerations of pharmacodynamics need to become more comprehensive to explain subsets of patients at risk.

molecules and especially aliphatic tertiary amines are physicochemically and structurally close to the parent molecule (figure 3). Thus in some cases the circulating metabolites can contribute not only just to the desired pharmacology, but also to the undesired effects including cardiovascular. Traditional ADME has focussed on mainly the excreted metabolites rather than on the circulating species. It is relatively simple to quantitate the major species in urine and faeces and portray it as a proportion of dose absorbed. With circulating materials the matter is much more complex. For instance, the circulating concentrations of metabolites depend on the proportion of the dose of drug converted, the clearance of the metabolite, and the volume of distribution of the metabolite.

Superficial examination of data is misleading. Many basic drugs with moderate plasma protein binding and clearance, and high volumes of distribution are converted to acidic metabolites with high plasma protein binding, low clearance and low volumes of distribution. Thus, the major circulating species in terms of total concentration may actually be one which is minor in terms of percentage conversion and also minor when free unbound concentration is considered. ADME now needs to be assessed in terms of metabolite identification and then metabolite characterization in terms of physicochemistry, pharmacokinetics, pharmacology and pharmacodynamics. This will be accomplished by incorporating theoretical considerations rather than just practical ones. Such considerations are needed to define risk groups such as the renal impaired (renally cleared active metabolite(s) not just renally cleared parent), AIDS patients (cocktail of drugs includes Pgp and P450 inhibitors and P450 inducers), epileptics (P450 inducers), etc.

Areas of concern in the future: is the academic base being maintained and are we growing the drug metabolism leaders of the future?

Industrial drug metabolism has seen large investment in terms of people and instrumentation over the last decade. In contrast, academia has struggled to maintain funding. The competitive nature of recruitment within the pharmaceutical industry has pushed salaries higher and to a level that universities now cannot compete. This gulf has been growing for the last two decades and has created an unbalanced discipline with academia largely reliant on senior mature staff and PhD students with no middle-level academics; the would be research associates, lecturers, etc., mainly choosing the fertile grounds of pharmaceutical research. Moreover, much of the industry's work requires bioanalytical and chemical backgrounds being associated with structures and reactions. Funding for such work is difficult to obtain in academia, in contrast to 20–30 years ago, and much of the focus has moved to more molecular biology-based projects. The worry is that the imbalances are not self-limiting and once the present cluster of academic heads of departments, who represent drug metabolism, leave the field, the edifice will crumble, having dire effects on our discipline. At this time we will be forced into stopping at the data stage while pharmacologists, chemists, etc., possess the knowledge and wisdom. The solution to this problem is not obvious, but those who hold the subject dear to their heart and have found the science uplifting and fun, need to put their minds and bodies to this problem before it is too late.

References

- BELL, J. A. and TARBIT, M. H., 1992, Comment: Twenty years on: a review of the current practice of drug metabolism and pharmacokinetic studies in the pharmaceutical industry. *Xenobiotica*, **22**, 735–742.
- BEUDRY, F., LeBLANC, J. C., COUTU, M. and BROWN, N. K., 1998, *In vivo* pharmacokinetic screening in cassette dosing experiments, the use of on-line Prospekt liquid chromatography/atmospheric pressure chemical ionization tandem mass spectrometry technology in drug discovery. *Rapid Communications in Mass Spectrometry*, **12**, 1216–1222.
- CARTER, B. L., NOYES, M. A., DEMMIER, R. W., BERNETTE, D. J. and PIEPER, J. A., 1996, Pharmacokinetics of verapamil and its metabolites in elderly and young volunteers. *Journal of Applied Therapeutics*, **1**, 85–91.
- CASE, D. E., 1981, The state of the art: a commentary on the current practice of metabolism and pharmacokinetic studies in the pharmaceutical industry. *Xenobiotica*, **11**, 803–814.
- COLE, R. O., LAWS, K. A., HILLER, D. L., KIPLINGER, J. P. and WARE, R. S., 1998, Reduced dimension HPLC columns as a preclinical tool for rapid separations in drug discovery. *American Laboratory*, **30**, 15–20.
- DiMASI, J., 1995, Success rates for new drugs entering clinical testing in the United States. *Clinical Pharmacology and Therapeutics*, **58**, 1–14.
- LIPINSKI, C. A., LOMBARDO, F., DOMINY, B. W. and FEENEY, P. J., 1997, Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. *Advanced Drug Delivery Reviews*, **23**, 3–25.
- PRENTIS, R. A., LIS, Y. and WALKER, S. R., 1988, Pharmaceutical innovation by the seven UK-owned pharmaceutical companies (1964–1985). *British Journal of Clinical Pharmacology*, **25**, 387–396.
- ROY, M. L., DUMAINE, R. and BROWN, A. M., 1996, HERG, a primary human ventricular target of the non-sedating antihistamine terfenadine. *Circulation*, **94**, 817–823.
- SMITH, D. A. and VAN DE WATERBEEMD, H., 1999, Pharmacokinetics and metabolism in early drug discovery. *Current Opinion in Chemical Biology*, **3**, 373–378.
- WALDEGGERS, S., NIEMEYER, G., MORIKE, K. and WAGNER, C. A., 1999, Effect of verapamil enantiomers and metabolites on cardiac K^+ channels expressed in *Xenopus*. *Cell Physiology and Biochemistry*, **9**, 81–89.
- WILLIAMS, R. T., 1947, *Detoxication Mechanisms: The Metabolism and Detoxication of Drugs, Toxic Substances and Other Organic Compounds* (London: Chapman & Hall).

- TANII, H. and HASHIMOTO, K., 1982, Structure-toxicity relationship of acrylates and methacrylates. *Toxicology Letters*, **11**, 125–129.
- VERHAAR, H. J. M., MORRONI, J. S., REARDON, K. F., HAYS, S. M., GAVER, D. P., JR., CARPENTER, R. L. and YANG, R. S. H., 1997, A proposed approach to study the toxicology of complex mixtures of petroleum products: the integrated use of QSAR, lumping analysis and PBPK/PD modeling. *Environmental Health Perspectives*, **105** (suppl. 1), 179–195.
- VODICKA, P., GUT, I. and FRANTIŠEK, E., 1990, Effects of inhaled acrylic acid derivatives in rats. *Toxicology*, **65**, 209–221.
- YANG, R. S., THOMAS, R. S., GUSTAFSON, D. L., CAMPAIN, J., BENJAMIN, S. A., VERHAAR, H. J. and MUMTAZ, M. M., 1998, Approaches to developing alternative and predictive toxicology based on PBPK/PD and QSAR modeling. *Environmental Health Perspectives*, **106** (suppl. 6), 1385–1393.

Role of transport proteins in drug absorption, distribution and excretion

A. AYRTON[†] AND P. MORGAN^{‡*}

[†] Mechanism and Extrapolation Technologies, DMPK, GlaxoSmithKline, The Frythe, Welwyn, UK

[‡] Department of Drug Metabolism, Pfizer Global Research and Development, Ramsgate Road, Sandwich CT13 9NJ, UK

Received 4 March 2001

1. The molecular and functional characterization of transport proteins is emerging rapidly and significant numbers of drugs have been shown to be substrates or inhibitors. The purpose of this review is to highlight the *in vivo* preclinical and clinical evidence that supports a role for transport proteins in attenuating the absorption, distribution and excretion (ADE) of drugs.

2. For absorption, a clear role has emerged for P-glycoprotein in limiting permeability across the gastrointestinal tract. As a result, a wide variety of drugs suffer from incomplete, variable and non-linear absorption. Similarly, at the blood–brain barrier a range of drugs has limited brain penetration due to P-glycoprotein-mediated efflux, which can limit therapeutic effectiveness of CNS agents. In the liver, transport proteins are present on the sinusoidal membrane that can be the rate-limiting step in hepatic clearance for some drugs. Mechanistic studies clearly suggest a key role and broad substrate specificity for the OATP family of sinusoidal transporters. Mainly ATP-dependent transport proteins such as P-glycoprotein and MRP2 govern active biliary excretion.

3. Drug–drug interactions have been demonstrated involving inhibition or induction of transport proteins. Clinically significant interactions in the gastrointestinal tract and kidney have been observed with inhibitors such as ketoconazole, erythromycin, verapamil, quinidine, probenecid and cimetidine. Clinically significant inhibition at the blood–brain barrier is more difficult to demonstrate, relying on pharmacodynamic and toxicodynamic changes, but an example is quinidine increasing loperamide-induced central effects in humans.

4. This review highlights the emerging role of transport proteins in ADE of drugs and suggests these need to be considered, in drug discovery and development, with respect to variability in drug disposition and response.

Introduction

Over the last 5 years or so, there has been a significant increase in the molecular characterization of transport proteins in animals and man (Oude Elferink *et al.* 1995, Meijer *et al.* 1997, Müller and Jansen 1998, Kim 2000). This has led to a better understanding of the importance of such transport proteins in the disposition of endogenous compounds, drugs and other xenobiotics in many organs such as the intestine, liver, kidney and brain. This review will focus on the role of transport proteins in the absorption, distribution and excretion (ADE) of therapeutic drugs with particular emphasis on highlighting the supportive *in vivo* preclinical and clinical evidence. As the characterization of transport protein

*Author for correspondence; e-mail: paul_morgan@sandwich.pfizer.com

families and their nomenclature is still emerging, there is no attempt in this review to describe these efforts in any detail. However, for a description of the recent classification of transport proteins, the following reviews are recommended: Borst *et al.* (1999), Klein *et al.* (1999), König *et al.* (1999), Hooiveld *et al.* (2000), Inui *et al.* (2000) and Suzuki and Sugiyama (2000). It is hoped that the present review will be of particular relevance to drug discovery and development given the current debate about the role of transport proteins in drug ADE.

The distribution of drugs in the body is controlled by permeability across tissue membranes. For example, the gastrointestinal tract and blood–brain barrier (BBB) provide efficient barriers to the permeability of drugs (Goldstein and Betz 1986, Smith *et al.* 1996). The extent of membrane permeability is largely governed by the physicochemical properties of drugs, and lipophilicity is generally considered as a key determinant in extent of drug absorption, hepatic transport and brain penetration. However, increased lipophilicity is not always predictive of increased permeability due to the contribution of transport proteins. The tissue localization and probable role of transport proteins in drug ADE is depicted in figure 1. In the liver, the role of carrier proteins on the canalicular membrane in biliary excretion of drugs and metabolites has long been recognized (Petzinger 1994, Müller and Jansen 1997). However, it is now clear that there are many transport proteins involved in the uptake of drugs from blood to liver, across the sinusoidal membrane, that need to be considered in the context of hepatobiliary distribution and excretion (Yamazaki *et al.* 1996b, Kullak-Ublick 1999, Kim 2000). In the kidney the carrier-mediated processes at the basolateral and luminal membranes, which are involved in active renal secretion of drugs, are now better understood (Inui *et al.* 2000). In the intestine and brain it is now appreciated that transport proteins, such as P-glycoprotein, play a significant role in the extrusion of drugs from these organs thereby attenuating drug absorption and brain penetration (Schinkel 1999, Suzuki and Sugiyama 2000). As the importance of

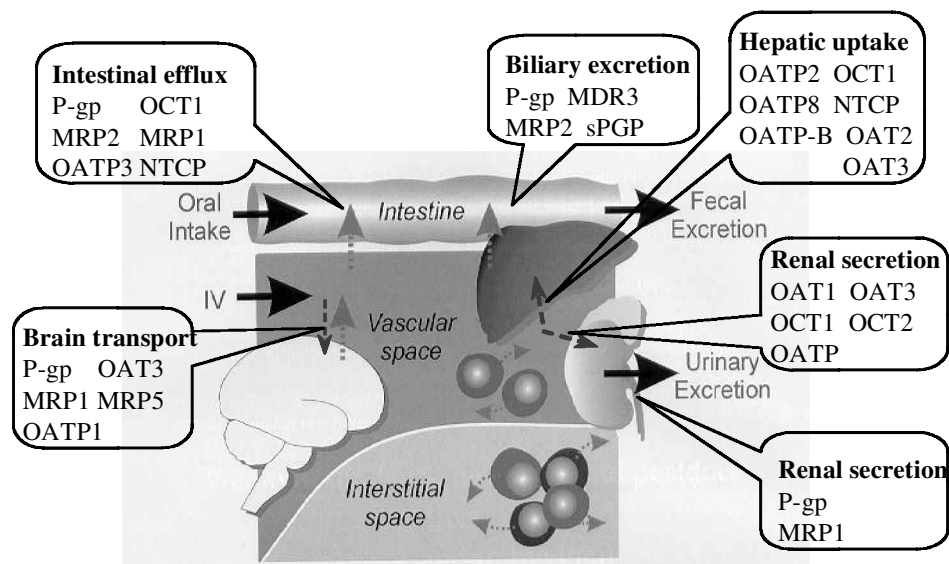


Figure 1. Tissue localization and role of transport proteins in drug disposition.

transport proteins in drug ADE emerges, it is also clear that these transport proteins are saturable, inducible, can be inhibited and display some degree of polymorphism – factors that need to be considered with respect to variability in drug disposition and response (Ito *et al.* 1998, Greiner *et al.* 1999, Watkins 1999, Hoffmeyer *et al.* 2000).

Role of transport proteins in drug absorption

Drug absorption across the gastrointestinal tract can be highly dependent upon transport protein affinity as well as lipophilicity. Transport proteins can be involved in the active absorptive influx of compounds, such as amino acids, oligopeptides, monosaccharides, mono- and di-carboxylic acids, bile acids, and several water-soluble vitamins, from the lumen into the portal bloodstream (Thwaites *et al.* 1995, Tsuji and Tamai 1996). Conversely, there are other transport proteins responsible for the active efflux of drugs and xenobiotics from gut epithelial cells back into the lumen (Benet *et al.* 1996, Zhang *et al.* 1998a). Molecular characterization of transport proteins present in the gut epithelial plasma membrane has identified a number of transport protein families such as MDR (Watkins 1997), MRP (Kool *et al.* 1997), OATP (Tamai *et al.* 2000), OCT (Koepsell 1998) and OAT (Sekine *et al.* 1999). However, it is the role of P-glycoprotein (MDR1) in the active efflux of drugs in the gastrointestinal tract that is the most widely studied area (Wacher *et al.* 1996, Hunter and Hirst 1997). P-glycoprotein is present on the villus tip of the apical brush border membrane of gut enterocytes and is orientated to pump substrates from inside the cells back into the lumen of the intestine (Watkins 1997). The distribution of P-glycoprotein is not uniform along the length of the intestine or along the villi within a cross-section of the mucosa. Thiebaut *et al.* (1987) used the monoclonal antibody MRK16 to study the distribution of P-glycoprotein in human jejunum and colon. Both tissues showed high levels of P-glycoprotein on the apical surface of superficial columnar epithelial cells but not in crypt cells. In another study, the content of mRNA expression of P-glycoprotein was measured over the total length of the human gastrointestinal tract. The levels of mRNA appeared to increase progressively from the stomach to the colon (Fojo *et al.* 1987, Fricker *et al.* 1996). Variable expression of P-glycoprotein mRNA and protein levels in the gut have been demonstrated with an estimated 8–10-fold variation (Lown *et al.* 1995, 1997) suggesting variability in P-glycoprotein effects on drug absorption is likely. More recently, polymorphisms of the human P-glycoprotein gene have been detected in Caucasians and African-Americans (Hoffmeyer *et al.* 2000, Kim *et al.* 2000) and attempts have been made to correlate these polymorphisms with variations in P-glycoprotein protein levels and activity. A correlation was observed between the MDR1 genotype and duodenal P-glycoprotein levels (for $n = 21$ individuals) with the homozygous T/T genotype resulting in more than 2-fold lower mean expression levels compared with homozygous C/C samples. In addition, in eight volunteers from this study, an inverse correlation was also observed between an exon 26 polymorphism and oral digoxin plasma levels with higher digoxin levels in volunteers having the T/T genotype compared with those with the C/C genotype. The implication from this study is that absorption of other P-glycoprotein substrates could be affected by this polymorphism with more variable absorption as the result.

The use of P-glycoprotein knockout (*mdr1a* $-/-$) mouse has provided significant evidence supporting a role for P-glycoprotein in limiting intestinal absorption. A good example of this was the study of the pharmacokinetics of paclitaxel (taxol) in the P-glycoprotein-knockout (*mdr1a* $-/-$) and wild-type (*mdr1a* $+/+$) mouse (Sparreboom *et al.* 1997). Plasma AUC was shown to increase 2- and 6-fold in the knockout versus the wild-type mouse after intravenous (i.v.) and oral dosing, respectively. Consequently, the oral bioavailability increased from 11% in the *mdr1a* $(+ / +)$ to 35% in the *mdr1a* $(- / -)$ mouse. In addition, intestinal secretion of paclitaxel, after i.v. dosing, decreased from 40% of dose in the wild-type to 1.5% in the knockout mouse. Collectively these results indicate that the oral absorption of paclitaxel was effectively limited by P-glycoprotein-mediated efflux from the intestinal epithelial cells back into the lumen. Intestinal P-glycoprotein efflux of a wide range of other drugs, such as vinblastine, digoxin, indinavir, saquinavir, nelfinavir and talinolol, has been demonstrated in the P-glycoprotein knockout mouse (Mayer *et al.* 1996, van Asperen *et al.* 1996, Sparreboom *et al.* 1997, Kim *et al.* 1998, R. J. Mitchell personal communication, 2000). Similar to mouse, the involvement of P-glycoprotein in intestinal absorption in rat has been demonstrated for talinolol by the increased oral bioavailability in the presence of the P-glycoprotein inhibitor verapamil (Spahn-Langguth *et al.* 1998). The increased bioavailability is more likely to be due to P-glycoprotein inhibition rather than inhibition of cytochrome P450 as talinolol is a metabolically inert drug (Trausch *et al.* 1995).

In human studies, cyclosporin provides a good example of the impact of P-glycoprotein on oral absorption. Cyclosporin transport has been shown to be impaired by P-glycoprotein in a variety of P-glycoprotein-containing *in vitro* systems (Saeki *et al.* 1993, Gan *et al.* 1996). Similarly, cyclosporin absorption in man is decreased by intestinal P-glycoprotein (Watkins 1997) with a greater decrease in absorption from the colon, plus a concurrent increase in coefficient of variation in oral blood levels, versus the stomach and jejunum, which appears to correlate with higher relative colonic levels of P-glycoprotein (Fricker *et al.* 1996). A similar finding was made for talinolol (Gramatté *et al.* 1996) where oral AUC in blood decreased with increasing distance from the teeth, after administration of oral doses in different regions of human gut, consistent with the premise that intestinal levels of P-glycoprotein increase along the length of the gut. In addition for cyclosporin, Lown *et al.* (1997) demonstrated that 30% of the variability in oral C_{max} and 17% of the variability in oral clearance of cyclosporin in humans can be accounted for by the individual variation in intestinal P-glycoprotein levels. Therefore, P-glycoprotein not only limits the absorption of cyclosporin, but also contributes to the interindividual variation in absorption and consequently variability in blood concentrations after oral administration. Intestinal P-glycoprotein is thought to contribute significantly to the limited absorption of a range of other drugs such as tacrolimus (Hebert 1997), digoxin (Hoffmeyer *et al.* 2000), talinolol (Wetterich *et al.* 1996), fexofenadine (Robbins *et al.* 1998, Cvetkovic *et al.* 1999), saquinavir (Noble and Faulds 1996) and UK-224,671 (Beaumont *et al.* 2000). Another interesting aspect of the attenuation of absorption by P-glycoprotein is that of non-linear absorption most probably due to saturation of P-glycoprotein-mediated efflux in the gastrointestinal tract as dose increases. This phenomenon has been observed for a number of drugs such as cyclosporin (Shibata *et al.* 1998), talinolol (Wetterich *et al.* 1996), celioprolol (Milne and Buckley 1991), fexofenadine

(Robbins *et al.* 1998), saquinavir (Noble and Faulds 1996) and UK-343,664 (Abel *et al.* 2001). The pharmacokinetic consequence of this is dose-dependent bioavailability, which has been shown to increase up to 2-fold with talinolol, celiprolol and cyclosporin, for example, as dose increases. UK-343,664 displays a 247- and 287-fold increase in $C_{\max}$ and AUC, respectively, for a 27-fold increase in dose. It is difficult to attribute this dose-dependent bioavailability purely to saturation of P-glycoprotein for some drugs such as cyclosporin and saquinavir due to the large contribution of CYP3A4 to their first-pass effect. However, in other cases it is more clearly a P-glycoprotein-only effect as these drugs (talinolol, celiprolol, fexofenadine) are metabolically inert (Milne and Buckley 1991, Lippert *et al.* 1995, Trausch *et al.* 1995).

Therefore, preclinical and clinical studies clearly demonstrate that P-glycoprotein-mediated intestinal efflux not only limits absorption of drugs, but also can result in variable and non-linear oral pharmacokinetics.

Role of transport proteins in the hepatobiliary excretion of drugs

The liver plays a key role in the clearance and excretion of many drugs and hepatobiliary excretion of drugs from blood, through the hepatocyte, and into the bile can be considered a three-step process. The first step involves the uptake of drugs from blood into the hepatocyte and the site of entry is the sinusoidal (basolateral) membrane. This membrane contains many microvilli that expand the surface area and as a result the sinusoidal membrane accounts for at least 70% of the hepatocyte surface area (Keppler and Arias 1997). The physicochemical properties of many drugs enable them to cross the sinusoidal membrane by passive diffusion. However, for a significant number of drugs, entry into the hepatocyte can be achieved via a variety of sinusoidal carrier systems that augment or replace the passive diffusion component (LeBlanc 1994). Such drugs tend to be ionized (either anionic or cationic) with molecular weights > 400 and contain further polar (H-bonding) groups (Meijer *et al.* 1990). A wide variety of active carrier systems are known to be present at the sinusoidal membrane that facilitate the active uptake of a diverse range of drugs (Hagenbuch 1997, Meijer *et al.* 1997, Kullak-Ublick 1999). Once inside the hepatocyte the second step of hepatobiliary excretion involves transfer of drugs to metabolic sites and/or the biliary canalicular membrane, which is mediated by intracellular transfer proteins and passive diffusion (LeBlanc 1994). The degree of metabolic biotransformation of drugs is highly dependent upon their physicochemical properties and structure-metabolism relationships (Smith and Jones 1992, Smith *et al.* 1996). Consequently, the third step of biliary excretion at the canalicular membrane can involve mainly unchanged drug, mainly metabolites or a combination of both parent drug and metabolites. The canalicular membrane constitutes a smaller percentage (10–15%) of the hepatocyte surface area compared with the sinusoidal membrane (at least 70%) and thus active transport provides the most efficient means of excretion from the hepatocyte into the bile. Many carrier proteins have been shown to be present on the canalicular membrane to mediate this process (Keppler and Arias 1997, Müller and Jansen 1997, Stieger and Meier 1998, Hooiveld *et al.* 2000). A summary of the major hepatic sinusoidal and canalicular membrane transporters involved in transport of therapeutic drugs is provided in table 1.

Table 1. Summary of the major liver sinusoidal and canalicular membrane transporters involved in transport of therapeutic drugs.

Common name of transporter	Gene family name	Species	Subcellular location	Known drug substrates
OATP-A	SLC21A3	human	S	fexofenadine, UK-191,005
OATP-C	SLC21A6	human	S	pravastatin, benzylpenicillin, BSP
OATP-8	SLC21A8	human	S	BSP
OATP-B	SLC21A9	human	S	fexofenadine, UK-191,005
Oatp1	SLC21A1	rat	S	fexofenadine, UK-191,005, temocaprilat, CRC-220, ouabain, BSP, BQ-123
Oatp2	SLC21A5	rat	S	digoxin, fexofenadine, ouabain, BQ-123
Lst-1	SLC21A10	rat	S?	ND
OCT1	SLC22A1	human	S	ND
Oct1		rat	S	ND
OAT2	SLC22A7	human	S?	ND
Oat2		rat	S?	methotrexate
OAT3	SLC22A8	human	S?	cimetidine
Oat3		rat	S?	cimetidine
MDR1	ABCB1	human	C	Cyclosporine, taxol, vincristine, vinblastine, doxorubicin, digoxin, talinolol, loperamide, erythromycin, UK-343,664, UK-224,671
Mdr1a/1b	ABCB1a/1b	rat	C	As for MDR1
MRP2	ABCC2	human	C	pravastatin, methotrexate, grepafloxacin, cefodizime, irinotecan, temocaprilat + conjugates of a variety of drugs
Mrp2		rat	C	As for MRP2
MRP1	ABCC1	human	L	Anticancer agents, anionic conjugates with glutathione, sulfate or glucuronide
Mrp1		rat	L	As for MRP1

S, sinusoidal; C, canalicular; L, lateral membranes.
ND, not determined.

Several studies suggest that active hepatic uptake needs to be considered as a key process in hepatic clearance of drugs (Meijer *et al.* 1990, 1997, Yamazaki *et al.* 1996a, b) and identification of the key hepatic uptake transporters is essential to understand this process in more detail at the molecular and functional level. Several members of the OATP family of transporters have been shown to be specifically located on the liver sinusoidal membrane in rodents (Bergwerk *et al.* 1996, Reichel *et al.* 1999) and humans (König *et al.* 2000a, b). In addition to being only on the sinusoidal membrane of the hepatocyte, some of the OATP transporters, such as OATP-C and OATP-8, have been shown to be expressed exclusively in liver (Tamai *et al.* 2000). Functional transport of a wide variety of drugs has been demonstrated in cell lines expressing members of the human and rat OATP family. Pravastatin, a non-metabolized hydrophilic acid, is transported by human OATP-C with $K_m = 34 \mu M$ (Hsiang *et al.* 1999). Other substrates of human OATP-C include other acids such as bilirubin, BSP, benzylpenicillin and 17 β -oestradiol glucuronide (Cui *et al.* 2000, König *et al.* 2000a). All studies reported to date suggest that OATP-C, and also OATP-8, transport only acidic compounds but other members of the OATP family (OATP-A, OATP-B, Oatp1, Oatp2) have been shown to transport basic, zwitterionic and neutral compounds such as UK-

191,005, rocuronium, *N*-methylquinidine, fexofenadine, CRC 220 and digoxin (Eckhardt *et al.* 1996, Cvetkovic *et al.* 1999, Morgan *et al.* 1999, van Montfoort *et al.* 1999). Therefore, the OATP family clearly has broader substrate specificity than just acid compounds which highlights the misleading nature of its name. A potential explanation for the greater acid substrate affinity of human OATP-C is that this is a more basic protein than other OATP family members (Hsiang *et al.* 1999) and thus may be expected to have greater affinity for acidic substrates. The mechanism of hepatic uptake transporters and the cofactors involved have been the focus of many studies (Meier *et al.* 1997, Meijer *et al.* 1997, Müller and Jansen 1997, Kullak-Ublick 1999) but the process is not clearly defined for many of the transport proteins. For the OATP family, studies with rat Oatp1 have illustrated that transport is saturable, bi-directional, highly temperature dependent and glutathione can be an effective cofactor for uptake (Shi *et al.* 1995, Li *et al.* 1998). More recent studies have demonstrated that rat Oatp2 also mediates bi-directional transport for which glutathione can be the driving force (Li *et al.* 2000). Clearly, the OATP family appears to have a key role in hepatobiliary excretion of drugs particularly in mediating hepatic uptake.

In contrast to OATP transporters, far less data are available on hepatic uptake of drugs by other transport families. OCT proteins are expressed in epithelial cells of kidney, liver and intestine and appear to be expressed on the basolateral membranes of the cells (Zhang *et al.* 1998a). Human and rat OCT1 are expressed mainly in liver and kidney whereas human and rat OCT2 are present mainly in kidney (Koepsell 1998, Zhang *et al.* 1998a). Small organic cations, such as TEA, MPP⁺, choline and *N*-methylnicotinamide, have been shown to be substrates for OCT proteins (Nagel *et al.* 1997, Zhang *et al.* 1998c). However, although several basic drugs, such as clonidine, quinine, quinidine and verapamil, inhibit the OCT protein-mediated transport of small organic cations (Urakami *et al.* 1998), no firm evidence exists for the transport of these or other basic drugs by this family of transporters. Hence, the role of OCT transport proteins in hepatobiliary excretion of drugs is considered limited. Another family of transporters is the OAT proteins, which are expressed in liver, kidney and brain, and display similarity in amino acid sequence to the OCT family (Sekine *et al.* 1999). Of the three main family members identified, OAT1 is expressed only in kidney, OAT2 is expressed only in liver and OAT3 has broader tissue distribution including liver. The main role identified to date for OAT proteins is in active renal secretion of substrates such as cimetidine, methotrexate, cidofovir and adefovir (Cundy 1999, Inui *et al.* 2000). A wide variety of drugs, such as probenecid, β -lactam antibiotics, non-steroidal anti-inflammatory drugs, can also interact with the transporter (Sekine *et al.* 1997, Cihlar *et al.* 1999, Kusuhara *et al.* 1999). However, the role and subcellular location of the OAT proteins in hepatobiliary excretion remains to be fully elucidated.

Hepatic uptake also appears to play a key role in defining hepatic clearance. Fexofenadine (zwitterionic) and UK-191,005 (basic) are both metabolically stable, high molecular weight, high H-bonding compounds whose *in vivo* clearance is mediated by rapid hepatic uptake and biliary excretion (Lippert *et al.* 1995, Morgan *et al.* 1999). Both compounds are substrates for rat and human hepatic uptake transporters from the OATP family (Cvetkovic *et al.* 1999, Morgan *et al.* 1999). Correlation of *in vivo* and *in vitro* clearance processes in rat demonstrated a significant contribution of hepatic uptake to clearance of temocaprilat (Ishizuka

et al. 1998) and grepafloxacin (Sasabe *et al.* 1997). The IPRL has also been used to provide mechanistic evidence that active hepatic uptake is the rate-limiting step for clearance in a series of combined thromboxane synthetase inhibitors/thromboxane A₂ receptor antagonists (Gardner *et al.* 1995). Metabolism of these compounds subsequent to the uptake process occurs to a varying extent but does not change the rate of disappearance from the circulation, although it determines the ultimate excreted form.

In vitro and *in situ* systems expressing transport proteins are essential as they provide additional tools key to the assessment of the importance of active hepatic transport processes in pharmacokinetics. Interestingly, good agreement has been found between K_m values obtained for active uptake into fresh rat hepatocytes and those obtained using expression systems for rat transport proteins (Meier *et al.* 1997). One such compound is pravastatin, which provides an interesting example of the apparent importance of hepatic uptake in the determination of hepatic clearance. Pravastatin is actively transported into rat hepatocytes with $K_m = 29 \mu\text{M}$ (Yamazaki *et al.* 1993) and into rat Oatp1-expressing cells with $K_m = 30 \mu\text{M}$ (Hsiang *et al.* 1999). In addition, pravastatin is actively transported into human hepatocytes, although enzyme kinetics were not determined in this study (van Vliet *et al.* 1995). Similar enzyme kinetics ($K_m = 34 \mu\text{M}$) to those *in vitro* have been determined for an active, saturable clearance process after i.v. steady-state infusions in rat (Yamazaki *et al.* 1996a). Further support for the importance of hepatic uptake of pravastatin to hepatic clearance comes from the accurate prediction of rat *in vivo* hepatic clearance from *in vitro* rates of hepatic uptake in hepatocytes and IPRL (Gardner *et al.* 2000). The conclusion from these studies is that hepatic clearance of pravastatin is considered to be regulated by an efficient uptake process followed by extensive excretion of unchanged pravastatin into bile with minimal efflux out of the hepatocyte. However, this series of studies with pravastatin illustrates the difficulty in deconvoluting from *in vivo* data the contribution of hepatic uptake and biliary excretion of unchanged drug towards hepatic clearance. In such cases, supportive evidence from *in vitro* studies needs to be used to aid understanding of the likely rate-determining processes *in vivo*, as is the case with pravastatin. Identification of the contribution of transport proteins in drug clearance would be significantly advanced by the availability of specific probe substrates and inhibitors and by the generation of specific genetic knockout animals.

In many instances the passage of drugs and/or their metabolites across the canalicular membrane into the bile is achieved or facilitated by the action of transporter proteins. Members of the ATP-binding cassette family of transporters are the main transport proteins involved in active drug secretion into bile and to date three subfamilies have been identified that mediate endobiotic and xenobiotic transport (Meijer *et al.* 1997, Hooiveld *et al.* 2000). One subfamily is the P-glycoproteins encoded by various multidrug-resistant genes (MDR). A second protein is responsible for the ATP-dependent transport of bile acids and is termed the bile salt export pump (BSEP). A third, distinct ATP-dependent transport system is referred to as cMOAT or MRP2.

The functional role of P-glycoprotein on the bile canalicular membrane was originally demonstrated by Kamimoto *et al.* (1989) in a study that demonstrated the ATP-dependent uptake of daunomycin by isolated bile canalicular membranes, uptake that was inhibited by competitive substrates of P-glycoprotein.

Subsequently a number of studies have provided support for the hypothesis that P-glycoprotein is one of the major cation carriers responsible for the biliary excretion of amphiphilic cationic drugs (Schinkel *et al.* 1994, 1995, Smit *et al.* 1996, 1998, Meijer *et al.* 1997). Although many studies have been performed to investigate the effect of P-glycoprotein inhibitors on the plasma or tissue concentrations of drugs, there are only a limited number of reports describing changes in biliary clearance and/or tissue to blood:plasma ratio. Milne *et al.* (2000) reported that erythromycin, a competitive substrate for P-glycoprotein, significantly decreases the biliary clearance of the P-glycoprotein substrate fexofenadine when co-administered in the IPRL. This work helps explain the unexpected clinical finding of drug-drug interactions between fexofenadine and the P-glycoprotein inhibitors, erythromycin and ketoconazole. Similarly, the inhibitory effect of the P-glycoprotein inhibitor, PSC833, on the biliary clearance of colchicine and doxorubicin in rat (Speeg and Madonado 1994) and digoxin in mouse (Mayer *et al.* 1997). Investigations with the *mdr1a* (-/-) knockout mouse have been hampered by the discovery that *mdr1b*, also present in the canalicular membrane, is up-regulated (Schinkel *et al.* 1994). However, studies with these mice have clearly demonstrated that *mdr1a* P-glycoprotein significantly contributes to the biliary secretion of both doxorubicin and vinblastine (van Asperen *et al.* 2000). In addition, work with the dual knockout mouse *mdr1a/b* (-/-) has demonstrated a significant role for P-glycoprotein in the biliary excretion of digoxin (Mayer *et al.* 1996). Furthermore, Schrenk *et al.* (1993) demonstrated that P-glycoprotein, both at the mRNA level and protein levels, was induced in rat by 2-acetylaminofluorene and that this increase resulted in a significant increase in the biliary excretion of vinblastine, which was inhibited by verapamil. Again, these results clearly indicate a significant role of P-glycoprotein in the biliary excretion of xenobiotics. Mouse *mdr2*, and its human orthologue MDR3, are not thought to play a significant role in drug transport (Hooiveld *et al.* 2000). Instead, they serve as the phosphatidylcholine translocator in the biliary canalicular membrane, and the absence of the murine *mdr2* P-glycoprotein results in chronic progressive liver disease (Smit *et al.* 1993, 1998). However, recent *in vitro* work has demonstrated that MDR3 can mediate transport of digoxin, vinblastine and paclitaxel, albeit at lower rates compared with MDR1, but not cyclosporin or dexamethasone (Smith *et al.* 2000).

MRP2 (human) and *mrp2* (rat) have been shown to mediate the transport of endobiotics (e.g. bilirubin and bilirubin conjugates) as well as many anionic drugs and drug conjugates. Grepafloxacin (Sasabe *et al.* 1998) pravastatin (Yamazaki *et al.* 1997), cefodizime (Sathirakul *et al.* 1994), methotrexate (Hooijberg *et al.* 1999) and irinotecan (Chu *et al.* 1997) are examples of drugs that are substrates of MRP2/*mrp2*. Temocaprilat, the active metabolite of the ACE inhibitor temocapril (Ishizuka *et al.* 1997), and SN-38, the active metabolite of irinotecan (Chu *et al.* 1997), are examples of metabolites that are substrates of MRP2/*mrp2*. Acetaminophen glucuronide (Xiong *et al.* 2000), E3040 glucuronide, the glucuronide metabolite of E3040 (Takenaka *et al.* 1995a, b), glucuronide conjugate of telmisartan (Kato *et al.* 2000), grepafloxacin glucuronide (Sasabe *et al.* 1998) and SN-38 glucuronide (Chu *et al.* 1997) are examples of drug conjugates that are substrates for MRP2/*mrp2*. Although several *in vitro* models have been used to identify substrates of MRP2/*mrp2*, elucidation of the key *in vivo* role of MRP2/*mrp2* in the hepatobiliary disposition and elimination of drug and their conjugates has been facilitated by the discovery of three different mutant rats with an hereditary defect

resulting in the loss of expression of functional rat mrp2; the TR⁻ (Jansen *et al.* 1985) and GY rat (Kuipers *et al.* 1988) derived from the Wistar strain, and the EHBR rat derived from the Sprague–Dawley strain (Mimaki *et al.* 1986). When [¹⁴C]-temocapril was given as an i.v. bolus (1 mg kg⁻¹) to the bile-duct cannulated EHBR rat (Ishizuka *et al.* 1997) the AUC_{0–6h} of total radioactivity was increased 6-fold, and biliary clearance of total radioactivity was decreased 20-fold when compared with the control Sprague–Dawley rat. As temocaprilat accounted for > 95% of the total radioactivity in both media, these results provide convincing evidence for the central role of mrp2 in both the disposition and hepatobiliary elimination of this active metabolite. Using this model, a clear *in vivo* role for mrp2 in the hepatobiliary disposition and elimination has been demonstrated for several drugs and their conjugates, such as grepafloxacin and its glucuronide metabolite (Sasabe *et al.* 1998), the carboxylate form of irinotecan, its active metabolite SN-38 and the lactone and carboxylate forms of the glucuronide conjugate of SN-38 (Chu *et al.* 1997), cefodizime (Sathirakul *et al.* 1993) and acetaminophen glucuronide (Xiong *et al.* 2000). Studies using the EHBR rat have indicated a partial role for mrp2 in the biliary excretion of pravastatin (Yamazaki *et al.* 1997) and also the glucuronide conjugate of telmizatan (Kato *et al.* 2000). However, the results clearly indicate a role for other, unidentified, biliary transporters in EHBR. The complexity introduced by multiple roles of transporters in the elimination of drugs emphasizes the need to have appropriate *in vivo* models to evaluate the significance of an individual drug transporter in the disposition and elimination of drugs. In the absence of highly specific and potent inhibitors of drug transporters, the use of mutant strains and knockout models is clearly pivotal in the understanding of this role.

An understanding of the role of MRP2 in the hepatobiliary disposition and elimination of drugs is essential as mutations in the human MRP2 gene have been discovered that result in a lack of expression of MRP2 (Paulusma *et al.* 1997, Kajihara *et al.* 1998, Wada *et al.* 1998, Toh *et al.* 1999, Tsuji *et al.* 1999). The Dubin–Johnson syndrome in humans is an autosomal recessively inherited disorder, characterized by conjugated hyperbilirubinemia and pigment deposition, which is attributed to the absence of MRP2 at the canalicular membrane (Dubin and Johnson 1954, Sprinz and Nelson 1954, Roy Chowdhury *et al.* 1994, Kartenbeck *et al.* 1996). To date, no information on impaired drug elimination in these individuals has been published and there have been no reports linking impaired biliary excretion with inhibition of MRP2. Interestingly, the up-regulation of the basolaterally expressed MRP3 (König *et al.* 1999b, Kool *et al.* 1999), with a similar substrate profile as MRP2, has been reported in the EHBR rat (Hirohashi *et al.* 1998, 1999). This compensatory up-regulation of MRP3 probably represents a mechanism for protection of the hepatocytes from drug and endobiotic exposure when MRP2 is compromised.

As the role of transport protein-mediated processes in hepatobiliary disposition and elimination emerges, an important consideration is whether species differences occur between animals and humans, particularly for drug clearance and excretion. For some drugs, which are cleared unchanged primarily by hepatobiliary excretion, significant species differences in clearance occur between animals and humans, which is not attributable to protein-binding differences. An example of such species differences is the 20- and 7-fold overestimation of human clearance for susalimod (Pahlman *et al.* 1998) and napsagatran (Lavé *et al.* 1999), respectively,

from animal clearance data. For drugs excreted primarily by active renal secretion (e.g. lamifiban, atenolol, famotidine, β -lactam antibiotics, ACE inhibitors) there can also be species differences in clearance but they tend to be less marked, than with hepatic clearance, with only up to 2-fold overestimation of renal clearance from animals to humans (Lin 1995, Lavé *et al.* 1996). One possible explanation for such species differences in pharmacokinetics is that transport proteins are not well conserved across species. However, where data are available on rat and human orthologues, sequence identity is reasonably high (70–80%) for OATP (Hsiang *et al.* 1999), OCT (Zhang *et al.* 1998c), MDR (Klein *et al.* 1999) and MRP (König *et al.* 1999a) transport families. There is some evidence that suggests that the rate of transport by rat transporters is greater than by the equivalent human transporters. For example, uptake rates for some basic compounds were shown to be ~ 10 -fold faster in rat when compared with human hepatocytes (Sandker *et al.* 1994). Also, K_m and K_i for a range of bases were significantly lower for rat OCT1 than human OCT1 (Zhang *et al.* 1998c) suggesting a greater substrate affinity in rat. If these *in vitro* data were to translate into the *in vivo* situation, higher intrinsic clearance would be expected in rat compared with man, which could explain the overestimation of human clearance based upon animal data.

Role of transporter proteins in modulating brain penetration of drugs

The blood–brain barrier (BBB) is located between the blood and brain tissue and consists primarily of brain capillary endothelial cells (BCECs), which are linked by tight junctions to form a lipophilic physical barrier that limits the passive entry of molecules into the brain. In general, BBB permeability increases with increasing lipophilicity (Levin 1980, Sha *et al.* 1989). However, transporter proteins have been identified that facilitate the BBB penetration of many poorly lipophilic compounds, like many essential nutrients (Tamai and Tsuji 2000). Conversely, a number of highly lipophilic drugs, for example cyclosporin, doxorubicin, teniposide, vinblastine and vincristine, all demonstrate unexpectedly poor BBB permeation (Levin 1980, Cefalu and Pardridge 1985, Begley 1992, Saeki *et al.* 1993). Complex interrelationships could be responsible for this phenomenon, e.g. plasma-protein binding, tissue affinity and/or molecular weight. However, there is increasing realisation that the unexpectedly poor BBB permeation of lipophilic drugs is a result of the action of drug transporter efflux proteins such as P-glycoprotein (MDR1) present in the BBB (Tamai and Tsuji 2000).

Molecular characterization of the transport proteins present in the apical and basolateral plasma membrane of BCECs has identified a number of representatives of drug transporter protein families such as MDR, MRP and OATP (Cordon-Cardo *et al.* 1989, Huai-Yun *et al.* 1998, Kusuha *et al.* 1998, Gao *et al.* 1999). Of the identified BBB transport proteins, it is P-glycoprotein, which has been the focus of most recent investigations. P-glycoprotein is expressed in the apical (luminal) membrane of BCEC's and is orientated such that it pumps substrates from inside the cells back into the blood (Schinkel *et al.* 1995).

Several *in vitro* models have been established to investigate the role of P-glycoprotein in the BBB and have identified a wide range of drugs from various therapeutic areas as substrates for this transporter protein. The lack of well-characterized, specific, potent inhibitors have impeded the progress of elucidation of the role P-glycoprotein in the BBB *in vivo*. However, a key tool for investigation

of its role in the BBB has been the generation of the P-glycoprotein knockout mouse (*mdr1a* $(-/-)$) (Schinkel *et al.* 1994). The *mdr1a* $(-/-)$ mouse completely lacks *mdr1a* coded P-glycoprotein, the form expressed in the BBB (Schinkel *et al.* 1994, 1995, Borst and Schinkel 1996, van Asperen *et al.* 1996) due to homozygous disruption of the *mdr1a* gene. Several studies have investigated the brain penetration of drugs in the *mdr1a* $(-/-)$ mouse and the results from these studies have provided unequivocal *in vivo* evidence to support a central role for this transporter protein in protection of the brain from a number of lipophilic drugs (table 2).

As shown in table 2, P-glycoprotein is responsible for limiting the brain penetration of a wide range of structurally diverse compounds from various therapeutic classes and in several instances brain drug levels in the *mdr1a* $(-/-)$ mouse are more than 20 times higher than the brain levels achieved in the wild-type mouse (*mdr1a* $(+/+)$). In addition to the high brain penetration seen in the *mdr1a* $(-/-)$ mouse, lack of P-glycoprotein also appears to have a significant impact on the rate of release of some drugs from the brain once penetrated. For example, following a single i.v. dose of vinblastine, release of drug from the brain of the *mdr1a* $(-/-)$ mouse is very slow and following a single i.v. dose of digoxin there is a gradual accumulation of drug in the brain of the *mdr1a* $(-/-)$ mouse compared with *mdr1a* $(+/+)$ over > 2 days (Schinkel *et al.* 1994, Mayer *et al.* 1996). In addition to the marked increases in brain penetration of P-glycoprotein substrates in the *mdr1a* $(-/-)$ mouse, a similar increase in sensitivity to centrally mediated side-effects for some drugs has also been observed. For example, the *mdr1a* $(-/-)$ mouse is ~ 50 – 100 times more sensitive than *mdr1a* $(+/+)$ to the neurotoxicity of ivermectin, which correlates well with 87-fold increase in brain levels (Schinkel *et al.* 1994).

Table 2. Drugs for which brain penetration is regulated by P-glycoprotein. The brain penetration ratio is determined as the ratio of total radioactivity or drug levels* in the brain of the *mdr1a* $(-/-)$ mouse: the levels determined in the *mdr1a* $(+/+)$ mouse.

Drug	Brain level ratio <i>mdr1a</i> $(-/-)$: <i>mdr1a</i> $(+/+)$	Therapeutic category	Reference
Amprenavir	27	HIV protease inhibitor	Polli <i>et al.</i> (1999)
Asimadoline*	11	analgesic	Jonker <i>et al.</i> (1999)
Azasetron	7	anti-emetic	Tamai and Tsuji (2000)
Carebastin	8	antihistamine	Tamia and Tsuji (2000)
Cyclosporin	17	immune suppressant	Schinkel <i>et al.</i> (1995)
Dexamethasone	3	glucocorticoid	Schinkel <i>et al.</i> (1995)
Digoxin	35	cardiotonic	Schinkel <i>et al.</i> (1995)
Doxorubicin*	3	antineoplastic	van Aperen <i>et al.</i> (1997)
Ebastine	7	antihistamine	Tamia and Tsuji (2000)
Grepafloxacin	3	antibacterial	Tamia and Tsuji (2000)
Indinavir	11	HIV protease inhibitor	Kim <i>et al.</i> (1998)
Ivermectin	87	anthelmintic	Schinkel <i>et al.</i> (1994)
Loperamide	14	antidiarrheal	Schinkel <i>et al.</i> (1996)
Morphine	2	analgesic	Schinkel <i>et al.</i> (1995)
Nelfinavir	36	HIV protease inhibitor	Kim <i>et al.</i> (1998)
Ondansetron	4	anti-emetic	Schinkel <i>et al.</i> (1996)
Paclitaxel*	12	antineoplastic	Van Aperen <i>et al.</i> (1997)
Quinidine	29	anti-arrhythmic	Fromm <i>et al.</i> (1999)
Saquinavir	7	HIV protease inhibitor	Kim <i>et al.</i> (1998)
Tacrolimus*	33	immunosuppressant	Yokagawa <i>et al.</i> (1999)
Verapamil	10	antihypertensive	Hendrikse <i>et al.</i> (1999)
Vinblastine*	22	antineoplastic	Schinkel <i>et al.</i> (1994)

Based on these results, it appears that for certain drugs, the role of P-glycoprotein in the BBB can have a decisive effect on the clinical usage of a drug. For example loperamide, an opioid agent widely used in the treatment of diarrhoea, is hypothesized to be peripherally acting without centrally mediated opiate side-effects solely due to the role of P-glycoprotein in maintaining low brain penetration. In support of this hypothesis, pronounced morphine-like, centrally mediated side-effects were observed in the *mdr1a* ($-/-$) mouse following administration of loperamide at dose levels that showed no side-effects in the *mdr1a* ($+/+$) mouse. Clearly, these results demonstrate a key function of P-glycoprotein in the BBB of preclinical species. However, assessment of the role of P-glycoprotein in BBB function in humans is problematic as brain concentrations cannot be measured readily. Thus, assessment of the role of P-glycoprotein in humans requires the assessment of either centrally mediated pharmacodynamic or toxicodynamic effects in individuals where P-glycoprotein is compromised, either through inhibition or as the result of a genetic polymorphism (Hoffmeyer *et al.* 2000, Kim *et al.* 2000). A recent clinical study investigating loperamide (Sadeque *et al.* 2000) demonstrated that the lack of centrally mediated opiate effects (respiratory depression), which allows it to be used safely in humans, can be reversed when loperamide is co-administered with quinidine, a competitive inhibitor of P-glycoprotein (Fromm *et al.* 1999). A key area for future investigation of the role of human P-glycoprotein in the BBB will be the development of a suitable probe substrate appropriately labelled for continuous monitoring of brain penetration using positron emission tomographic imaging techniques.

The realisation of the impact that P-glycoprotein has on limiting the brain penetration of many drugs has given rise to the possibility that drug delivery to the brain could be enhanced by co-administration with an effective inhibitor of P-glycoprotein. Several studies in animal models have shown that co-administration of an inhibitor or competitive substrate of P-glycoprotein with a substrate can lead to significant increases in brain penetration of the substrate. In Hendrikse *et al.* (1998), the brain levels of [^{11}C]-verapamil (based on total radioactivity) were shown to be ~ 10 fold higher in *mdr1a* ($-/-$) compared with the *mdr1a* ($+/+$) mouse (figure 2).

Following 30-min pretreatment of the *mdr1a* ($+/+$) mouse with cyclosporin (50 mg kg^{-1} i.v.), the effect of *mdr1a* was attenuated, and brain levels of [^{11}C]-verapamil, similar to those achieved in the *mdr1a* ($-/-$) mouse, were observed. Enhanced brain penetration has also been demonstrated for quinidine, digoxin and vinblastine when co-administered with PSC833, a potent inhibitor of P-glycoprotein in animal models (Lemaire *et al.* 1996, Kusuhara *et al.* 1997, Mayer *et al.* 1997). Although these results demonstrate that brain penetration can be enhanced by modulation of P-glycoprotein, two questions arise: will central efficacy be enhanced?; and will effective inhibition of P-glycoprotein at the BBB occur in humans? With respect to the first question, several studies with morphine, a substrate of P-glycoprotein, have demonstrated that the antinociceptive action of morphine is enhanced by co-administration of GF-120918, a potent and selective inhibitor of P-glycoprotein (Letrent *et al.* 1998, 1999). In Zong and Pollack (2000), brain penetration of morphine was increased ~ 2 -fold in mouse when P-glycoprotein was compromised either through genetic knockout or when co-administered with the competitive substrate verapamil. In both cases where P-glycoprotein was compromised the ED_{50} for antinociception was ~ 2 -fold lower

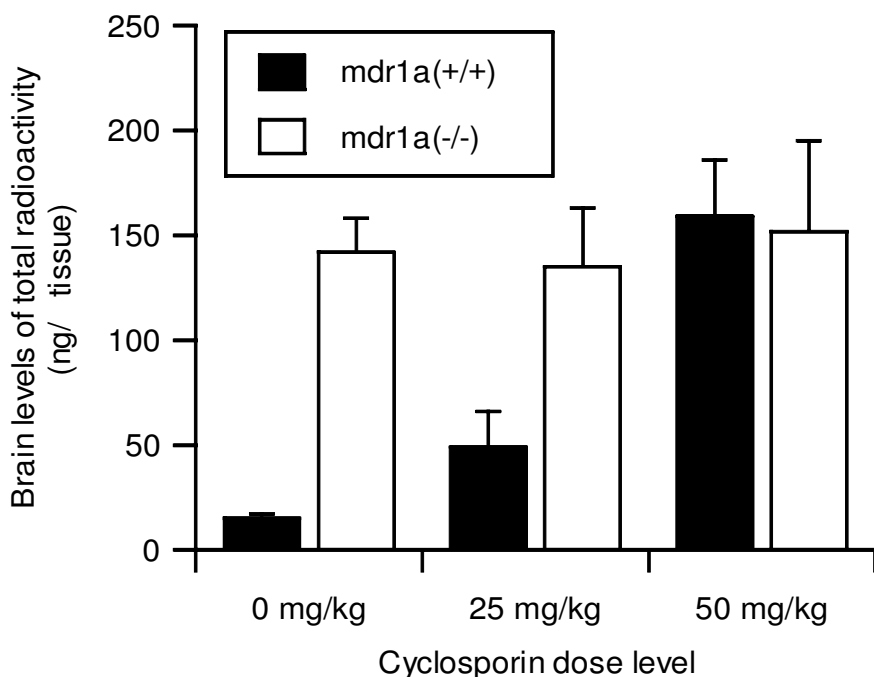


Figure 2. Brain levels of radioactivity in mouse 1 h after an intravenous injection of 0.1 mg kg^{-1} [^{14}C]-verapamil and 30-min pretreatment with various dose levels of cyclosporin A (adapted from Hendrikse *et al.* 1998).

than in the appropriate controls. As the estimated brain EC_{50} 's were similar in both the *mdr1a* (-/-) and *mdr1a* (+/+) mouse, and also in the *mdr1a* (+/+) mouse following co-administration with verapamil, the enhanced antinociceptive effect of morphine in these animals was accounted for by the improved brain penetration of morphine when P-glycoprotein was either absent or inhibited. P-glycoprotein also limits the brain penetration of HIV protease inhibitors amprevir, indinavir, saquinavir and nelfinavir (table 2). Several investigators have speculated that improved brain penetration of HIV protease inhibitors, through the use of selective and potent P-glycoprotein inhibitors, could be used to reduce AIDS-induced dementia complex. Indeed, Polli *et al.* (1999) demonstrated a 13-fold increase in the brain levels of [^{14}C]-amprenavir in the *mdr1a* (+/+) mouse pretreated with GF-120918 when compared with appropriate controls. However, such approaches to improving drug delivery to the brain in AIDS treatment are yet to be proven effective in the clinic, which brings us to the second question. Indeed there are very few examples of clinical interactions involving transport proteins at the BBB that result in increased drug exposure in the brain. One such example is from a recent study showing that central adverse effects (respiratory depression) were observed with the P-glycoprotein substrate loperamide when co-administered with quinidine (Sadeque *et al.* 2000), which was not due to increased loperamide plasma concentrations. Thus, the increased central effects of loperamide can be attributed to inhibition of P-glycoprotein-mediated efflux by quinidine at the BBB. Assessment of the systemic exposure of quinidine at the dose used (600 mg) in the loperamide study suggests that free drug concentrations

could be as high as $2.4\text{ }\mu\text{M}$ at this dose (Rahkit *et al.* 1984), which is close to the quoted $\text{IC}_{50} = 2.2\text{ }\mu\text{M}$ for quinidine inhibition of P-glycoprotein (Wandel *et al.* 1999). Therefore, there is a clear rationale, supported by clinical pharmacokinetic data, that such doses of quinidine can cause inhibition of P-glycoprotein-mediated efflux at the BBB in humans and thus lead to increased drug delivery to the brain. However, for other well-documented P-glycoprotein inhibitors, such as ketoconazole, verapamil, erythromycin and protease inhibitors, which have $\text{IC}_{50} = 1 - 10\text{ }\mu\text{M}$ (Kim *et al.* 1999, Wandel *et al.* 1999), systemic exposure in humans ($\sim 0.015\text{--}0.4\text{ }\mu\text{M}$ free drug C_{max} concentrations) even at maximum therapeutic doses is significantly less than concentrations needed for effective inhibition as judged by the IC_{50} . In addition, these P-glycoprotein inhibitors are even less effective inhibitors of drug uptake by other transporters, such as the human OATPs (Cvetkovic *et al.* 1999). Therefore, despite the observation of P-glycoprotein inhibition in preclinical *in vivo* models (Mayer *et al.* 1997, Polli *et al.* 1999, Choo *et al.* 2000), the lack of reported clinical interactions involving P-glycoprotein at the BBB for drugs such as ketoconazole, verapamil, erythromycin and protease inhibitors can be rationalized by the low systemic exposure achieved in humans at therapeutic doses. In addition, it has to be taken into account that effective inhibitors of P-glycoprotein activity at the BBB will increase not only the brain penetration of the target drug, but also the brain penetration of other co-administered P-glycoprotein substrates. This in turn may give rise to unexpected hypersensitivity to other co-administered P-glycoprotein substrates, in particular those with centrally mediated effects, as exemplified by the interaction between quinidine and loperamide (Sadeque *et al.* 2000). Interestingly, P-glycoprotein has been demonstrated to be inducible in preclinical species and humans (Gant *et al.* 1995, Greiner *et al.* 1999). Although little is known with respect to the inducibility of P-glycoprotein in the BBB, Aquilante *et al.* (1999) showed that chronic administration of morphine induces brain levels of P-glycoprotein and as a result may reduce morphine's antinociceptive effect. This induction of P-glycoprotein may present a potential mechanism to explain development of morphine tolerance.

In summary, P-glycoprotein is an integral element of the BBB and plays a key role in limiting the brain penetration of drugs which are substrates of this transporter. As a result, the degree of expression and functionality of P-glycoprotein, either as a result of substrate affinity, drug-drug interaction (induction or inhibition) or through a genetic polymorphism, may directly affect the therapeutic effectiveness, safety and target specificity of P-glycoprotein substrates.

Potential for drug-drug interactions due to attenuation of drug transport

Clinically significant drug interactions occur when the efficacy or toxicity of a drug is altered by administration of another drug or substance. Traditionally, a change in metabolic clearance of a drug, particularly via cytochrome P450-mediated metabolism, has been considered as the cause of many clinically important drug interactions (von Moltke *et al.* 1998, Dresser *et al.* 2000). However, increasing evidence suggests that some drug interactions believed to be due to changes in metabolic clearance may in part result from changes in the activity of transport proteins. A summary of the main drug-drug interactions thought to involve transport proteins is presented in table 3.

Table 3. Drug-drug interactions due to inhibition of transport proteins.

Inhibitee	Inhibitor	Effect measured	Potential transport proteins
Digoxin	quinidine, verapamil, itraconazole	↑ plasma concentrations; ↓ renal clearance	P-glycoprotein; OATP
Fexofenadine	ketoconazole, erythromycin	↑ plasma concentrations	P-glycoprotein; OATP
Talinolol	verapamil	↑ or ↓ plasma concentrations; ↓ intestinal secretion	P-glycoprotein
Loperamide	quinidine	↑ central adverse effects	P-glycoprotein
Dofetilide, Procainamide, Levofloxacin	cimetidine	↑ in AUC; ↓ renal clearance	OCT; OAT; OATP
Penicillins, ACE inhibitors, antiviral drugs	probenecid	↓ renal clearance; prolong $t_{1/2}$	OAT

In support of the role of transport proteins in drug interactions, several *in vitro* studies have demonstrated that there is overlapping specificity between CYP3A4 and P-glycoprotein substrates and inhibitors (Wacher *et al.* 1995, Zhang *et al.* 1998b, Kim *et al.* 1999). These studies have shown that there are several drugs that are inhibitors for both CYP3A4 and P-glycoprotein including erythromycin, ketoconazole, itraconazole, ritonavir, saquinavir and verapamil. However, no correlation was found between the extent of P-glycoprotein and CYP3A4 inhibition in Wandel *et al.* (1999), where the ratio of the IC₅₀ for CYP3A4 inhibition to the IC₅₀ for P-glycoprotein inhibition varied from 1.1 to 125. Indeed, some P-glycoprotein inhibitors have also been shown to inhibit other transport proteins *in vitro* such as OATP (Cvetkovic *et al.* 1999) and MRP2 (Gutmann *et al.* 1999).

The clearest clinical evidence that drug-drug interactions can be mediated by attenuation of drug transport comes from the fact that the oral exposures of several poorly metabolized drugs, such as fexofenadine, talinolol and digoxin, have been shown to be significantly increased by inhibitors such as ketoconazole, verapamil and erythromycin. For example, ~95% of orally administered fexofenadine is excreted unchanged in humans (Lippert *et al.* 1995), yet ketoconazole and erythromycin co-administration have been shown to cause up to 2.5-fold increases in oral fexofenadine plasma exposure (product information, Aventis). Fexofenadine has been shown to be a substrate for OATP and P-glycoprotein transport proteins (Cvetkovic *et al.* 1999) and thus its oral absorption and subsequent elimination, via hepatic uptake and biliary excretion, are thought to be mediated by transport proteins. Hence, inhibitors of OATP and P-glycoprotein can attenuate fexofenadine pharmacokinetics probably via inhibition of transport-mediated efflux in the gastrointestinal tract and/or inhibition of hepatobiliary excretion (Milne *et al.* 2000). Similarly, the β -blocker talinolol has been shown to be essentially metabolically inert with metabolism accounting for < 5% of total clearance (Trausch *et al.* 1995) and absorption and disposition of talinolol believed to be controlled by P-glycoprotein-mediated transport (Grammaté *et al.* 1996, Wetterich *et al.* 1996). Preclinical studies in rat (Spahn-Langguth *et al.* 1998) and mouse (R. J. Mitchell, personal communication), investigating the effects of oral co-administration with the P-glycoprotein inhibitor verapamil, observed a 4–6-

fold increase in oral exposure of talinolol in the presence of verapamil. The inhibition of intestinal secretion of i.v. talinolol in humans by intraluminal perfusion of verapamil agreed with the preclinical studies (Gramatté and Oertel 1999). Interestingly, Schwarz *et al.* (1999) demonstrated that concomitant oral administration of talinolol and verapamil to healthy volunteers resulted in a decrease in talinolol oral AUC compared with placebo. The authors attributed this result to intestinal metabolism of the relatively low single dose (120 mg) of verapamil rendering it an ineffective P-glycoprotein inhibitor in this study. Digoxin is also relatively metabolically stable with > 76% of a radiolabelled dose in humans being excreted as unchanged digoxin (Hinderling and Hartmann 1991) via renal and biliary secretion thought to be mediated by P-glycoprotein (Ito *et al.* 1992, Schinkel *et al.* 1995, Fromm *et al.* 1999). Several clinically significant drug interactions have been reported with digoxin with ~ 50–300% increases in plasma concentrations after concomitant administration with P-glycoprotein inhibitors such as quinidine (Dahlqvist *et al.* 1980, Pedersen *et al.* 1983, Angelin *et al.* 1987), verapamil (Verschraagen *et al.* 1999), nifedipine (Belz *et al.* 1983) amiodarone (Fenster *et al.* 1985), talinolol (Westphal *et al.* 2000a), PSC833 (Kovarík *et al.* 1999) and itraconazole (Partanen *et al.* 1996, Jalava *et al.* 1997). The interactions can be attributed largely to inhibition of P-glycoprotein-mediated transport of digoxin in the gastrointestinal tract and/or systemic (biliary/renal) elimination, frequently resulting in symptoms of digoxin toxicity.

P-glycoprotein has also been shown to be inducible *in vitro* and *in vivo* by xenobiotics such as rifampicin (Westphal *et al.* 2000b), phenobarbital, dexamethasone, rifampicin, isosafrole, clotrimazole and reserpine (Lin *et al.* 1999), and herbal extracts from St John's Wort (Johne *et al.* 1999, Durr *et al.* 2000). For example, rifampicin pretreatment caused a 4–13-fold increase in hepatic P-glycoprotein in monkey (Gant *et al.* 1995). Similarly, in humans rifampicin pretreatment resulted in a 3.5-fold increase in intestinal P-glycoprotein levels, which correlated with a decrease in oral exposure of both digoxin (Greiner *et al.* 1999) and talinolol (Westphal *et al.* 2000b). The inducibility of some other transport proteins has also been observed with rifampicin pretreatment causing induction of MRP2 in human intestine (Fromm *et al.* 2000).

The kidney plays an important role in the elimination of many drugs which can involve a component of active tubular secretion in renal clearance. It has been demonstrated that various transport proteins mediate the active secretion of drugs, mainly hydrophilic cations (Pritchard and Miller 1997) and hydrophilic anions (Dantzler and Wright 1997), in the renal tubule. Recently the molecular characterization of transport proteins in the kidney has demonstrated that an increasing number of transport protein families, such as OAT, OCT, OATP, MDR and MRP, are represented in the kidney (Inui *et al.* 2000). Consequently, the many examples of drug interactions due to modulation of renal excretion of drugs can be explained, at least in part, by attenuation of active tubular secretion. Thus, the decrease in renal excretion caused by probenecid co-administration with a variety of anionic drugs (penicillins, ACE inhibitors, antiviral drugs) can be attributed, at least in part, to inhibition of OAT-mediated secretion at the basolateral membrane of renal proximal tubules (de Miranda *et al.* 1989, Massarella *et al.* 1996, Somogyi 1996, Sekine *et al.* 1999). For cationic drugs, inhibition of renal excretion by co-administration with drugs such as cimetidine can be partly explained by inhibition of basolateral active secretion by OCT proteins (Christian *et al.* 1984, Somogyi

1996, Abel *et al.* 2000). In addition, inhibition of P-glycoprotein at the apical membrane of renal proximal tubules provides part of the explanation for the digoxin interactions with inhibitors such as quinidine, verapamil, etc. (Dahlqvist *et al.* 1980, Verschraagen *et al.* 1999). Whereas the majority of renal drug interactions have the potential to cause adverse effects and result in dosage decreases for the inhibitee, some interactions have a positive impact such as probenecid inhibition of cidofovir renal clearance that significantly reduces incidences of nephrotoxicity (Cundy 1999).

While there are many clinical examples of drug interactions due to inhibition of transport proteins in the gastrointestinal tract and liver (as discussed above), these occur despite the fact that inhibitors are relatively weak against transport proteins, such as P-glycoprotein, with usually $IC_{50} > 1 \mu M$ (Choo *et al.* 2000). This can be rationalized by the fact that oral drug administration will usually result in high concentrations ($> 1 \mu M$) of inhibitors in the intestinal lumen which will generally exceed the IC_{50} and thus facilitate effective inhibition of transport proteins in gut enterocytes. It follows that after absorption across the gastrointestinal tract, drug concentrations, in the hepatic portal vein, entering the liver will be relatively high as well. In addition, it has been shown that concentrative hepatic uptake of inhibitors significantly increases liver concentrations (Ito *et al.* 1998, Yamano *et al.* 1999, 2000) and thus effective inhibitory concentrations are probably reached in the liver. However, there are very few examples of clinical interactions involving transport proteins at the BBB which result in increased drug exposure in the brain. This lack of examples may result from the fact that alterations in brain disposition may not be reflected in systemic pharmacokinetics, a common end-point in drug-drug interaction studies, and will rely on changes in a pharmacodynamic or toxicodynamic end-point. Alternatively, as discussed previously, the lack of reported clinical interactions involving P-glycoprotein at the BBB for drugs such as ketoconazole, verapamil, erythromycin and protease inhibitors can be rationalized by the low systemic exposure achieved in humans at therapeutic doses. Indeed, Ibrahim *et al.* (2000) showed that for a range of antipsychotic, anti-emetic and Ca^{2+} channel-blocking drugs, ineffective P-glycoprotein inhibition was observed for the majority of drugs even when compared with total therapeutic blood concentrations. Some of the above drugs have also been shown to be ineffective in prevention of multidrug resistance in cancer chemotherapy (Sikic *et al.* 1997) probably because of the same reason of low systemic exposure. A new generation of apparently more potent and selective P-glycoprotein inhibitors, such as valspodar, LY-335979 and XR9576, are in clinical development and it remains to be seen whether these cause more clinically significant P-glycoprotein inhibition. However, the adverse central nervous system effects seen, for example, with cyclosporin (tremors, agitation, confusion) quinidine (cinchonism) and digoxin (confusion, dizziness, nervousness, agitation, amnesia) may have been exacerbated by inhibition or down-regulation of P-glycoprotein. This is an area for further investigation.

Conclusions

It is clear that the field of drug transporter science is a rapidly emerging area in drug metabolism and pharmacokinetics both in terms of molecular characterization of transport proteins and understanding their contribution to drug ADE and

drug response. This review demonstrates that there is increasing preclinical and clinical evidence that supports a key role for a number of transport proteins in drug ADE. However, progress in this field will require the identification of specific probe substrates and inhibitors for novel transporters to aid in the elucidation of the impact of these transporters on the ADE of drugs. The key to our understanding of the *in vivo* role of these transporters will also be the generation of specific genetic knockout animals or identification of inherited mutations similar to those which have significantly advanced our understanding of the roles of *mdr1a/b* and *mrp2* in rodents.

In many cases transporter protein affinity needs to be considered in conjunction with the impact of metabolizing enzymes such as the cytochrome P450s. For example, absorption and hepatobiliary excretion of cyclosporin and saquinavir are clearly influenced by affinity for both P-glycoprotein and CYP3A4. However, for a significant number of drugs, metabolism is negligible and in such cases transport protein affinity becomes the rate-determining step in elimination (for some compounds even when metabolism occurs access to the metabolic site may be rate determining). This is exemplified by the data presented here for fexofenadine, digoxin, talinolol and pravastatin where transport proteins clearly govern the ADE of these drugs. P-glycoprotein has been shown to be a key element in limiting the brain penetration of many substrates and thus plays a central role in determining therapeutic target (central or peripheral), central effectiveness and safety profile. Compelling clinical and preclinical evidence also exists for drug-drug interactions involving transport proteins, in particular for drugs that are poorly metabolized. These interactions have been shown to modulate absorption, brain penetration, hepatic and renal excretion. In some cases the interaction can be fatal, e.g. digoxin-quinidine interaction, or result in unexpected side-effects, e.g. loperamide-quinidine. Therefore, the potential for drug-drug interactions also needs to be considered for transport proteins as well as for metabolizing enzymes.

The evidence presented here makes a clear case for considering the role of transport proteins as a key determining factor in the ADE of a wide variety of structurally diverse drugs. As a consequence, the degree of expression and functionality of transport proteins, either as a result of substrate affinity, drug-drug interaction or through a genetic polymorphism, may directly affect the therapeutic effectiveness, safety and target specificity of substrates. Accordingly, the contribution of transport proteins to drug ADE and to drug response is an area that needs careful consideration in drug discovery and development.

Appendix:

Notations

The following notations were used in this paper.

ADE	absorption, distribution and excretion
CNS	central nervous system
OATP	organic anion transporting polypeptide
ATP	adenosine triphosphate
MRP2	multidrug resistance related protein
MDR	multidrug resistance
OCT	organic cation transporter

OAT	organic anion transporter
AUC	area under the curve
BSP	bromosulphophthalein
CYP3A4	cytochrome P450 3A4
TEA	triethylamine
MPP ⁺	1-methyl-4-phenylpyridinium
IPRL	isolated perfused rat liver
cMOAT	canalicular mulitspecific organic anion transporter
ACE	angiotensin converting enzyme
TR ⁻	transport deficient
GY	Groningen yellow
EHBR	Eisai hyperbilirubinaemic
BBB	blood-brain barrier
BCEC	brain capillary endothelial cells
HIV	human immunodeficiency virus
AIDS	acquired immune deficiency syndrome

Acknowledgements

The authors acknowledge the contribution of the many colleagues at Pfizer and GlaxoSmithKline who have contributed to this review through very helpful discussions.

References

- ABEL, S., BEAUMONT, K. C., CRESPI, C. L., FOX, L., HYLAND, R., JONES, B. C., MUIRHEAD, G. J., SMITH, D. A., VENN, R. F. and WALKER, D. K., 2001, A potential role for P-glycoprotein in the non-proportional pharmacokinetics of UK-343,664 in man. *Xenobiotica*, **31**, 595–606.
- ABEL, S., NICHOLS, D. J., BREARLEY, C. J. and EVE, M. D., 2000, Effect of cimetidine and ranitidine on pharmacokinetics and pharmacodynamics of a single dose of dofetilide. *British Journal of Clinical Pharmacology*, **49**, 64–71.
- ANGELIN, B., ARVIDSSON, A., DAHLQVIST, R., HEDMAN, A. and SCHENK-GUSTAFSSON, K., 1987, Quinidine reduces biliary clearance of digoxin in man. *European Journal of Clinical Investigation*, **17**, 262–265.
- AQUILANTE, C. L., LETRENT, S. P., POLLACK, G. M. and BROUWER, K. L. R., 2000, Increased brain P-glycoprotein in morphine tolerant rats. *Life Sciences*, **66**, PL47–51.
- BEAUMONT, K., HARPER, A., SMITH, D. A. and BENNETT, J., 2000, The role of P-glycoprotein in determining the oral absorption and clearance of the NK2 antagonist, UK-224,671. *European Journal of Pharmaceutical Sciences*, **12**, 41–50.
- BEGLEY, D. J., 1992, The interaction of some centrally active drugs with the blood–brain barrier and circumventricular organs. *Progress in Brain Research*, **91**, 163–169.
- BELZ, G. G., DOERING, W., MUNKES, R. and MATTHEWS, J., 1983, Interaction between digoxin and calcium antagonists and anti-arrhythmic drugs. *Clinical Pharmacology and Therapeutics*, **33**, 410–417.
- BENET, L. Z., WU, C.Y., HEBERT, M. F. and WACHER, V. J., 1996, Intestinal drug metabolism and antitransport processes: a potential paradigm shift in oral drug delivery. *Journal of Controlled Release*, **39**, 139–143.
- BERGWERK, A. J., SHI, X., FORD, A. C., KANAI, N., JACQUEMIN, E., BURK, R. D., BAI, S., NOVIKO, P. M., STIEGER, B., MEIER, P. J., SCHUSTER, V. L. and WOLKO, A. W., 1996, Immunologic distribution of an organic anion transport protein in rat liver and kidney. *American Journal of Physiology*, **271**, G231–G238.
- BORST, P., EVERS, R., KOOL, M. and WIJNHOLDS, J., 1999, The multidrug resistance protein family. *Biochimica et Biophysica Acta*, **1461**, 347–357.
- BORST, P. and SCHINKEL, A. H., 1996, What have you learnt thus far from mice with disrupted P-glycoprotein genes? *European Journal of Cancer*, **32A**, 985–990.

- CEFALU, W. T. and PARDRIDGE, W. M., 1985, Restrictive transport of a lipid soluble peptide (cyclosporin) through the blood-brain barrier. *Journal of Neurochemistry*, **45**, 1954-1956.
- CHOO, E. F., LEAKE, B., WANDEL, C., IMAMURA, H., WOOD, A. J. J., WILKINSON, G. R. and KIM, R. B., 2000, Pharmacological inhibition of P-glycoprotein transport enhances the distribution of HIV-1 protease inhibitors into brain and testes. *Drug Metabolism and Disposition*, **28**, 655-660.
- CHRISTIAN, C. D., MEREDITH, C. G. and SPEEG, K. V., 1984, Cimetidine inhibits renal procainamide clearance. *Clinical Pharmacology and Therapeutics*, **36**, 221-227.
- CHU, X. Y., KATO, Y. and SUGIYAMA, Y., 1997, Multiplicity of biliary excretion mechanisms for irinotecan, CPT-11, and its metabolites in rat. *Cancer Research*, **57**, 1934-1938.
- CIHLAR, T., LIN, D. C., PRITCHARD, J. B., FULLER, M. D., MENDEL, D. B. and SWEET, D. H., 1999, The antiviral nucleotide analogs didanosine and zalcitabine are novel substrates for human and rat renal organic anion transporter 1. *Molecular Pharmacology*, **56**, 570-580.
- CORDON-CARDO, C., O'BRIEN, J. P., CASALS, D., RITTMAN-GRAVER, L., BEIDLER, J. L., MELAMED, M. R. and BERTINO, J. R., 1989, Multidrug resistance gene P-glycoprotein is expressed by endothelial cells at blood-brain barrier sites. *Proceedings of the National Academy of Sciences, USA*, **86**, 695-698.
- CUNDY, K. C., 1999, Clinical pharmacokinetics of the antiviral nucleotide analogues didanosine and zalcitabine. *Clinical Pharmacokinetics*, **36**, 127-143.
- CUI, Y., KONIG, J., LEIER, I., BUCHHOLZ, U. and KEPPLER, D., 2000, Hepatic uptake of bilirubin and its conjugates by the human organic anion-transporting polypeptide SLC21A6. *Journal of Biological Chemistry* (in press).
- CVETKOVIC, M., LEAKE, B., FROMM, M. F., WILKINSON, G. R. and KIM, R. B., 1999, OATP and P-glycoprotein transporters mediate the cellular uptake and excretion of fexofenadine. *Drug Metabolism and Disposition*, **27**, 866-871.
- DAHLQVIST, R., EJVINSSON, G. and SCHENK-GUSTAFSSON, K., 1980, Effect of quinidine on plasma concentration and renal clearance of digoxin. A clinically important drug interaction. *British Journal of Clinical Pharmacology*, **9**, 413-418.
- DANTZLER, W. H. and WRIGHT, S. H., 1997, Renal tubular secretion of organic anions. *Advanced Drug Delivery Reviews*, **25**, 217-230.
- DE MIRANDA, P., GOOD, S. S., YARCHOAN, R., THOMAS, R. V., BLUM, M. R. and MYERS, C. E., 1989, Alteration of zidovudine pharmacokinetics by probenecid in patients with AIDS and AIDS-related complex. *Clinical Pharmacology and Therapeutics*, **46**, 494-500.
- DRESSER, G. K., SPENCE, J. D. and BAILEY, D. G., 2000, Pharmacokinetic-pharmacodynamic consequences and clinical relevance of cytochrome P450 3A4 inhibition. *Clinical Pharmacokinetics*, **38**, 41-57.
- DUBIN, I. N. and JOHNSON, F. B., 1954, Chronic idiopathic jaundice with unidentified pigment in liver cells: a new clinicopathologic entity with report of 12 cases. *Medicine*, **33**, 155-179.
- DURR, D., STEIGER, B., KULLAK-UBLUCK, G. A., RENTSCH, K. M., STEINERT, H. C., MEIER, P. J. and FATTINGER, K., 2000, St John's Wort induces intestinal P-glycoprotein/MDR1 and intestinal and hepatic CYP3A4. *Clinical Pharmacology and Therapeutics*, **68**, 598-604.
- ECKHARDT, U., STUBER, W., DICKNEITE, G., REERS, M. and PETZINGER, E., 1996, First-pass elimination of a peptidomimetic thrombin inhibitor is due to carrier-mediated uptake by the liver. *Biochemical Pharmacology*, **52**, 65-96.
- FENSTER, P. E., WHITE, N. W. and HANSON, C. D., 1985, Pharmacokinetic evaluation of the digoxin-amiodarone interaction. *Journal of American College Cardiology*, **5**, 108-112.
- FOJO, A. T., UEDA, K., SLAMON, D. J., POPLACK, D. G., GOTTESMAN, M. M. and PASTAN, I., 1987, Expression of a multidrug resistance gene in human tumours and tissues. *Proceedings of National Academy of Sciences, USA*, **84**, 265-269.
- FRICKER, G., DREWE, J., HUWYLER, J., GUTMANN, H. and BEGLINGER, C., 1996, Relevance of P-glycoprotein for the enteral absorption of cyclosporin A: *in vitro-in vivo* correlation. *British Journal of Pharmacology*, **118**, 1841-1847.
- FROMM, M. F., KAUFMANN, H. M., FRITZ, P., BURK, O., KROEMER, H. K., WARZOK, R. W., EICHELBAUM, M., SIEGMUND, W. and SCHRENK, D., 2000, The effect of rifampicin treatment on intestinal expression of human MRP transporters. *American Journal of Pathology*, **157**, 1575-1580.
- FROMM, M. F., KIM, R. B., STEIN, C. M., WILKINSON, G. R. and RODEN, D. M., 1999, Inhibition of P-glycoprotein-mediated drug transport: a unifying mechanism to explain the interaction between digoxin and quinidine. *Circulation*, **99**, 552-557.
- GAN, L. S. L., MOSELEY, G. M., KHOSLA, B., AUGUSTIJNS, P. F., BRADSHAW, T. P., HENDREN, R. W. and THAKKER, D. R., 1996, CYP3A-like cytochrome P450-mediated metabolism and polarised efflux of cyclosporin A in Caco-2 cells. *Drug Metabolism and Disposition*, **24**, 344-349.
- GANT, T. W., O'CONNOR, C. K., CORBITT, R., THORGEIRSSON, U. and THORGEIRSSON, S. S., 1995, *In vivo* induction of liver P-glycoprotein expression by xenobiotics in monkeys. *Toxicology and Applied Pharmacology*, **133**, 269-276.

- GAO, B., STEIGER, B., NOE, B., FRITSCHY, J. M. and MEIJER, P. J., 1999, Localisation of the organic anion transporting polypeptide 2 (Oatp2) in capillary endothelium and choroid plexus epithelium of rat brain. *Journal of Histochemistry and Cytochemistry*, **47**, 1255–1263.
- GARDNER, I. B., FENNER, K. and MORGAN, P., 2000, Using the model compound pravastatin to understand *in vitro-in vivo* correlations for transport proteins. *Drug Metabolism Reviews*, **32** (suppl. 1), 55.
- GARDNER, I. B., WALKER, D. K., LENNARD, M. S., SMITH, D. A. and TUCKER, G. T., 1995, Comparison of the disposition of two novel combined thromboxane synthase inhibitors/thromboxane A₂ receptor antagonists in the isolated perfused rat liver. *Xenobiotica*, **25**, 185–197.
- GOLDSTEIN, G. W. and BETZ, A. L., 1986, The blood–brain barrier. *Science American*, **255**, 70–79.
- GRAMATTE, T. and OERTEL, R., 1999, Intestinal secretion of intravenous talinolol is inhibited by luminal R-verapamil. *Clinical Pharmacology and Therapeutics*, **66**, 239–245.
- GRAMATTE, T., OERTEL, R., TERHAAG, B. and KIRCH, W., 1996, Direct demonstration of small intestinal secretion and site-dependent absorption of the β -blocker talinolol in humans. *Clinical Pharmacology and Therapeutics*, **59**, 541–549.
- GREINER, B., EICHELBAUM, M., FRITZ, P., KREICHGAUER, H. P., VON RICHTER, O., ZUNDLER, J. and KROEMER, H. K., 1999, The role of intestinal P-glycoprotein in the interaction of digoxin and rifampicin. *Journal of Clinical Investigation*, **104**, 147–153.
- GUTMANN, H., FRICKER, G., DREWE, J., TOEROEK, M. and MILLER, D. S., 1999, Interactions of HIV protease inhibitors with ATP-dependent drug export proteins. *Molecular Pharmacology*, **56**, 383–389.
- HAGENBUCH, B., 1997, Molecular properties of hepatic uptake systems for bile acids and organic anions. *Journal of Membrane Biology*, **160**, 1–8.
- HEBERT, M. F., 1997, Contributions of hepatic and intestinal metabolism and P-glycoprotein to cyclosporin and tacrolimus oral drug delivery. *Advanced Drug Delivery Reviews*, **27**, 201–214.
- HENDRIKSE, N. H., SCHINKEL, A. H., DE VRIES, E. G. E., FLUKS, E., VAN DER GRAAF, W. T. A., WIOLEMSSEN, A. T. M., VAALBURG, W. and FRANSSEN, E. J. F., 1998, Complete *in vivo* reversal of P-glycoprotein pump function in the blood–brain barrier visualised with positron emission tomography. *British Journal of Pharmacology*, **124**, 1413–1418.
- HINDERLING, P. H. and HARTMANN, D., 1991, Pharmacokinetics of digoxin and main metabolites/derivatives in healthy humans. *Therapeutic Drug Monitoring*, **13**, 381–401.
- HIROHASHI, T., SUZUKI, H., ITO, K., OGAWA, K., KUME, K., SHIMIZU, T. and SUGIYAMA, Y., 1998, Hepatic expression of multidrug resistance-associated protein-like proteins maintained in Eisai hyperbilirubinemic rats. *Molecular Pharmacology*, **53**, 1068–1075.
- HIROHASHI, T., SUZUKI, H. and SUGIYAMA, Y., 1999, Characterisation of the transport properties of cloned multidrug resistance-associated protein 3 (MRP3). *Journal of Biological Chemistry*, **274**, 15181–15185.
- HOFFMEYER, S., BURK, O., VON RICHTER, O., ARNOLD, H. P., BROCKMOLLER, J., JOHNE, A., CASCORBI, I., GERLO, T., ROOTS, I., EICHELBAUM, M. and BRINKMANN, U., 2000, Functional polymorphisms of the human multidrug-resistance gene: multiple sequence variations and correlation of one allele with P-glycoprotein expression and activity *in vivo*. *Proceedings of National Academy of Sciences, USA*, **97**, 3473–3478.
- HOOIJBERG, J. H., BROKTERMAN, H. J., KOOL, M., ASSARAF, Y. G., PETERS, G. J., NOORDHUIS, P., SCHEPER, R. J., BORST, P., PINEDO, H. M. and JANSEN, G., 1999, Antifolate resistance mediated by the multidrug resistance proteins MRP1 and MRP2. *Cancer Research*, **59**, 2532–2535.
- HOOVELD, G. J. E. J., VAN MONTFOORT, J. E., MEIJER, D. K. F. and MULLER, M., 2000, Function and regulation of ATP-binding cassette transport proteins involved in hepatobiliary transport. *European Journal of Pharmaceutical Sciences*, **12**, 13–30.
- HSIANG, B., ZHU, Y., WANG, Z., WU, Y., SASSEVILLE, V., YANG, W. P. and KIRCHGESSNER, T. G., 1999, A novel human hepatic organic anion transporting polypeptide (OATP2). *Journal of Biological Chemistry*, **274**, 37161–37168.
- HUAI-YUN, H., SECREST, D. T., MARK, K. S., CARNEY, D., BRANDQUIST, C., ELMQUIST, W. F. and MILLER, D. W., 1998, Expression of multidrug resistance-associated protein (MRP) in brain microvessel endothelial cells. *Biochemical and Biophysical Research Communications*, **243**, 816–820.
- HUNTER, J. and HIRST, B. H., 1997, Intestinal secretion of drugs. The role of P-glycoprotein and related drug efflux systems in limiting oral drug absorption. *Advanced Drug Delivery Reviews*, **25**, 129–157.
- IBRAHIM, S., PEGGINS, J., KNAPTON, A., LICHT, T. and ASZALOS, A., 2000, Influence of antipsychotic, antiemetic and Ca²⁺ channel blocker drugs on the cellular accumulation of the anticancer drug daunorubicin: P-glycoprotein modulation. *Journal of Pharmacology and Therapeutics*, **295**, 1276–1283.
- INUI, K. I., MASUDA, S. and SAITO, H., 2000, Cellular and molecular aspects of drug transport in the kidney. *Kidney International*, **58**, 944–958.

- ITO, K., IWATSUBO, T., KANAMITSU, S., UEDA, K., SUZUKI, H. and SUGIYAMA, Y., 1998, Prediction of pharmacokinetic alterations caused by drug-drug interactions: metabolic interaction in the liver. *Pharmacological Reviews*, **50**, 387-411.
- ITO, S., KOREN, G. and HARPER, P. A., 1992, Energy-dependent transport of digoxin across renal tubular cell monolayers (LLC-PK1). *Canadian Journal of Physiology and Pharmacology*, **71**, 40-47.
- ISHIZUKA, H., KONNO, K., NAGANUMA, H., NISHIMURA, K., KOUZUKI, H., SUZUKI, H., STIEGER, B., MEIER, P. J. and SUGIYAMA, Y., 1998, Transport of temocaprilat into rat hepatocytes: role of organic anion transporting polypeptide. *Journal of Pharmacology and Experimental Therapeutics*, **287**, 37-42.
- ISHIZUKA, H., KONNO, K., NAGANUMA, H., SASAHARA, K., KAWAHARA, Y., NIMURA, K., SUZUKI, H. and SUGIYAMA, Y., 1997, Temocaprilat, a novel angiotensin-converting enzyme inhibitor, is excreted in bile via an ATP-dependent active transporter (cMOAT) that is deficient in Eisai hyperbilirubinemic mutant rats (EHBR). *Journal of Pharmacology and Experimental Therapeutics*, **280**, 1304-1311.
- JANSEN, P. L. M., PETER, W. H. M. and LAMERS, W. H., 1985, Hereditary chronic conjugated hyperbilirubinaemia in mutant rats caused by defective hepatic anion transport. *Hepatology*, **5**, 573-579.
- JALAVA, K.M., PARTANEN, J. and NEUVONEN, P. J., 1997, Itraconazole decreases renal clearance of digoxin. *Therapeutic Drug Monitoring*, **19**, 609-613.
- JOHNE, A., BROCKMOLLER, J., BAUER, S., MAURER, A., LANGHEINRICH, M. and ROOTS, I., 1999, Pharmacokinetic interaction of digoxin with an herbal extract from St John's Wort (*Hypericum perforatum*). *Clinical Pharmacology and Therapeutics*, **66**, 338-345.
- JONKER, J. W., WEGENAAR, E., VAN DEEMETER, L., GOTTSCHILD, R., BENDER, H. M., DASENBROCK, J. and SCHINKEL, A. H., 1999, Role of the blood-brain barrier P-glycoprotein in limiting brain accumulation and sedative side-effects of asimadoline, a peripherally-acting analgesic drug. *British Journal of Clinical Pharmacology*, **127**, 43-50.
- KAJIHARA, S., HIZATOMI, A., MIZUTA, T., HARA, T., OZAKI, I., WADA, I. and YAMAMOTO, K., 1998, A splice mutation in the human canalicular multispecific organic anion transporter gene causes Dubin-Johnson syndrome. *Biochemical et Biophysical Research Communications*, **253**, 454-457.
- KAMIMOTO, Y., GATMAITAN, Z., HSU, J. and ARIAS, I. M., 1989, The function of Gp170, the multidrug resistance gene product, in rat liver canalicular membrane vesicles. *Journal of Biological Chemistry*, **264**, 11693-11698.
- KARTENBECK, J., LEUSCHNER, U., MAYER, R. and KEPPLER, D., 1996, Absence of the canalicular isoform of the MRP gene-encoded conjugate export pump from the hepatocytes in Dubin-Johnson syndrome. *Hepatology*, **23**, 1061-1066.
- KATO, Y., IGARASHI, T. and SUGIYAMA, Y., 2000, Both cMoat/MRP2 and another unknown transporter(s) are responsible for the biliary excretion of glucuronide conjugate of the nonpeptide angiotensin II antagonist, telmisaltan. *Drug Metabolism and Disposition*, **28**, 1146-1148.
- KEPPLER, D. and ARIAS, I. M., 1997, Transport across the hepatocyte canalicular membrane. *FASEB Journal*, **11**, 15-17.
- KIM, R. B., 2000, Transporters in drug disposition. *Current Opinion in Drug Discovery and Development*, **3**, 94-101.
- KIM, R. B., FROMM, M. F., WANDEL, C., LEAKE, B., WOOD, A. J. J., RODEN, D. M. and WILKINSON, G. R., 1998, The drug transporter P-glycoprotein limits oral absorption and brain entry of HIV-1 protease inhibitors. *Journal of Clinical Investigation*, **101**, 289-294.
- KIM, R. B., LEAKE, B., CHOO, E., DRESSER, G. K., KUBBA, S. V., SCHWARZ, U. I., TAYLOR, A., XIE, H. G., STEIN, C. M., WOOD, A. J. J., MCKINSEY, J., SCHUETZ, E. G., SCHUETZ, J. D. and WILKINSON, G. R., 2000, Identification of functionally important MDR1 variant alleles among African-American and Caucasian subjects. *Drug Metabolism Reviews*, **32** (suppl. 2), 199.
- KIM, R. B., WANDEL, C., LEAKE, B., CVETKOVIC, M., FROMM, M. F., DEMPSEY, P. J., RODEN, M. M., BELAS, F., CHAUDHARY, A. K., RODEN, D. M., WOOD, A. J. J. and WILKINSON, G. R., 1999, Interrelationship between substrates and inhibitors of human CYP3A and P-glycoprotein. *Pharmaceutical Research*, **16**, 408-414.
- KLEIN, I., SAKARDI, B. and VARADI, A., 1999, An inventory of the human ABC proteins. *Biochimica et Biophysica Acta*, **1461**, 237-262.
- KOEPSSELL, H., 1998, Organic cation transporters in intestine, kidney, liver and brain. *Annual Review of Physiology*, **60**, 243-266.
- KÖNIG, J., CUI, Y., NIES, A. T. and KEPPLER, D., 2000a, A novel human organic anion transporting polypeptide localised to the basolateral hepatocyte membrane. *American Journal of Physiology and Gastrointestinal Liver Physiology*, **278**, G156-G164.
- KÖNIG, J., CUI, Y., NIES, A. T. and KEPPLER, D., 2000b, Localisation and genomic organisation of a new hepatocellular organic anion transporting polypeptide. *Journal of Biological Chemistry*, **275**, 23161-23168.

- KÖNIG, J., NIES, A. T., CUI, Y., LEIER, I. and KEPPLER, D., 1999a, Conjugate export pumps of the multidrug resistance protein (MRP) family: localisation, substrate specificity, and MRP2-mediated drug resistance. *Biochimica et Biophysica Acta*, **1461**, 377–394.
- KÖNIG, J., ROST, D., CUI, Y. and KEPPLER, D., 1999b, Characterization of the human multidrug resistance protein isoform MRP3 localized to the basolateral hepatocyte membrane, *Hepatology*, **29**, 1156–1163.
- KOOL, M., DE HAAS, M., SCHEER, G. L., SCHEPER, R. J., VAN EIJK, M. J. T., JUIJN, J. A., BAAS, F. and BORST, P., 1997, Analysis of expression of cMOAT (MRP2), MRP3, MRP4 and MRP5, homologues of the multidrug resistance-associated protein gene (MRP1), in human cancer cell lines. *Cancer Research*, **57**, 3537–3547.
- KOOL, M., VAN DER LINDEN, M., DE HAAS, M., SCHEER, G. L., DE VREE, J. M. L., SMITH, A. J., JANSEN, G., PETERS, G. J., PONNE, N., SCHEPER, R. J., OUDE-ELFERINK, R. P. J., BAAS, F. and BORST, P., 1999, MRP3 an organic anion transporter able to transport anticancer drugs. *Proceedings of the National Academy of Sciences, USA*, **96**, 6914–6919.
- KOVARIK, J. M., RIGAUDY, L., GUERRET, M., GERBEAU, C. and ROST, K.L., 1999, Longitudinal assessment of P-glycoprotein-mediated drug interaction of valsopodar on digoxin. *Clinical Pharmacology and Therapeutics*, **66**, 391–400.
- KUIPERS, F., ENSERINK, E., VAN DER STEEN, A. B. M., HARDONK, M. J., FEVERY, J. and VONK, R. J., 1988, Separate transport systems for biliary excretion of sulfated and unsulfated bile acid in the rat. *Journal of Clinical Investigation*, **81**, 1593–1599.
- KULLAK-ÜBLICK, G.A., 1999, Regulation of organic anion and drug transporters of the sinusoidal membrane. *Journal of Hepatology*, **31**, 563–573.
- KUSUHARA, H., SEKINE, T., UTSUNOMIYA-TATE, N., TSUDA, M., KOJIMA, R., HO CHA, S., SUGIYAMA, Y., KANAI, Y. and ENDOU, H., 1999, Molecular cloning and characterisation of a new multispecific organic anion transporter from rat brain. *Journal of Biological Chemistry*, **274**, 13675–13680.
- KUSUHARA, H., SUZUKI, H., NAITO, M., TSURUO, T. and SUGIYAMA, Y., 1998, Characterisation of efflux transport of organic anions in a mouse brain capillary endothelial cell line. *Journal of Pharmacology and Experimental Therapeutics*, **285**, 1260–1265.
- KUSUHARA, H., SUZUKI, H., TERASAKI, T., KAKEE, A., LEMAIRE, M. and SUGIYAMA, Y., 1997, P-glycoprotein mediates the efflux of quinidine across the blood–brain barrier. *Journal of Pharmacology and Experimental Therapeutics*, **283**, 574–580.
- LAVE, T., PORTMANN, R., SCHENKER, G., GIANNI, A., GUENZI, A., GIROMETTA, M.A. and SCHMITT, M., 1999, Interspecies pharmacokinetic comparisons and allometric scaling of napsagatran, a low molecular weight thrombin inhibitor. *Journal of Pharmacy and Pharmacology*, **51**, 85–91.
- LAVE, T., SANER, A., COASSOLO, P., BRANDT, R., SCHMITT-HOMANN, A. H. and CHOU, R. C., 1996, Animal pharmacokinetics and interspecies scaling from animals to man of lamifiban, a new platelet aggregation inhibitor. *Journal of Pharmacy and Pharmacology*, **48**, 573–577.
- LEBLANC, G. A., 1994, Hepatic vectorial transport of xenobiotics. *Chemico-Biological Interactions*, **90**, 101–120.
- LEMAIRE, M., BRUELISAUER, A., GUNTZ, P. and SATO, H., 1996, Dose-dependent brain penetration of SDZ PSC833, a novel multidrug resistance reversing cyclosporin, in rat. *Cancer Chemotherapy and Pharmacology*, **38**, 481–486.
- LETRENT, S. P., POLLACK, G. M., BROUWER, K. R. and BROUWER, K. L. R., 1998, Effect of GF120918, a potent P-glycoprotein inhibitor, on morphine pharmacokinetics and pharmacodynamics in the rat. *Pharmaceutical Research*, **15**, 599–605.
- LETRENT, S. P., POLLACK, G. M., BROUWER, K. R. and BROUWER, K. L. R., 1999, Effects of a potent and specific P-glycoprotein inhibitor on the blood–brain barrier distribution and antinociceptive effect of morphine in the rat. *Drug Metabolism and Disposition*, **27**, 827–834.
- LEVIN, V. A., 1980, Relationship of octanol/water partition coefficient and molecular weight to rat brain capillary permeability. *Journal of Medicinal Chemistry*, **23**, 682–684.
- LI, L., LEE, T. K., MEIER, P. J. and BALLATORI, N., 1998, Identification of glutathione as a driving force and leukotriene C4 as a substrate for oap1, the hepatic sinusoidal organic solute transporter. *Journal of Biological Chemistry*, **273**, 16184–16191.
- LI, L., MEIER, P. J. and BALLATORI, N., 2000, Oap2 mediates bidirectional organic solute transport: a role for intracellular glutathione. *Molecular Pharmacology*, **58**, 335–340.
- LIN, J. H., 1995, Species similarities and differences in pharmacokinetics. *Drug Metabolism and Disposition*, **23**, 1008–1021.
- LIN, J. H., CHIBA, M. and BAILLIE, T. A., 1999, Is the role of the small intestine in first-pass metabolism overestimated? *Pharmacological Reviews*, **51**, 135–157.
- LIPPERT, C., LING, J., BROWN, P., BURNASTER, S., ELLER, M., CHENG, L., THOMPSON, R. and WIER, S., 1995, Mass balance and pharmacokinetics of MDL 16455A in healthy male volunteers. *Pharmaceutical Research*, **12** (suppl. 9), S390.

- LOWN, K. S., FONTANA, R. J., SCHMIEDLIN-REN, P., TURGEON, D. K. and WATKINS, P. B., 1995, Interindividual variation in intestinal *mdr1* expression: lack of short term dietary effects. *Gastroenterology*, **108**, A737.
- LOWN, K. S., MAYO, R. R., LEICHTMAN, A. B., HSIAO, H. L., TURGEON, D. K., SCHMIEDLIN-REN, P., BROWN, M. B., GUO, W., ROSSI, S. J., BENET, L. Z. and WATKINS, P. B., 1997, Role of intestinal P-glycoprotein (*mdr1*) in interpatient variation in the oral bioavailability of cyclosporine. *Clinical Pharmacology and Therapeutics*, **62**, 248–260.
- MASSARELLA, J. W., NAZARENO, L. A., PASSE, S. and MIN, B., 1996, The effect of probenecid on the pharmacokinetics of zalcitabine in HIV-positive patients. *Pharmaceutical Research*, **13**, 449–452.
- MAYER, U., WAGENAAR, E., BEIJNEN, J. H., SMIT, J. W., MEIJER, D. K. F., VAN ASPEREN, J., BORST, P. and SCHINKEL, A. H., 1996, Substantial excretion of digoxin via the intestinal mucosa and prevention of long-term digoxin accumulation in the brain by the *mdr1a* P-glycoprotein. *British Journal of Pharmacology*, **119**, 1038–1044.
- MAYER, U., WAGENAAR, E., DOROBOK, B., BEIJNEN, J. H., BORST, P. and SCHINKEL, A. H., 1997, Full blockade of intestinal P-glycoprotein and extensive inhibition of blood–brain barrier P-glycoprotein by oral treatment of mice with PSC833. *Journal of Clinical Investigation*, **100**, 2430–2436.
- MEIER, P. J., ECKHARDT, U., SCHROEDER, A., HAGENBUCH, B. and STIEGER, B., 1997, Substrate specificity of sinusoidal bile acid and organic anion uptake systems in rat and human liver. *Hepatology*, **26**, 1667–1677.
- MEIJER, D. K. F., MOL, W. E. M., MULLER, M. and KURZ, G., 1990, Carrier-mediated transport in the hepatic distribution and elimination of drugs with special reference to the category of organic cations. *Journal of Pharmacokinetics and Biopharmaceutics*, **18**, 35–70.
- MEIJER, D. K. F., SMIT, J. W. and MULLER, M., 1997, Hepatobiliary elimination of cationic drugs: the role of P-glycoproteins and other ATP-dependent transporters. *Advanced Drug Delivery Reviews*, **25**, 159–200.
- MILNE, R. J. and BUCKLEY, M. T., 1991, Celiprolol: an updated review of its pharmacokinetic properties and therapeutic efficacy in cardiovascular diseases. *Drugs*, **41**, 941–969.
- MILNE, R. W., LARSEN, L. A., JORGENSEN, K. L., BASTLUND, J. F., STRETCH, G. L. and EVANS, A. M., 2000, Fexofenadine: a substrate for OATP and P-GP in the liver. Paper presented at the Millennium World Congress of Pharmaceutical Sciences, San Francisco, CA, 16–20 April.
- MIMAKI, T., NOZAKI, T., TAGAYA, O., HOSOKAWA, S., NAKURA, T., MORI, H., KONDOU, S., MATSUBARA, Y. and OUGOH, T., 1986, The character of a new mutant in rats with hyperbilirubinuria syndrome. *Cong. Anom.*, **26**, 250–251.
- MORGAN, P., BEAUMONT, K., FENNER, K., LEAKE, B. and KIM, R. B., 1999, Identification of OATP-mediated uptake of a series of lipophilic bases. Paper presented at the 9th North American ISSX Meeting, Nashville, TN, 25–29 October.
- MÜLLER, M. and JANSEN, P. L. M., 1997, Molecular aspects of hepatobiliary transport. *American Journal of Physiology*, **272**, G1285–G1303.
- MÜLLER, M. and JANSEN, P. L. M., 1998, The secretory function of the liver: new aspects of hepatobiliary transport. *Journal of Hepatology*, **28**, 344–354.
- NADEMANEE, K., KANNAN, R., HENDRICKSON, J., OOKHTENS, M., KAY, I. and SINGH, B. N., 1984, Amiodarone-digoxin interaction: clinical significance, time-course of development, potential pharmacokinetic mechanisms and therapeutic implications. *Journal of American College of Cardiology*, **4**, 111–116.
- NAGEL, G., VOLK, C., FRIEDRICH, T., ULZHEIMER, J. C., BAMBERG, E. and KOEPSSELL, H., 1997, A reevaluation of substrate specificity of the rat cation transporter rOCT1. *Journal of Biological Chemistry*, **272**, 31953–31956.
- NOBLE, S. and FAULDS, D., 1996, Saquinavir: a review of its pharmacology and clinical potential in the management of HIV infection. *Drugs*, **52**, 93–112.
- OUDE ELFERINK, R. P. J., MEIJER, D. K. F., KUIPERS, F., JANSEN, P. L. M., GROEN, A. K. and GROOTHUIS, G. M. M., 1995, Hepatobiliary secretion of organic compounds; molecular mechanisms of membrane transport. *Biochimica et Biophysica Acta*, **1241**, 215–268.
- PAHLMAN, I., EDHOLM, M., KANKAANRANTHA, S. and ODELL, M. L., 1998, Pharmacokinetics of susalimod, a highly biliary-excreted sulphasalazine analogue, in various species. Nonpredictable human clearance by allometric scaling. *Pharmacy and Pharmacology Communications*, **4**, 493–498.
- PARTANEN, J., JALAVA, K. M. and NEUVONEN, P. J., 1996, Itraconazole increases serum digoxin concentrations. *Pharmacology and Toxicology*, **79**, 274–276.
- PAULUSMA, C. C., KOOL, M., BOSMA, P. J., SCHEER, G. L., TER BORG, F., SCHEPER, R. J., TYTGAT, G. N., BORST, P., BAAS, F. and OUDE ELFERINK, R. P. J., 1997, A mutation in the human canalicular multispecific organic anion transporter gene causes the Dubin–Johnson syndrome. *Hepatology*, **25**, 1539–1542.
- PEDERSEN, K. E., CHRISTIANSEN, B. D., KLITGAARD, N. A. and NIELSEN-KUDSK, F., 1983, Effect of quinidine on digoxin bioavailability. *European Journal of Clinical Pharmacology*, **24**, 41–47.

- PETZINGER, E., 1994, Transport of organic anions in the liver. An update on bile acid, fatty acid, monocarboxylate, anionic amino acid, cholephilic organic anion and anionic drug transport. *Review of Physiology and Biochemical Pharmacology*, **123**, 47–211.
- POLLI, J. W., JARRETT, J. L., STUDENBERG, S. D., HUMPHREYS, J. E., DENNIS, S. W., BROUWER, K. R. and WOOLLEY, J. L., 1999, Role of P-glycoprotein on the CNS disposition of amprenavir (141W94), an HIV protease inhibitor. *Pharmaceutical Research*, **16**, 1206–1212.
- PRITCHARD, J. B. and MILLER, D. S., 1997, Renal secretion of organic cations: a multistep process. *Advanced Drug Delivery Reviews*, **25**, 231–242.
- RAHKIT, A., HOLFORD, N. H., GUENTERT, T. W., MALONEY, K. and RIEGELMAN, S., 1984, Pharmacokinetics of quinidine and three of its metabolites in man. *Journal of Pharmacokinetics and Biopharmaceutics*, **12**, 1–21.
- REICHEL, C., GAO, B., VAN MONTFOORT, J., CATTORI, V., RAHNER, C., HAGENBUCH, B., STIEGER, B., KAMISAKO, T. and MEIER, P. J., 1999, Localisation and function of the organic anion transporting polypeptide Oatp2 in rat liver. *Gastroenterology*, **117**, 688–695.
- ROBBINS, D. K., CASTLES, M. A., PACK, D. J., BHARGAVA, V. O. and WEIR, S. J., 1998, Dose proportionality and comparison of single and multiple dose pharmacokinetics of fexofenadine (MDL 16455) and its enantiomers in healthy male volunteers. *Biopharmaceutics and Drug Disposition*, **19**, 455–463.
- ROY CHOWDHURY, J., ROY CHOWDHURY, N., WOLFKO, A. W. and ARIAS, I. M., 1994, Heme and bile pigment metabolism. In I. M. Arias, J. L. Boyer, N. Fausto, W. B. Jakoby, D. A. Schachter and D. A. Shafritz (eds), *The Liver: Biology and Pathobiology* (New York: Raven), pp. 471–504.
- SADEQUE, A. J. M., WANDEL, C., HE, H., SHAH, S. and WOOD, A. J. J., 2000, Increased drug delivery to the brain by P-glycoprotein inhibition. *Clinical Pharmacology and Therapeutics*, **68**, 231–237.
- SAEKI, T., UEDA, K., TANIGAWARA, Y., HORI, R. and KOMANO, T., 1993, Human P-glycoprotein transports cyclosporin A and FK506. *Journal of Biological Chemistry*, **268**, 6077–6080.
- SANDKER, G. W., WEERT, B., OLINGA, P., WOLTERS, H., SLOOF, M. J. H., MEIJER, D. K. F. and GROOTHUIS, G. M. M., 1994, Characterisation of transport in isolated human hepatocytes. *Biochemical Pharmacology*, **47**, 2193–2200.
- SATHIRAKUL, K., SUSUKI, H., YAMADA, T., HANANO, M. and SUGIYAMA, Y., 1994, Multiple transport systems for organic anions across the bile canalicular membrane. *Journal of Pharmacology and Experimental Therapeutics*, **268**, 65–73.
- SASABE, H., TERASAKI, T., TSUJI, A. and SUGIYAMA, Y., 1997, Carrier-mediated hepatic uptake of quinolone antibiotics in the rat. *Journal of Pharmacology and Experimental Therapeutics*, **282**, 162–171.
- SASABE, H., TSUJI, A. and SUGIYAMA, Y., 1998, Carrier-mediated mechanism for the biliary excretion of the quinolone antibiotic grepafloxacin and its glucuronide in rats. *Journal of Pharmacology and Experimental Therapeutics*, **284**, 1033–1039.
- SCHINKEL, A. H., 1999, P-glycoprotein, a gatekeeper in the blood–brain barrier. *Advanced Drug Delivery Reviews*, **36**, 179–194.
- SCHINKEL, A. H., SMIT, J. J., VAN TELLINGEN, O., BEIJEN, J. H., WAGENAAR, E., VAN DEEMTER, L., MOL, C. A. A. M., VAN DER VALK, M. A., ROBANUSMAANDAG, E. C. and BORST, P., 1994, Disruption of the mouse *mdr1a* P-glycoprotein gene leads to a deficiency in the blood–brain barrier and to increased sensitivity to drugs, *Cell*, **77**, 491–502.
- SCHINKEL, A. H., WAGENAAR, E., MOL, C. A. A. M., VAN DEEMTER, L., 1996, P-glycoprotein in the blood–brain barrier of mice influences the brain penetration and pharmacological activity of many drugs. *Journal of Clinical Investigation*, **97**, 2517–2524.
- SCHINKEL, A. H., WAGENAAR, E., VAN DEEMTER, L., MOL, C. A. A. M. and BORST, P., 1995, Absence of the *mdr1a* P-glycoprotein in mice affects tissue distribution and pharmacokinetics of dexamethasone, digoxin and cyclosporin A. *Journal of Clinical Investigation*, **96**, 1698–1705.
- SCHRENK, D., GANT, T. W., PREISEGGER, K. H., SILVERMAN, J. A., MARINO, P. and THORGEIRSSON, S. S., 1993, Induction of multidrug resistance gene expression during cholestasis in rats and nonhuman primates. *Hepatology*, **17**, 854–860.
- SCHWARZ, U. I., GRAMATTE, T., KRAPPWEIS, J., BERNDT, A., OERTEL, R., VON RICHTER, O. and KIRCH, W., 1999, Unexpected effect of verapamil on oral bioavailability of the β -blocker talinolol in humans. *Clinical Pharmacology and Therapeutics*, **65**, 283–290.
- SEKINE, T., CHA, S. H., KANAI, Y. and ENDOU, H., 1999, Molecular biology of multispecific organic anion transporter family (OAT family). *Clinical and Experimental Nephrology*, **3**, 237–243.
- SEKINE, T., WATANABE, N., HOSOYAMADA, M., KANAI, Y. and ENDOU, H., 1997, Expression cloning and characterisation of a novel multispecific organic anion transporter. *Journal of Biological Chemistry*, **272**, 18526–18529.
- SHA, M. V., AUDUS, K. L. and BORCHARDT, R. T., 1989, The application of bovine brain microvessel endothelial-cell monolayers grown onto polycarbonate membranes *in vitro* to estimate the potential permeability of solutes through the blood–brain barrier, *Pharmaceutical Research*, **6**, 624–627.

- SHI, X., BAI, S., FORD, A. C., BURK, R. D., JACQUEMIN, E., HAGENBUCH, B., MEIER, P. J. and WOLKO, A. W., 1995, Stable inducible expression of a functional rat liver organic anion transport protein in HeLa cells. *Journal of Biological Chemistry*, **270**, 25591–25595.
- SHIBATA, N., HOSHINO, N., MINOUCHI, T., YAMAI, A., PARK, K., TOMOYOSHI, T., ABE, H. and KODAMA, M., 1998, Relationship between area under the concentration versus time curve of cyclosporin A, creatinine clearance, hematocrit value, and other clinical factors in Japanese renal transplant patients. *International Journal of Clinical Pharmacology and Therapeutics*, **36**, 202–209.
- SIKIC, B. I., FISHER, G. A. and LUM, B. L., 1997, Modulation and prevention of multidrug resistance by inhibitors of P-glycoprotein. *Cancer Chemotherapy and Pharmacology*, **40** (suppl.), S13–S19.
- SMIT, J. J. M., SCHINKEL, A. H., OUDE ELFERINK, R. P. J., GROEN, A. K., WAGENAAR, E., VAN DEEMTER, L., MOL, C. A. A. M., OTTENHO, R., VAN DER LUGT, N. M., VAN ROON, M. A., VAN DER VALK, M. A., OERHAUS, G. J. A., BERNIS, A. J. M. and BORST, P., 1993, Homozygous disruption of the murine *mdr2* P-glycoprotein gene leads to a complete absence of phospholipid from bile and to liver disease. *Cell*, **75**, 451–462.
- SMIT, J. W., SCHINKEL, A. H., BORST, P. and MEIJER, D. K. F., 1996, Involvement of P-glycoprotein in the biliary and intestinal excretion of amphiphilic drugs: *mdr* gene knock out studies. *Hepatology*, **24**, 351.
- SMIT, J. W., SCHINKEL, A. H., WEERT, B. and MEIJER, D. K. F., 1998, Hepatobiliary and intestinal clearance of amphiphilic cationic drugs in mice in which both *mdr1a* and *mdr1b* genes have been disrupted. *British Journal of Pharmacology*, **124**, 416–424.
- SMITH, A. J., DEVREEE, J. M. L., OTTENHO, R., OUDE ELFERINK, R. P. J., SCHINKEL, A. H. and BORST, P., 1998, Hepatocyte-specific expression of the human MDR3 P-glycoprotein restores the biliary phosphatidylcholine excretion absent in *Mdr2*($-/-$) mice. *Hepatology*, **28**, 530–536.
- SMITH, A. J., VAN HELVOORT, A., VAN MEER, G., SZABO, K., WELKER, E., SZAKACS, G., VARADI, A., SARKADI, B. and BORST, P., 2000, MDR2 P-glycoprotein, a phosphatidylcholine translocase, transports several cytotoxic drugs and directly interacts with drugs as judged by interference with nucleotide trapping. *Journal of Biological Chemistry*, **275**, 23530–23539.
- SMITH, D. A. and JONES, B. C., 1992, Speculations on the substrate structure–activity relationships of cytochrome P450 enzymes. *Biochemical Pharmacology*, **44**, 2089–2098.
- SMITH, D. A., JONES, B. C. and WALKER, D. K., 1996, Design of drugs involving concepts and theories of drug metabolism and pharmacokinetics. *Medicinal Research Reviews*, **16**, 243–266.
- SOMOGYI, A., 1996, Renal transport of drugs: specificity and molecular mechanisms. *Clinical and Experimental Pharmacology and Physiology*, **23**, 986–989.
- SPAHN-LANGGUTH, H., BAKTIR, G., RADSCHUWEIT, A., OKYAR, A., TERHAAG, B., ADER, P., HANAFY, A. and LANGGUTH, P., 1998, P-glycoprotein transporters and the gastrointestinal tract: evaluation of the potential *in vivo* relevance of *in vitro* data employing talinolol as model compound. *International Journal of Clinical Pharmacology and Therapeutics*, **36**, 16–24.
- SPEEG, K. V. and MALDONADO, A. L., 1994, Effect of the nonimmunosuppressive cyclosporin analog SDZ PSC-833 on colchicine and doxorubicin biliary secretion by the rat *in vivo*. *Cancer Chemotherapy and Pharmacology*, **34**, 133–136.
- SPRINZ, H. and NELSON, R. S., 1954, Persistent nonhemolytic hyperbilirubinemia associated with lipochrome-like pigment in liver cells; report of four cases. *Annals of Internal Medicine*, **41**, 952–962.
- SPARREBOOM, A., VAN ASPEREN, J., MAYER, U., SCHINKEL, A. H., SMIT, J. W., MEIJER, D. K. F., BORST, P., NOOIJEN, W. J., BEIJNEN, J. H. and VAN TELLINGEN, O., 1997, Limited oral bioavailability and active epithelial excretion of paclitaxel (taxol) caused by P-glycoprotein in the intestine. *Proceedings of the National Academy Sciences, USA*, **94**, 2031–2035.
- STIEGER, B. and MEIER, P., 1998, Bile acid and xenobiotic transporters in liver. *Current Opinions in Cell Biology*, **10**, 462–467.
- SUZUKI, H. and SUGIYAMA, Y., 2000, Role of metabolic enzymes and efflux transporters in the absorption of drugs from the small intestine. *European Journal of Pharmaceutical Sciences*, **12**, 3–12.
- TAMAI, I., NEZU, J., UCHINO, H., SAI, Y., OKU, A., SHIMANE, M. and TSUJI, A., 2000, Molecular identification and characterisation of novel members of the human organic anion transporter (OATP) family. *Biochemical and Biophysical Research Communications*, **273**, 251–260.
- TAMAI, I. and TSUJI, A., 2000, Transporter-mediated permeation of drugs across the blood–brain barrier. *Journal of Pharmaceutical Sciences*, **89**, 1371–1388.
- TAKENAKA, O., HORIE, T., KOBAYASHI, K., SUZUKI, H. and SUGIYAMA, Y., 1995a, Kinetic analysis of hepatobiliary transport for conjugated metabolites in the perfused liver of mutant rats (EHBR) with hereditary conjugated hyperbilirubinemia. *Pharmaceutical Research*, **12**, 1746–1755.
- TAKENAKA, O., HORIE, T., SUZUKI, H. and SUGIYAMA, Y., 1995b, Different biliary excretion systems for glucuronide and sulfate of a model compound; study using Eisai hyperbilirubinemic rats. *Journal of Pharmacology and Experimental Therapeutics*, **274**, 13262–1369.
- THIEBAUT, F., TSURURO, T., HAMADA, H., GOTTESMAN, M. M., PASTAN, I. and WILLINGHAM, M. C., 1987, Cellular localisation of the multidrug resistance gene product in normal human tissues. *Proceedings of National Academy of Sciences, USA*, **84**, 7735–7738.

- THOMPSON, S. J., KOSZDIN, K. and BERNARDS, C. M., 2000, Opiate-induced analgesia is increased and prolonged in mice-lacking P-glycoprotein. *Anaesthesiology*, **92**, 1392–1399.
- THWAITES, D. T., CAVET, M., HIRST, B. H. and SIMMONS, N. L., 1995, Angiotensin-converting enzyme (ACE) inhibitor transport in human intestinal epithelial (Caco-2) cells. *British Journal of Pharmacology*, **114**, 981–986.
- TOH, S., WADA, M., UCHIUMI, T., INOKUCHI, A., MAKIN, Y., HORIE, Y., ADACHI, Y., SAKIZAKA, S. and KUWANO, M., 1999, Genomic structure of the canalicular multispecific organic anion-transporter gene (MRP2/cMOAT) and mutations in the ATP-binding-cassette region in Dubin–Johnson syndrome. *American Journal of Human Genetics*, **64**, 739–746.
- TRAUSCH, B., OERTEL, R., RICHTER, K. and GRAMATTE, T., 1995, Disposition and bioavailability of the β_1 -adrenoceptor antagonist talinolol in man. *Biopharmaceutics and Drug Disposition*, **16**, 403–414.
- TSUJI, A. and TAMAI, T., 1996, Carrier-mediated intestinal transport of drugs. *Pharmaceutical Research*, **13**, 963–977.
- TSUJI, H., KONIG, J., ROST, D., STOCKEL, B., LEUSCHNER, U. and KEPPLER, D., 1999, Exon–intron organization of the human multidrug-resistance protein 2 (MRP2) gene mutated in Dubin–Johnson syndrome. *Gastroenterology*, **117**, 653–660.
- URAKAMI, Y., OKUDA, M., MASUDA, S., SAITO, H. and INUI, K. I., 1998, Functional characteristics and membrane localisation of rat multispecific organic cation transporters, OCT1 and OCT2, mediating tubular secretion of cationic drugs. *Journal of Pharmacology and Experimental Therapeutics*, **287**, 800–805.
- VAN ASPEREN, J., MAYER, U., VAN TELLINGEN, O. and BEIJNEN, J. H., 1997, The functional role of P-glycoprotein in the blood–brain barrier. *Journal of Pharmaceutical Sciences*, **86**, 881–884.
- VAN ASPEREN, J., SCHINKEL, A. H., BEIJNEN, J. H., NOOIJEN, W. J., BORST, P. and VAN TELLINGEN, O., 1996, Altered pharmacokinetics of vinblastine in mdr1a P-glycoprotein-deficient mice. *Journal of National Cancer Institute*, **88**, 994–999.
- VAN ASPEREN, J., VAN TELLINGEN, O. and BEIJNEN, J. H., 2000, The role of MDR1A P-glycoprotein in the biliary and intestinal secretion of doxorubicin and vinblastine in mice. *Drug Metabolism and Disposition*, **28**, 264–267.
- VAN MONTFOORT, J. E., HAGENBUCH, B., FATTINGER, K. E., MULLER, M., GROOTHUIS, G. M. M., MEIJER, D. K. F. and MEIER, P. J., 1999, Polyspecific organic anion transporting polypeptides mediate hepatic uptake of amphipathic type II organic cations. *Journal of Pharmacology and Experimental Therapeutics*, **291**, 147–152.
- VAN VLIET, A. K., VAN THIEL, C. F., HUSIMAN, R. H., MOSHAGE, H., YAP, S. H. and COHEN, L. H., 1995, Different effects of 3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitors on sterol synthesis in various human cell types. *Biochimica et Biophysica Acta*, **1254**, 105–111.
- VERSCRAAGEN, M., KOKS, C. H. W., SCHELLENS, J. H. M. and BEIJNEN, J. H., 1999, P-glycoprotein system as a determinant of drug interactions: the case of digoxin-verapamil. *Pharmacological Research*, **40**, 301–306.
- VON MOLTKE, L. L., GREENBLATT, D. J., SCHMIDER, J., WRIGHT, C. E., HARMATZ, J. S. and SHADER, R. I., 1998, *In vitro* approaches to predicting drug interactions *in vivo*. *Biochemical Pharmacology*, **55**, 113–122.
- WACHER, V. J., SALPHATI, L. and BENET, L. Z., 1996, Active secretion and enterocytic drug metabolism barriers to drug absorption. *Advanced Drug Delivery Reviews*, **20**, 99–112.
- WACHER, V. J., WU, C.Y. and BENET, L. Z., 1995, Overlapping substrate specificities and tissue distribution of cytochrome P450 3A and P-glycoprotein: implications for drug delivery and activity in cancer chemotherapy. *Molecular Carcinogenesis*, **13**, 129–134.
- WADA, M., TOH, S., TANIGUCHI, K., NAKAMURA, T., UCHIUMI, T., KOHNO, K., YOSHIDA, I., KIMURA, A., SAKZAKA, S., ADACHI, Y. and KUWANO, M., 1998, Mutations in the canalicular multispecific organic anion transporter (cMOAT) gene, a novel ABC transporter, in patients with hyperbilirubinemia II Dubin–Johnson syndrome. *Human Molecular Genetics*, **7**, 203–207.
- WANDEL, C., KIM, R. B., KAJIJI, S., GUENGERICH, F. P., WILKINSON, G. R. and WOOD, A. J. J., 1999, P-glycoprotein and cytochrome P-450 3A inhibition: dissociation of inhibitory potencies. *Cancer Research*, **59**, 3944–3948.
- WASHINGTON, C. B., WILTSHIRE, H. R., MAN, M., MOY, T., HARRIS, S. R., WORTH, E., WEIGL, P., LIAND, Z., HALL, D., KROEMER, H. and BLASCHKE, T. F., 2000, The disposition of Saquinavir in normal and P-glycoprotein deficient mice, rats, and in cultured cells, *Drug Metabolism and Disposition*, **28**, 1058–1062.
- WATKINS, P. B., 1997, The barrier function of CYP3A4 and P-glycoprotein in the small bowel. *Advanced Drug Delivery Reviews*, **27**, 161–170.
- WATKINS, P. B., 1999, *Approaches to Studying the Role of Transporters in Drug Interactions in Man*. International Congress Series 1178 (Variability in Human Drug Response), pp. 55–67.
- WESTPHAL, K., WEINBRENNER, A., GIESSMANN, T., STUHR, M., FRANKE, G., ZSCHIESCHE, M., OERTEL, R., TERHAAG, B., KROEMER, H. K. and SIEGMUND, W., 2000a, Oral bioavailability of digoxin is enhanced by talinolol: Evidence for involvement of intestinal P-glycoprotein. *Clinical Pharmacology and Therapeutics*, **68**, 6–12.

- WESTPHAL, K., WEINBRENNER, A., ZSCHIESCHE, M., FRANKE, G., KNOKE, M., OERTEL, R., FRITZ, P., VON RICHTER, O., WARZOK, R., HACHENBERG, T., KAUMANN, H.M., SCHRENK, D., TERHAAG, B., KROEMER, H. K. and SIEGMUND, W., 2000b, Induction of P-glycoprotein by rifampin increases intestinal secretion of talinolol in human beings: a new type of drug/drug interaction. *Clinical Pharmacology and Therapeutics*, **68**, 345–355.
- WETTERICH, U., SPAHN-LANGGUTH, H., MUTSCHLER, E., TERHAAG, B., ROSCH, W. and LANGGUTH, P., 1996, Evidence for intestinal secretion as an additional clearance pathway of talinolol enantiomers: concentration and dose-dependent absorption *in vitro* and *in vivo*. *Pharmaceutical Research*, **13**, 514–522.
- XIE, R., HAMMARLUND-UDENAES, M., DE BOER, A. G. and DE LANGE, E. C. M., 1999, The role of P-glycoprotein in blood–brain barrier transport of morphine: transcranial microdialysis studies in *mdr1a*(–/–) and *mdr1a*(+/+) mice. *British Journal of Pharmacology*, **128**, 563–568.
- XIONG, H., TURNER, K. C., WARD, E. S., JANSEN, P. L. M. and BROUWER, K. L. R., 2000, Altered hepatobiliary disposition of acetaminophen glucuronide in isolated perfused livers from multidrug resistance-associated protein 2-deficient TR⁺ rats. *Journal of Pharmacology and Experimental Therapeutics*, **295**, 512–518.
- YAMANO, K., YAMAMOTO, K., KOTAKI, H., SAYURI, T., MATSUO, H., SAWADA, Y. and IGA, T., 1999, Correlation between *in vivo* and *in vitro* hepatic uptake of metabolic inhibitors of cytochrome P-450 in rats. *Drug Metabolism and Disposition*, **27**, 1225–1231.
- YAMANO, K., YAMAMOTO, K., KOTAKI, H., SAYURI, T., MATSUO, H., SAWADA, Y. and IGA, T., 2000, Quantitative prediction of metabolic inhibition of midazolam by erythromycin, diltiazem and verapamil in rats: implication of concentrative uptake of inhibitors into liver. *Journal of Pharmacology and Therapeutics*, **292**, 1118–1126.
- YAMAZAKI, M., AKIYAMA, S., NISHIGAKI, R. and SUGIYAMA, Y., 1996a, Uptake is the rate-limiting step in overall hepatic elimination of pravastatin at steady-state in rats. *Pharmaceutical Research*, **13**, 1559–1564.
- YAMAZAKI, M., AKIYAMA, S., NIINUMA, K., NISHIGAKI, R. and SUGIYAMA, Y., 1997, Biliary excretion of pravastatin in rats: contribution of the excretion pathways mediated by the canalicular multispecific organic anion transporter. *Drug Metabolism and Disposition*, **25**, 1123–1129.
- YAMAZAKI, M., SUZUKI, H., HANANO, M., TOKUI, T., KOMAI, T. and SUGIYAMA, Y., 1993, Na⁺-independent multispecific anion transporter mediates active transport of pravastatin into rat liver. *American Journal of Physiology*, **27**, G36–G44.
- YAMAZAKI, M., SUZUKI, H. and SUGIYAMA, Y., 1996b, Recent advances in carrier-mediated hepatic uptake and biliary excretion of xenobiotics. *Pharmaceutical Research*, **13**, 497–513.
- YOKOGAWA, K., TAKAHASHI, M., TAMAI, I., KONISHI, H., NOMURA, M., MORITANI, S., MIYAMOTO, K. and TSUJI, A., 1999, P-glycoprotein-dependent disposition kinetics of tacrolimus: studies in *mdr1a* knockout mice. *Pharmaceutical Research*, **16**, 1213–1218.
- ZHANG, L., BRETT, C. M. and GIACOMINI, K. M., 1998a, Role of organic cation transporters in drug absorption and elimination. *Annual Review of Pharmacology and Toxicology*, **38**, 431–460.
- ZHANG, L., SCHANER, M. E. and GIACOMINI, K. M., 1998c, Functional characterisation of an organic cation transporter (hOCT1) in a transiently transfected human cell line. *Journal of Pharmacology and Experimental Therapeutics*, **286**, 354–361.
- ZHANG, Y., GUO, X., TIN, E. T. and BENET, L. Z., 1998b, Overlapping substrate specificities of cytochrome P450 3A and P-glycoprotein for a novel cysteine protease inhibitor. *Drug Metabolism and Disposition*, **26**, 360–366.
- ZONG, J. and POLLACK, G. M., 2000, Morphine antinociception is enhanced in *mdr1a* gene-deficient mice. *Pharmaceutical Research*, **17**, 749–753.

Assessment of drug–drug interactions: concepts and approaches

R. J. WEAVER*

Division of Metabolism & Pharmacokinetics, Servier Research and Development Ltd, Fulmer Hall, Windmill Road, Slough SL3 6HH, UK

Received 28 February 2001

1. *A priori* knowledge of the enzyme inhibitory potential of new drug entities and the drug-metabolizing enzymes involved can be used in support of important decisions on the future progress of a drug in clinical development.

2. Important advances in the knowledge of human drug-metabolizing enzymes have largely fuelled the integration of *in vitro* drug metabolism and clinical drug interaction studies for use in drug development programmes.

3. The likelihood of correctly predicting *in vivo* drug–drug interactions appears highly dependent on selecting the correct enzyme inhibition model for use in deriving the inhibitor constant (K_i) and correctly determining the available concentration of inhibitor at the active site of the enzyme(s) of interest.

4. The uncertainty and inaccuracy of predicting the extent and duration of *in vivo* drug interactions currently stems from a lack of definitive models by which to assess likely substrate and inhibitor concentrations at the active site of metabolism. Additional issues contributing to the uncertainty of predicting drug interactions include assumptions of the contribution of presystemic drug extraction and the effect of inhibitors on the processes involved.

5. This review considers the practical aspects of *in vitro* enzyme inhibition studies and the use of *in vitro*–*in vivo* correlation approaches described in the literature to predict *in vivo* drug–drug interactions.

Introduction

Drug–drug interaction studies provide an essential means of understanding, predicting and avoiding adverse drug reactions (ADR). Of the total number of ADR reported, > 90% are associated with dose-dependent pharmacokinetic and pharmacodynamic events, and therefore are potentially avoidable with appropriate dose adjustment or simply by contraindication of the drugs concerned. ADR associated with pharmacokinetic drug interactions can be defined as a change in the anticipated exposure to a drug administered in combination with one or more other drugs. The incidence of ADR associated with multiple drug therapies is reported to rise sharply from between 0.3–4.7% and > 80% dependent on the number of drugs used in co-therapy (Jankel and Fitterman 1993, Malaty and Kuper 1999, Roos and Merk 2000). Therefore, multiple drug therapies whilst greatly improving the outlook for a number of patient groups, such as those with diabetes mellitus, HIV or other chronic diseases, patients are nevertheless at a far higher risk of developing clinically relevant ADR.

* Author for correspondence; e-mail: weaver@netgrs.demon.co.uk

The incidence of clinically relevant pharmacokinetic drug interactions is generally low, however several factors relating to the individual patient, as well as the therapeutic index and steep dose–response curve of a drug, will affect the magnitude and duration of the intended therapeutic action and the severity of the ADR. Pharmacokinetic drug interactions can affect drug disposition through changes in absorption, distribution, metabolism and excretion (Johnson *et al.* 1999). Most orally administered drugs are absorbed in the small intestine, so co-administered drugs affecting gastric pH, gastric motility and emptying can lead to delayed or variable systemic availability (Wood 1991). Interactions associated with plasma protein binding rarely prove clinically significant unless the drugs concerned exhibit high protein binding, low volume of distribution and are slowly eliminated, e.g. tolbutamide, warfarin and methotrexate (Bernard and Bruera 2000). Drug interactions affecting active renal excretion are associated with changes in tubular reabsorption and the secretion of drugs (Verschraagen *et al.* 1999). Drug combinations that modify excretion, systemic drug availability following oral drug delivery or the tissue distribution of drugs across membrane barriers are increasingly found to occur as a consequence of active transport processes involving P-glycoprotein (p-gp) efflux and the organic ion transporters (Meijer *et al.* 1999, Yu 1999). The function and clinical consequences of interactions associated with these proteins are covered in detail elsewhere in this issue of *Xenobiotica*. Lastly, the inhibition and to a lesser extent induction of the drug-metabolizing enzymes provide the most common occurrences of drug–drug interactions to be found in current literature and is the main focus of this review.

Pharmacological and toxicological effects are often explained in terms of the pharmacokinetics of a drug and show relationships with parameters such as the area under the blood concentration–time curve (AUC), peak concentration ($C_{\max}$) and steady-state blood concentration (C_{ss}). Such parameters are useful indicators of important pharmacokinetic drug interactions that can be readily determined from drug clearance and hepatic availability when administered alone and in combination with other drugs (Ganneman *et al.* 1995, Thummel *et al.* 1996, Azie *et al.* 1998, Davit *et al.* 1999). *A priori* knowledge of the enzymes, metabolic pathways and kinetics governing metabolism-based inhibition can be used in support of important decisions on the future progress of new drug candidates during drug discovery and lead optimization. Furthermore, metabolism-based drug interactions identified early in preclinical drug development can be proactively managed and may provide an important marketing edge, especially with certain classes of drug such as the antacids, antihistamines and antibiotics. Therefore, the use and interpretation of data gathered from preclinical drug metabolism and enzyme inhibition studies help enormously in the quest to forecast likely drug interactions in the clinic and in turn has formed the basis for current expanding drug metabolism and pharmacokinetic (DMPK) research and development.

Drug metabolism in drug discovery and development

Knowledge of the various drug-metabolizing enzymes has rapidly expanded over recent years, mainly through advances in molecular biology, use of *in vitro* models and the availability of more sensitive analytical methods for the detection and characterization of metabolites. These and other advances in DMPK have

contributed greatly to the integration of a variety of *in vitro* metabolism studies for the estimation of *in vivo* metabolic drug clearance and routes of metabolic elimination during early drug discovery and lead optimization of new drug entities (NDE). Furthermore, assessments of potential metabolism-based drug interactions can be used to ensure the safer conduct of clinical programmes that are directed to relevant questions raised during early preclinical screening programmes regarding the safety of NDE. This compares with the less favourable 'classical' approaches of measuring drug interactions late in Phase II and III clinical programmes using a strategy based on the therapeutic indices of drugs and possible concurrent use. Traditionally, Phase II/III drug interaction studies will have used cimetidine (probe inhibitor) and antipyrine (probe substrate), drugs of narrow therapeutic index such as warfarin, digoxin, phenytoin and theophylline, and drug combinations likely to be co-administered in therapy, without prior knowledge of the drug-metabolizing enzymes involved. The '*ad hoc*' testing of drug combinations is both complex, time consuming and without *a priori* knowledge of the enzymes involved, gives little assurance that important and potentially fatal drug combinations are contraindicated prior to marketing. Fortunately, with the variety of *in vitro* and *in situ* techniques now available to the DMPK scientist, understanding and anticipating likely drug interactions early in drug discovery and lead optimization are largely achievable, thereby justifying the integration of a whole variety of *in vitro* metabolic studies in DMPK. Finally, the importance of these *in vitro* metabolic drug interaction studies for use in the drug safety evaluation of new drug entities are recognized by regulatory authorities with the publication of several guidelines for the conduct of *in vitro* and *in vivo* drug-drug interaction studies (CDER 1997, 1998, CPMP 1998).

Strategies used to screen new drug entities as potential inhibitors of important drug-metabolizing enzymes have been widely adopted as one of several approaches used to select potential NDE in drug discovery (Jurima-Romet *et al.* 1996, Bertz and Granneman 1997, Iwasubo *et al.* 1997, Obach *et al.* 1997, von Moltke *et al.* 1998). However, whilst it is the goal of drug discovery to identify new drug entities that are not inhibitors of drug-metabolizing enzymes, it is also worth recognizing that many of the more successful drugs widely prescribed are themselves inhibitors of drug-metabolizing enzymes (Wrighton and Ring 1994, Azie *et al.* 1998, Davit *et al.* 1999, Greenblatt *et al.* 1999). It is therefore more likely that future drug discovery programmes will identify new NDE that exhibit some inhibitory potential thereby requiring more detailed studies designed to characterize fully the mechanism of inhibition and a prediction of the likely extent of drug interaction in the clinic. The value of the *in vitro* drug interaction studies and predictions derived from them can greatly depend on the correct identification of the enzymes inhibited, the extent of inhibition and the contribution each of the enzymes make to the overall elimination of the drug under investigation.

Drug-metabolizing enzymes

Drug-metabolizing enzymes comprise of groups of multiple enzymes responsible for the oxidation (Phase I) and conjugation (Phase II) of a vast array of drug compounds. Phase I and II enzymes are arbitrarily grouped into families and subfamilies based on the structural homology of the enzymes. The Phase I drug-metabolizing enzymes comprise the cytochromes P450 enzyme system, pivotal in

the oxidation of > 90% of all known oxidations and related reactions, and the flavin monooxygenases (Ziegler 1991, Lewis 1996a). The Phase II enzymes include *N*-acetyl transferases, UDP-glucuronosyltransferases and sulphotransferases (Mulder *et al.* 1990a, b, Weber *et al.* 1990, Spielberg 1996). In the majority of cases, *in vitro* studies focus on human liver microsomal cytochrome P450 enzymes as our present knowledge of these enzymes largely exceed those of other drug-metabolizing enzymes. As knowledge of the hepatic Phase II enzymes increase, it will become equally important to include these enzymes as part of the overall assessment of drug biotransformation and potential drug-drug interactions during drug discovery and development. Furthermore, the role of extrahepatic-drug metabolizing enzymes, particularly those of the gastrointestinal tract, are achieving greater importance as knowledge of these enzymes improve, thus helping to facilitate explanations of high first-pass metabolism (interaction) due to the presence of high levels of enzyme in extrahepatic tissues as well as the liver.

Cytochrome P450

The cytochromes P450 (CYP) comprise a superfamily of haem-thiolate enzymes subdivided into families and subfamilies based on the amino acid sequence homology (Nelson *et al.* 1993). Of > 55 human CYP enzymes belonging to 17 gene families and 42 subfamilies described (<http://drnelson.utmem.edu/nelsonhomepage>), three gene families, CYP1, CYP2 and CYP3, are responsible for the vast majority of known drug-oxidative and related Phase I reactions. The CYP-dependent metabolism of the majority of drugs in clinical use is associated with the hepatic metabolism by one or more of the following: CYP1A2, CYP2A6, CYP2B6, CYP2C8/9/19, CYP2D6, CYP2E1 and CYP3A4. Although there are large interindividual variations in the content of each hepatic CYP enzyme expressed (see below), CYP3A4 and the subfamily of CYP2C enzymes are quantitatively the most important, accounting for > 47-81% of total hepatic CYP (table 1) (Shimada *et al.* 1994, Lewis 1996a). Each CYP enzyme, as with many other drug-metabolizing enzymes described, all exhibit characteristics of

Table 1. Cytochrome P450 content in the human liver.

CYP enzyme	Level of expression (%)*	Variability (fold)
1A2	~ 13 (9)	40
1B1	~< 1	–
2A6	~ 4	30–100
2B6	~< 1	50
2C	20 (20)	150
2D6	2.5 (2)	> 1000
2E1	7	20
3A4	28 (63)	20
2C8	4.5	150
2C9	34	8
2C19	6.9	30

* Mean percent expression of total hepatic CYP enzymes (Shimada *et al.* 1994), CYP2C8, CYP2C9, CYP2C19 (Lasker *et al.* 1998). Levels of expression in parentheses (Lewis 1996, Renic and di Carlo 1997, *passim*).

broad substrate specificity with occasionally overlapping substrate selectivity. In fact, substrate specificity is so broad that CYP3A4, CYP2D6 and the subfamily of CYP2C enzymes account for > 77% of hepatic CYP-dependent drug metabolism (Lewis 1996a, Rendic and Carlo 1997). For many drugs the rate and routes of hepatic CYP-dependent clearance will be determined by only a few CYP enzymes, often supporting reactions that are described by high-affinity (low K_m) low-capacity enzyme-substrate kinetics. It is clearly evident therefore that multiple drug therapies will inevitably lead to a cohort of potentially competing metabolic reactions, the extent and duration of which will be determined by the fraction of total drug clearance inhibited and the mechanism of enzyme inhibition. There are many excellent papers describing in detail many of these reactions, the physico-chemical properties of CYPs and, substrates and inhibitors of CYP available in the literature of which only a handful are cited here.

Whilst the hepatic first-pass extraction of orally administered drugs has been recognized for sometime, it has only been recently acknowledged that there is a significant contribution by the intestine to the overall first-pass extraction of several drugs in clinical use (Doherty and Pang 1997, Thummel *et al.* 1997, Lin *et al.* 1999). For many orally administered CYP3A4 drug-substrates, there are appreciable levels of intestinal metabolism by virtue of the high levels of CYP3A4 expressed in the gastrointestinal tract (Watkins *et al.* 1987, Kolars *et al.* 1992). The presence of CYP3A in the gastrointestinal mucosa contributes to the overall first-pass metabolism of several drugs predominantly cleared by CYP3A4-dependent metabolism, such as cyclosporin, midazolam and nifedipine, are also responsible for a number of clinically important oral drug-drug interactions. Most notable among the CYP3A4 drug-drug interactions are terfenadine-ketoconazole (Honig *et al.* 1993), midazolam-saquinavir and midazolam-macrolide antibiotics (Olkkola *et al.* 1993, Yeates *et al.* 1996, Merry *et al.* 1997, Gorski *et al.* 1998). Furthermore, significant gender differences in oral and systemic metabolism of midazolam, consistent with the expression of higher levels of CYP3A4 expression in women, are reported (Watkins *et al.* 1989, Gorski *et al.* 1998). Besides CYP3A4 expression in the gastrointestinal mucosa, low levels of CYP1A1, CYP2C and CYP2D6 proteins are also detected by immunoblotting, and mRNAs of CYP1B1 and CYP2E1 have been measured but as yet no detectable levels of the corresponding proteins have been reported (Madani *et al.* 1999, Paine *et al.* 1999, Zang *et al.* 1999).

Probe substrates and inhibitors of cytochrome P450

Reported experimental data obtained on CYP-probe substrates and inhibitors are often difficult to compare when differences in experimental approach, variability in human liver microsomal preparations and occasionally dissenting data confound the interpretation of experimental results. Nevertheless, the correct assignment of the individual enzymes responsible for the metabolism of drugs is a pivotal stage in the rationale prediction of drug interactions. Several approaches can be employed in the characterization of the enzymes including heterologous expression of enzymes, selective chemical inhibitors, inhibitory antibodies raised against individual enzymes, and correlation of enzyme activities (Wrighton *et al.* 1995, Guengerich 1996, Parkinson 1996a, Clarke 1998, McGinnity *et al.* 2000). The characterization of probe substrate selectivity and specificity of individual

drug-metabolizing enzyme reactions has facilitated a clearer understanding of the role of different enzymes in the metabolic elimination of drugs and in providing the rationale for the occurrence of drug-drug interactions. Probe substrates and inhibitors are widely employed, sometimes in conjunction with heterologous enzyme expression systems, to support the identification of enzymes involved in the metabolism of NDE (figure 1). When using chemical inhibitors to confirm the characterization of the enzymes involved in the metabolism of a drug, it is equally important to assess the inhibitory potential of NDE on selective CYP-probe substrate activities; NDE may inhibit CYP activity that are themselves not substrates for the enzymes inhibited. For example, quinidine is not a substrate for CYP2D6 and yet is a potent inhibitor of CYP2D6, and mexiletine is a substrate for CYP2D6 but inhibits CYP1A2 activity (Hurwitz *et al.* 1991).

SKF 525A, metyrapone and DPEA are among some of the first *in vitro* CYP chemical inhibitors to be used in metabolic studies (Testa and Jenner 1981, Murray 1992). Whilst these inhibitors are themselves too general for the selective identification of individual CYP-dependent reactions, they nevertheless prove useful tools in early enzyme studies where *a priori* assumptions about the class of enzyme(s) involved cannot be presumed. Newer generations of CYP chemical inhibitor are often more selective as probes for the selective measure and inhibition of individual CYPs when used at the appropriate concentration and under optimal incubation conditions at $\sim K_m$ and at $10 \times K_i$ for substrate and inhibitors respect-

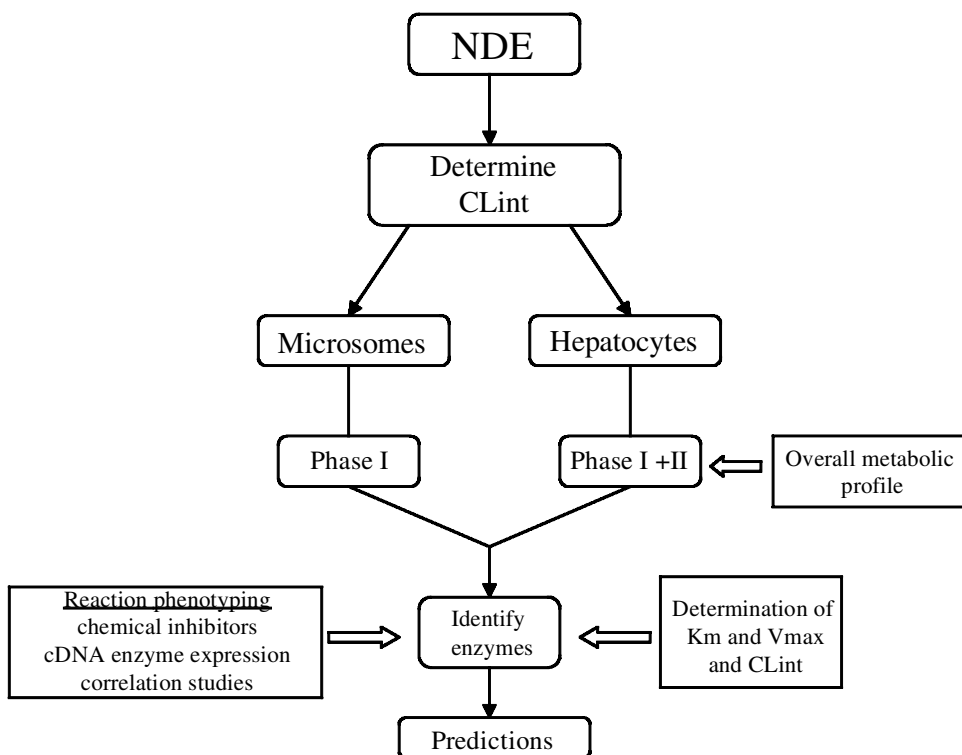


Figure 1. Scheme for the identification of enzymes involved in the metabolism of new drug entities (NDEs). Definition of K_m = Michealis-Menten constant, V_{max} = maximal initial reaction velocity and CL_{int} = intrinsic clearance.

ively (Bourrie *et al.* 1996, Eagling *et al.* 1998, Hickman *et al.* 1998, Carlile *et al.* 1999, Ong *et al.* 2000). The availability of inhibitory anti-CYP antibodies is also a useful additional tool in enzyme identification studies (Weaver *et al.* 1993, 1999, Edwards 1998). Several studies have evaluated the more commonly used CYP *in vitro* probe substrates and inhibitors. Using substrate concentrations close to that of the K_m for the CYP enzyme of interest, the specificity of a number of commonly used CYP chemical inhibitors have been evaluated. In general, furafylline, sulphaphenazole, quinidine and TAO are selective inhibitors for CYP1A2, 2C9, 2D6 and 3A4 respectively (Newton *et al.* 1995, Hickman *et al.* 1998). 8-Methoxypsoralen is a relatively selective inhibitor of coumarin 7-hydroxylation by CYP2A6 but also inhibits CYP1A2 and to a much lesser extent CYP2C19, CYP2D6 and CYP2E1 (Hickman *et al.* 1998). *S*-mephenytoin is used as a specific inhibitor of CYP2C19. However, recent studies have reported the potent inhibition of CYP2C19 by an N-3 substituted phenobarbital derivative, (*R*)-3-benzylphenobarbital (Suzuki *et al.* 2000), and a usable selective inhibition of CYP2C19 by a sesquiterpene nootkatone (Tassaneeyakul *et al.* 2000). Ketoconazole and TAO are widely used as selective inhibitors of CYP3A4 (Watkins *et al.* 1985, Brian *et al.* 1990, Guengerich *et al.* 1990, Maurice *et al.* 1992). However, the use of inappropriately high concentrations of ketoconazole also implicates ketoconazole in the inhibition of CYP2D6, CYP2C8 and CYP2C9 (Back *et al.* 1988, Ward and Black 1993, Ong *et al.* 2000). Diethyldithiocarbamate (DDC) is used as a selective inhibitor of CYP2E1, however studies using heterologously expressed CYPs show moderate inhibition of CYP2C8 (Ong *et al.* 2000), CYP1A1, CYP1A2, 2A6, 2B6 and 3A4 (Chang *et al.* 1994, Ono *et al.* 1996). Studies using human liver microsomes have demonstrated the selective inhibition of CYP2E1-mediated chlorzoxazone 6'-hydroxylation by DDC (Guengerich *et al.* 1991, Tassaneeyakul *et al.* 1993, Newton 1995) whilst other studies also report the inhibition of testosterone 6 β -hydroxylation by DDC (Gorski *et al.* 1997, Eagling *et al.* 1998). Contradictory data reported in the literature, such as those described for DDC, highlight some of the difficulties in the interpretation of reported experimental data suggesting occasionally marked differences in enzyme selectivity and specificity between different studies. Some of these differences can be attributed to the use of alternative probe substrates and substrate concentrations, but also from the use of expression systems. Expression systems, while enabling the evaluation of the inhibitory effects of NDE in a single enzyme system, will not necessarily permit the significance of the inhibition or evaluate the contribution of other enzymes to the overall metabolic clearance of a NDE.

Caffeine N3-demethylation is cited as a relevant measure of *in vitro* and *in vivo* CYP1A2 activity (Butler *et al.* 1989, Gu *et al.* 1992, Tassaneeyakul *et al.* 1992, Hickman *et al.* 1998). However, caffeine N3-demethylation exhibits biphasic kinetics in human liver microsomes associated with a high-affinity CYP1A2 and a low-affinity CYP3A4 component (Campbell *et al.* 1987, Fuhr *et al.* 1992, Gu *et al.* 1992, Hickman *et al.* 1998). The low-affinity CYP3A4 component contributes < 5% to the overall rate of caffeine N3-demethylation formation in human liver microsomes using caffeine concentrations (1000 μM) close to the K_m for the high-affinity CYP1A2 enzyme (Hickman *et al.* 1998). Other studies have, by contrast, reported lower K_m (200–240 μM) for caffeine N3-demethylation, that are possibly attributable to interindividual variability in the estimates of K_m or to differences in the conditions of incubation and solvents used to solubilize drug substrates.

Tolbutamide 4-methyl hydroxylation is primarily catalysed by CYP 2C9, and to a much lesser extent 2C8 and 2C19 (Veronese *et al.* 1993, Inoue *et al.* 1997, Venkatakrishnan *et al.* 1998, Wester *et al.* 2000); the kinetics governing 4-methyl hydroxylation of tolbutamide by human liver microsomes are consistent with the involvement of more than one enzyme all having a similar K_m (Relling *et al.* 1990, Srivastava *et al.* 1991, Veronese *et al.* 1993). Of the available *in vivo* probe substrates, tolbutamide is more widely used as an *in vivo* CYP2C9 probe substrate. Phenytoin 4'-hydroxylation is primarily hydroxylated by CYP2C9 (Doecke *et al.* 1991) and is believed to account for 80% of the *in vivo* clearance of phenytoin in man (Miners and Birkett 1998). *In vitro* studies have shown a significant correlation between phenytoin and tolbutamide hydroxylase activities (Veronese *et al.* 1991).

S-mephenytoin 4'-hydroxylation is primarily catalysed by, and is correlated with, CYP2C19 content in human liver microsomes (Goldstein *et al.* 1994, Lasker *et al.* 1998). In man, (S)-(R)-mephenytoin undergoes rapid hydroxylation to form (S)-4 hydroxy mephenytoin (SOHP) with the slower formation of (R)-ethylhydantoin (PEH). The ratio of (S)-4 hydroxy mephenytoin and (R)-ethylhydantoin is used as a measure of CYP2C19 activity, albeit issues regarding the assessment of CYP2C19 activity based on the ratio of S-OHM/R-PEH have been raised (Zhang *et al.* 1991).

Dextromethorphan O-demethylation is well established as a measure of CYP2D6 activity *in vivo* and is highly correlated with CYP2D6 expression in human liver microsomes (Rodrigues *et al.* 1994). The urinary ratio of dextromethorphan/dextrophan is most commonly used as an index of CYP2D6 activity and previous studies have shown a good correlation with other CYP2D6 probe substrates such as desipramine (Spina *et al.* 1997) and debrisoquine (Mortimer *et al.* 1989, Kupfer *et al.* 1994). In addition, sparteine, debrisoquine and metoprolol have also been used as *in vitro* and *in vivo* probe substrates, but for reasons of poor analytical detection, co-segregation with other CYP2D6 substrates, or lack of safety as *in vivo* probe substrates, are less commonly used.

Chlorzoxazone 6'-hydroxylation is the most commonly used CYP2E1 *in vitro* probe substrate and is the only available *in vivo* probe. There is evidence in the literature to suggest, however, that CYP1A1, CYP1A2 and CYP3A4 also support 6'-hydroxylation of chlorzoxazone. As an *in vivo* probe substrate, the fractional clearance of chlorzoxazone is the preferred indicator of CYP2E1 activity (Streetman *et al.* 2000).

Complex CYP3A4 structure-activity relationships associated with substrates and inhibitors of CYP3A4 have recently been described in detail (Kenworthy *et al.* 1999, Wang *et al.* 2000). In these studies, CYP3A4 substrates appear to form three distinct groups based on the linear regression analyses of pairs of CYP3A4 substrates. The recognition of groups of CYP3A4 substrates has helped enormously to rationalize previous attempts to explain often poorly correlating pairs of substrates in the literature and the failure of some CYP3A4 probes to predict CYP3A4-dependent metabolism or inhibition of CYP3A4 activity (Hunt *et al.* 1992, Watkins *et al.* 1992, Kinirons *et al.* 1994, Lown *et al.* 1995). The complex reaction kinetics associated with CYP3A4 is believed to occur as a result of multiple substrate binding sites and consequently a single probe substrate is unlikely to be representative of drug interactions observed with other CYP3A4

probe substrates. For this reason, it is viewed that at least one probe substrate from each group is selected to fully characterize potential CYP3A4 enzyme inhibition.

Several probe substrates are available for the purposes of phenotyping *in vivo* CYP3A4 activity, each reportedly with advantages and disadvantages (Streetman *et al.* 2000). The most widely accepted *in vivo* CYP3A4 probe substrates are ^{14}C -erythromycin and midazolam (Kinirons *et al.* 1993, Lown *et al.* 1993, Ahonen *et al.* 1995). Other than the disadvantage of requiring Administration of Radiolabelled Substances Advisory Committee (ARSAC) approval for the administration of radiolabelled erythromycin, the intravenous administration of erythromycin is widely adopted for use in the measurement of CYP3A4 activity. An important consideration, especially if an oral erythromycin test were to be developed, is that erythromycin is subject to efflux transport by p-glycoprotein, thereby potentially limiting the usefulness of orally administered erythromycin as a selective CYP3A4 probe substrate (Tanako *et al.* 1998). Oral administration of midazolam undergoes hydroxylation by both liver and the intestine, but only hepatic CYP3A4 is presumed to be involved in the elimination of midazolam following intravenous administration and is often cited for use as a marker of CYP3A4 activity (Thummel *et al.* 1994a, b, von Moltke *et al.* 1996a, Streetman *et al.* 2000). Other CYP3A4 probes used to phenotype *in vivo* CYP3A activity include lidocaine, dapsone, nifedipine, dextromethorphan and cortisol however, each of these CYP3A substrates have a number of drawbacks associated with their use (Streetman *et al.* 2000).

Non-CYP drug metabolizing enzymes

The flavin monooxygenases, UDP-glucuronosyl transferases, sulphotransferases and *N*-acetyl transferases are involved in the metabolism of many drugs in clinical use. Phase II conjugations are more often associated with supporting catalytic reactions characteristic of low-affinity (high K_m), high-capacity (high $V_{\max}$) pathways compared with CYPs (Mistry and Houston 1987, Magdalou *et al.* 1992, Sweeny *et al.* 1992, Stevens *et al.* 1999, Störmer *et al.* 2000). Examples of inhibition of these Phase II enzymes by competing substrates are few compared with CYP enzymes because of the high metabolic capacity (high $V_{\max}$) associated with many of these reactions, or simply because drug interactions associated with the Phase II enzymes have yet to be identified and characterized.

The UDP-glucuronosyltransferases (UDPGTs) comprise of a multigene family of enzymes responsible for the conjugation of a large number of substrates to form readily excreted water-soluble glucuronides (Burchell and Coughtrie 1989, Dutton *et al.* 1990, Mulder *et al.* 1990, Chiu and Huskey 1998). The glucuronidation of drugs containing amine functions such as amitriptyline, imipramine, chlorpromazine (Lehman *et al.* 1983) and ketotifen (Le Bigot *et al.* 1983) is an important route of biotransformation for these drugs. The human UDPGT family of enzymes contains more than 12 isoenzymes arbitrarily divided into two subfamilies (Mackenzie *et al.* 1997). UGT1A and B, expressed in the liver and extrahepatic tissues, are responsible for the glucuronidation of most drugs. UGT1A1, 1A6 and 2B7 are expressed in both human liver and intestine and are potentially important enzymes in the first-pass metabolism of orally administered drugs (McDonnell *et al.* 1996, Bock *et al.* 1999, Tukey and Strassburg 2000). Interestingly, acetaminophen *O*-glucuronidation (UGT1A6), morphine 3-glucur-

onidation (UGT2B7) and oestradiol 3-glucuronidation in human intestinal microsomes all show similar or even higher activity (nmolmg^{-1} protein) than for the same reactions measured in human liver microsomes (Fisher *et al.* 2000).

Besides conjugation of drug compounds, UDPGTs are responsible for the conjugation of a wide range of endogenous compounds associated with cellular function (Dutton *et al.* 1980, Burchell Coughtrie 1989, Mulder *et al.* 1990). Interactions, between not only concomitantly administered drugs, but also endogenous substrates of UDPGTs, therefore present a potential mechanism of cellular toxicity and ADR associated with the inhibition of drug-metabolizing enzymes (Heath and Dingell 1974, Burchell *et al.* 1987). It is postulated that a number of adverse side-effects are linked with the frequent use of certain drugs containing amine or amide functions such as the tricyclic antidepressants, antihistamines and phenothiazine neuroleptics, and disruption of steroid hormone glucuronidation (Sharp *et al.* 1992). However, studies designed to characterize inhibitors of UDPGTs are often complex because of the location of these enzymes within the microsomal membrane and consequently very few compounds have been identified as specific inhibitors of UDPGT (Magdalou *et al.* 1992, Golovinsky *et al.* 1998).

Sulphotransferases are important in the sulphation of drugs and endogenous compounds in human liver and the wall of the gastrointestinal tract (Muler and Jackoby 1990, Pang 1990, Matsui and Homma 1994). The most common acceptors for sulphation are hydroxyl groups in phenols and aliphatic alcohols, and in many instances competition between sulphotransferases and glucuronosyltransferase for a substrate are found to occur *in vivo* (Mulder 1990). Examples of sulphotransferase inhibitors include 2,6-dichloro-4-nitrophenol (DCNP), pentachlorophenol (PCP) (Mulder and Scholtens 1977) and salicylamide (Moldéus *et al.* 1979, Zaleski *et al.* 1983). DCNP and PCP are suicide inhibitors of most sulphotransferases with $K_i = 0.1\text{--}1\text{ }\mu\text{M}$ (Duffel and Jakoby 1981, Campbell *et al.* 1987) and are commonly used as *in vitro* and *in vivo* inhibitors of sulphotransferase activity (Koster *et al.* 1982, Boberg *et al.* 1983, Meerman *et al.* 1983, Furlong *et al.* 1987).

Human *N*-acetyltransferases (NAT1 and NAT2) (Grant *et al.* 1991) catalyse the acetyl coenzyme A-dependent *N*-acetylation of a broad range of arylamines and hydrazines (Hein 1988). NAT2 is responsible for the polymorphic metabolism of drugs such as isoniazid and sulphamethazine, and NAT1 for the polymorphic metabolism of *p*-aminosalicylic acid and *p*-aminobenzoic acid *N*-acetylation (Grant *et al.* 1991). The apparent K_m can differ with phenotypic status for example, the reported K_m for rapid and slow acetylator status are 210 and 63 μM , respectively, for *p*-aminobenzoic acid. Acetylator status can be characterized using probe substrates such as sulphamethazine, dapsone and caffeine (Hein *et al.* 1994, Streetman *et al.* 2000), and by use of irreversible inhibitors of NAT activity such as *N*-ethylmaleimide, iodoacetate and *p*-chloromercuribenzoate (Weber *et al.* 1990). Several compounds are reversible inhibitors of NAT activity and are often structurally similar to substrates or products of *N*-acetylation of amides, heterocyclic carboxamides, salicylamide analogues and carboxyhydrazides (Weber *et al.* 1990) of which, several are potentially useful inhibitors of NAT activity.

The flavin-containing monooxygenases (FMO) are a family of microsomal enzymes responsible for the biotransformation of a number of drugs in clinical use. Five mammalian isoforms of FMO have been characterized (FMO1–5) (Lawton *et al.* 1994, Lang *et al.* 1998) of which FMO3 and to a much lesser extent FMO5

are the primary isoforms expressed in human liver (Yeung *et al.* 2000). The oxidative metabolism of thiobenzamide, tamoxifen, olanzapine, clozapine, ranitidine and cimetidine are all examples of drugs reported to be catalysed by FMO3. Caffeine is widely used as an *in vivo* probe substrate for CYP1A2, NAT2 and xanthine oxidase (Kalow and Tang 1991, Butler *et al.* 1992, Miners and Birkett 1996) and more recently as a probe substrate for FMO3 (Park *et al.* 1999). However, Rettie and Lang (2000) have questioned the usefulness of caffeine *N*-demethylation as a measure of *in vivo* human FMO3 activity and suggest benzydamine *N*-oxide formation (Störmer *et al.* 2000) is a more useful measure of human FMO3 activity. There are no selective mechanism-based inhibitors of FMO, as substrates are oxidized to form ionic rather than reactive intermediate substrates and do not form the tightly bound transition-states familiar to CYP-catalysed reactions (Ziegler 1991). Currently, the general lack of selective FMO inhibitors restricts detailed studies to investigate the role of FMO in drug biotransformation and drug-drug interaction studies (Narimatsu *et al.* 1999), although the recent availability of expressed FMO may prove helpful in the future characterization of FMO-dependent metabolism and drug-drug interactions. To help distinguish between the contribution of liver microsomal FMO- and CYP-dependent metabolite formation, the addition of detergent (e.g. Lubrol PX) to a microsomal reaction mixture, or alternatively by incubating microsomes at 45°C for ~5 min, will abrogate the component of CYP-dependent metabolite formation in liver microsomes whilst still retaining FMO activity (Sadeque *et al.* 1992, Grothusen *et al.* 1996).

Variability of enzyme expression

Interindividual variability in drug metabolism is well documented in the literature (Shimada *et al.* 1994, Weaver *et al.* 1995, Bourrié *et al.* 1996, Fisher *et al.* 2000). Owing in part to environmental and physiological factors, variability is also attributable to genetic factors resulting in enzyme variants (polymorphisms) with higher, lower or no activity of drug-metabolizing enzymes, or variants of gene regulation affecting the levels of the enzyme expressed. Differences in the levels of catalytic activity or expression of enzymes result in an increase or decrease in the rate of metabolite formation with subsequent changes in the pharmacological or toxicological response to a drug. By characterizing the enzymes involved in the metabolism of a drug, including those associated with genetic polymorphisms, it is possible to evaluate interindividual variability in the hepatic metabolism of an investigational drug. In practice, liver microsomes are used to characterize the range of individual CYP enzyme activities in human livers. It is also of interest to note that the variability of a CYP-dependent reaction measured in a bank of human liver microsomes rarely reflects the variation for the same drug reaction following *in vivo* administration (Bertz and Granneman 1997, Iwatsudo *et al.* 1997a, b, Carlile *et al.* 1999). However, Thummel *et al.* (1994a) have shown that the range of interindividual variability of a reaction measured *in vivo* is comparable with the range variability of intrinsic clearance (CL_{int}) measured in liver homogenates prepared from biopsy samples taken from the same individuals. This suggests that the differences in the observed interindividual *in vivo* and *in vitro* variability of enzyme-catalysed reactions may be attributable to the variability of CL_{int} associated with the use of different *in vivo* and *in vitro* sampling pools or as

an artefact of the *in vitro* methodologies used to determine CL_{int} . It is just as likely, however, that the quality of the donor tissue used in *in vitro* studies is compromised by less than optimal or inconsistent methods of tissue handling from procurement to preparation and storage of liver microsomes.

Mechanisms of enzyme inhibition

The specificity and mechanism of enzyme inhibition are important determinants in the evaluation of metabolism-based drug interactions. Any drug that reduces the initial rate of an enzyme-catalysed reaction occurs as the result of either the reversible or irreversible inhibition of the enzyme. Reversible inhibition occurs as a result of the inhibitor binding to the active site to prevent substrate binding (competitive inhibition), binding of the inhibitor without affecting the binding of the substrate to the active site (non-competitive inhibition) and binding of the inhibitor to only the enzyme-substrate complex (uncompetitive inhibition) (Segal 1993).

The velocity (v) of an enzyme-catalysed reaction in the absence of an inhibitor is usually described in its simplest form by hyperbolic (Michaelis-Menten) kinetics, where V_{max} is the maximum velocity of the reaction and K_m is the Michaelis-Menten constant reflecting the affinity of the enzyme-substrate complex. Derivation of the equations used to describe the relative velocity of a reaction in the presence of competitive, non-competitive and uncompetitive inhibitors are described in detail by Segal (1993). The relative velocity of a reaction can be usefully expressed as the fractional inhibition (i) or per cent inhibition ($i \times 100\%$) of competitive, non-competitive or uncompetitive inhibition of simple hyperbolic kinetics in equations (1–3) respectively. For a competitive inhibitor, K_i is the concentration of inhibitor that is required to double the apparent K_m of a reaction, and $[S]$ and $[I]$ are the substrate and inhibitor concentrations. The relative velocity or fractional inhibition is dependent on $[S]$, $[I]$, K_m and K_i for competitive and uncompetitive inhibition, whereas non-competitive inhibition is only dependent on $[I]$ and K_i for non-competitive inhibitors:

$$i = \frac{[I]}{[I] + K_i \left(1 + \frac{[S]}{K_m} \right)} \quad (1)$$

$$i = \frac{[I]}{K_i + [I]} \quad (2)$$

$$i = \frac{[I]}{K_i \left(1 + \frac{K_m}{[S]} \right) + [I]} \quad (3)$$

Kinetically, a competitive inhibitor is characterized by an increase in the apparent K_m , a non-competitive inhibition by a decrease in V_{max} , and uncompetitive inhibition by a decrease in both K_m and V_{max} . Drugs often associated with potent reversible inhibition of CYPs typically include nitrogen-containing compounds such as the imidazoles (e.g. ketoconazole) (Meredith *et al.* 1985, von Moltke *et al.* 1996b), quinolones (e.g. ellipticine) (Lesca *et al.* 1979), and azabenzene (e.g. metyrapone) (Jonen *et al.* 1974). The potency of enzyme inhibition is largely

determined by the lipophilicity and strength of the bond between the nitrogen lone pairs and iron moiety of the drug-CYP complex (Wilkinson *et al.* 1974, Rogerson *et al.* 1997).

Irreversible inhibition is characterized by a time-dependent loss of enzyme activity not seen with the reversible inhibition of enzyme-catalysed reactions. The individual rate constants used to describe a mechanism-based inactivation of an enzyme will determine the rate of enzyme inactivation and consequently the half-life of a particular enzyme activity. In an irreversible reaction, the enzyme catalyses the inhibitor (I) to its reactive form (IR) and the reactive species can either be released (P) or react with the enzyme to form an inactive enzyme–inhibitor complex (E–IA) (figure 2) (Silverman 1988). K_i is the concentration of inactivator that is half the maximal rate of inactivation and K_{inact} is the rate of inactivation – the shorter the half-life the greater potency of the inhibitor. K_{inact} and K_i can be determined experimentally (equation 4) by measuring the half-life of enzyme inactivation. A time-dependent decrease in the activity of an enzyme will result in a decrease in V_{max} but will not affect K_m , and consequently irreversible and non-competitive inhibition would be graphically indistinguishable (Silverman 1988):

$$t_{\frac{1}{2}} = \frac{0.693}{K_{\text{inact}}} \left[1 + \frac{K_i}{I} \right]. \quad (4)$$

Drugs containing terminal double or triple bonds (acetylenes) such as allylisopropylacetamide, ethinyloestradiol and chloramphenicol (Halpert 1981, Guengerich 1988), are typical examples of irreversible inhibitors of the cytochromes P450. Evidence of irreversible inhibition is given by the equimolar loss of enzyme activity and haem through the formation of radical intermediates with subsequent covalent binding (Ortiz de Montellano 1988). Mayhew *et al.* (2000) proposed an *in vitro* model enabling the *in vivo* prediction of CYP3A4 metabolite intermediate complex (MIC) formation and inactivation of CYP3A4 for three widely prescribed drugs, *N*-desmethyl diltiazem, clarithromycin and fluoxetine. From the experi-

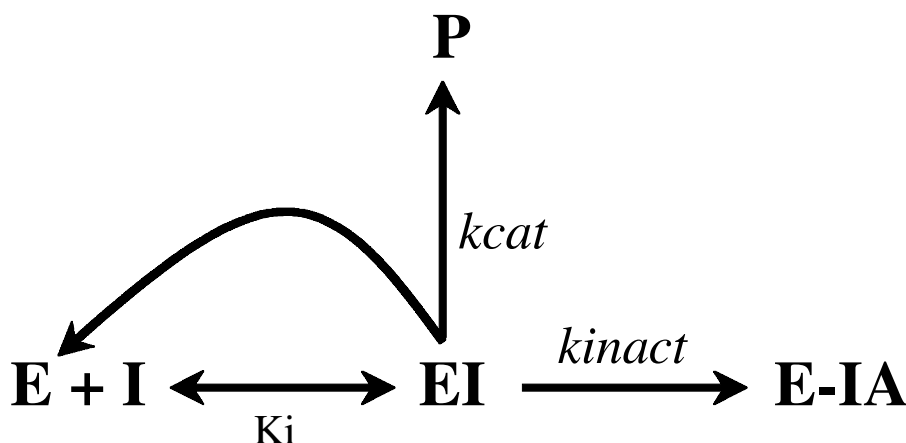


Figure 2. Scheme of enzyme inactivation. K_i is the concentration of inhibitor required to achieve half-maximal rate of inactivation and comprises of several rate constants. K_{inact} is the maximal rate of inactivation and formation of E-IA. K_{cat} is equal to the rate of product formation.

mental determination of K_b , the rate of enzyme inactivation (k_{inact}) and degradation of CYP3A4 (k_{degrad}), they correctly predicted the increase in AUC for all three reactions.

Occasionally, quasi-irreversible inhibition of enzyme reactions are described resulting from the formation of stable complexes with the prosthetic haem of CYP forming metabolic intermediates and rendering the enzyme inactive (Pessayre *et al.* 1982). Whilst the inhibitory complex can be reversed *in vitro* by the addition of highly lipophilic compounds to the incubation mixture (Dickins *et al.* 1979), these complexes are stable *in vivo* and therefore effectively irreversible. Classes of drugs frequently shown to exhibit quasi-irreversible inhibition include the alkylamines such as orphenadrine and proadifen (SKF-525A) (Murray *et al.* 1988, Bensoussan *et al.* 1995), 1,1-hydrazines such as isoniazid and macrolide antibiotics such as troleandomycin and erythromycin (Pessayre *et al.* 1982).

Enzyme activation

A simple hyperbolic relationship (Michaelis–Menten kinetics) is often used to describe adequately the relationship between substrate and inhibitor concentrations used in *in vitro* drug interaction studies. However, there are increasing reports highlighting instances where the relationship between substrate and inhibitor concentration is more appropriately described by a sigmoidal (third-order polynomial) curve to account for inhibition, stimulation of enzyme activity, and multiple K_m and K_i (Korzekwa *et al.* 1998, Fisher *et al.* 2000, Houston and Kenworthy 2000). Non-Michaelis–Menten or ‘allosteric kinetics’ arise as a consequence of autoactivation or autoinhibition and are typically characterized by an initial lag phase in the reaction kinetics of a concentration–time profile and decreasing V_{max} with increasing substrate concentration. Conceptually, it can be considered that the enzyme exists in two conformational forms or alternatively there exists more than one binding site (Segal 1993). In either case, the sigmoidicity arising from positive cooperativity can be more clearly interpreted by using an Eadie–Hofstee plot. The data are modelled using the Hill equation (equation 5) where K' is the sum of the number of binding sites and n is the Hill coefficient. Eadie–Hofstee plots describing a concave line are likely to be explained by the presence of more than one enzyme with different values of K_m for the reaction (Hill coefficient, $n < 1$):

$$\frac{v}{V_{\text{max}}} = \frac{[S]^n}{K' + [S]^n}. \quad (5)$$

The impact of approximating inhibition data towards hyperbolic kinetics is potentially to generate poor estimates of CL_{int} , IC_{50} and K_i at low substrate concentrations that might typically reflect clinically relevant concentrations. Allosteric effects are particularly well described in the literature for CYP3A4, but also for a number of other CYPs, sulphotransferases and glucuronosyl transferases (Schmider *et al.* 1996, Beckman *et al.* 1998, Korzekwa *et al.* 1998, Fisher *et al.* 2000, Houston and Kenworthy 2000, *passim*). A two-site model in which the enzyme can bind more than a single molecular entity simultaneously (i.e. effector and substrate, or two substrates), is used to describe the kinetic properties associated with activation, autoactivation, partial inhibition and biphasic kinetics for CYP enzymes (Korzekwa *et al.* 1998, Houston and Kenworthy

2000). There is to date at least one study to suggest that the phenomenon of allosteric kinetics observed *in vitro* with a number of the drug-metabolizing enzymes may possibly occur *in vivo* with the CYP-probe substrate diclofenac following co-administration with quinidine (Tang *et al.* 1999, Ngui *et al.* 2000).

Enzyme induction

Two primary issues concerning enzyme induction are the reduction in the pharmacological activity resulting from increased drug metabolism and the enhanced toxicity and genotoxic potential resulting from an increase in the rates of active or reactive metabolite formation. Not all drug-metabolizing enzymes are inducible and their response to inducers is highly dependent on the duration and level of exposure to the inducing agent. In addition, it is well established that the regulation and response of drug-metabolizing enzymes to enzyme inducers can occasionally be very different between toxicology species and human, thus enzyme induction in toxicological species may bear little relevance to the clinical situation. For example, omeprazole and rifampacin are inducers of CYP1A and CYP3A, respectively, in humans but not in mouse (Diaz *et al.* 1990, Pitchard *et al.* 1990). In contrast, mephenytoin and pregnenolone 16- α -carbonitrile are both inducers of CYP in rat but do not appear to induce human CYP. Phenobarbital is an inducer of both rat and human CYP, however in rat phenobarbital induces CYP2B1/2 whereas in human phenobarbital induces CYP3A and the CYP2C subfamily of enzymes.

Enzyme induction is most apparent with high doses or prolonged exposure to an inducing agent. Enzyme induction, like enzyme inhibition, is a dose-dependent adaptive response of drug-metabolizing systems that is likely to have evolved as a protective mechanism against the accumulation of xenobiotic agents. In most cases, induction of drug-metabolizing enzymes leads to increased detoxification of drugs, but occasionally enzyme induction leads to the formation of active metabolites or reactive intermediates (Conney 1982). For example, enzyme inducers of CYP2E1, such as ethanol and isoniazid, lead to the enhanced metabolism of acetaminophen to form the hepatotoxic metabolite, *N*-acetylbenzoquinoneimine. With drugs that are both inhibitors and inducers of drug-metabolizing enzymes, the processes of drug-drug interaction become more complex. For example, ethanol is both an inhibitor and inducer of CYP2E1 (Zand *et al.* 1993). The initial effect of co-administering ethanol and acetaminophen is the observed inhibition of CYP2E1-dependent metabolism of acetaminophen. This is followed by activation of acetaminophen because of the time-dependent increase in CYP2E1 levels leading to an overall induction of acetaminophen metabolism. Not all inducers of drug-metabolizing enzymes necessarily lead to the formation of genotoxic events. For example, the chronic treatment of rodents with phenobarbital-type inducers (inducers of CYP2B) and peroxisome proliferators (inducers of CYP4A) results in the formation of liver and thyroid tumours (Parkinson 1996b). In the case of thyroid tumours, the induction of UDPGT activity leads to an increase in the glucuronidation of thyroid hormones which, in turn, results in chronic stimulation of the thyroid by a compensatory increase in the formation of thyroid stimulating hormone. These events are not observed in human. The prolonged elevation of thyroid stimulating hormone in humans leads to the reversible enlargement of the thyroid gland, which is associated with iodide deficiency.

Evidence that a drug is an inducer of drug-metabolizing enzyme activity usually derives from preclinical metabolism and toxicological data. To establish the significance of enzyme induction requires an understanding of the dose-response relationship of the induction process, the level of induction, the enzymes involved and how enzyme induction in toxicology species is likely to reflect the clinical situation. The important challenge is to extrapolate data from toxicology species to the clinical situation by deriving the likely human exposure and induction potential from the minimum drug exposure level(s) in animals that are required to achieve measurable enzyme induction (Park *et al.* 1996). It may be reasonable to assume that a drug is unlikely to induce drug-metabolizing enzymes when there is no evidence of enzyme induction in toxicology species and no change in the pharmacokinetic properties of a drug following repeat dosing to human volunteers. Depending on the intended therapeutic use of the drug, safety pharmacology and *a priori* knowledge of related therapeutic drugs, it may be necessary to follow-up preclinical and clinical findings with an appropriately designed clinical drug-drug interaction study. The concomitant administration of several probe-substrates (cocktail approach) can provide a useful means to measure simultaneously the activity of multiple drug-metabolizing enzymes in human subjects and to assess the likelihood of enzyme induction in human subjects (Frye *et al.* 1997, Streetman *et al.* 2000).

The use of *in vitro* models provides an important adjunct to assessing enzyme induction in toxicology species. Currently, the most widely accepted *in vitro* model for the measurement of enzyme induction uses primary cultured hepatocytes (Donato 1995, Li *et al.* 1997). The use of primary cultured human hepatocytes, in particular, provides a useful tool in the early detection and assessment of human enzyme induction. Several methods appear in the literature describing the evaluation of enzyme induction and inhibition using primary human hepatocytes (Pitchard *et al.* 1990, Jurima-Romet *et al.* 1996, Li *et al.* 1997). Unfortunately, the use of primary culture hepatocytes can show considerable qualitative and quantitative variation in results depending on either the quality of the tissue procured or the techniques used in the isolation and culturing of human hepatocytes. The period of cell culture before initiation of inducer treatment, the extent of cell differentiation and the seeding density of hepatocytes, are all found to influence the expression of drug-metabolizing enzymes. One potential problem with using cultured human hepatocytes is the rapid loss of drug-metabolizing enzyme activity, although several methods are described in the literature by which improvements in CYP maintenance can be achieved. Examples of predictive drug interactions arising from enzyme induction, and the practical aspects and scientific merit of using hepatocytes for predictive DMPK are discussed elsewhere in this issue of *Xenobiotica*.

CYP enzyme induction and transcriptional control involve a complex array of regulatory elements controlling gene expression (Lewis 1996b, Park *et al.* 1996, Pascucci *et al.* 2000). The regulatory elements of CYP genes vary, even within the same gene family or subfamily. This could help explain why closely related genes can differ in their response to inducing agents and level of expression (i.e. constitutive versus inducible expression of CYP enzymes). Furthermore, multiple genes with xenobiotic regulatory element (XRE)-like sequences, or the presence of several response elements within the same gene may provide explanations for multiple enzyme induction by a single agent and the induction of a single CYP

enzyme by several different inducing agents (Lewis 1996, Pascussi *et al.* 2000). In most examples of CYP induction, transcriptional activation of CYP genes is among the most common mechanism of CYP induction. Other mechanisms of CYP induction include mRNA processing, mRNA stabilization, increased translation and enzyme stabilization.

The ability of drugs to alter gene expression can be exploited *in vitro* by use of reporter gene constructs (Ogg *et al.* 1997, 1999, El-Sankary *et al.* 2000). The establishment of cell lines of test systems is challenging because of the complexity of the regulatory machinery controlling gene expression, and multiple mechanisms by which induction can occur. In using this approach, however, the regulatory elements of the gene of interest are incorporated into a plasmid vector together with an assay reporter gene. The use of reporter gene constructs is not only a useful tool in helping to elucidate the mechanisms and regulatory elements of CYP expression, but also as a potential tool for the rapid assessment of new chemical entities as potential inducers of CYP enzymes (Giddings *et al.* 1997).

Practical aspects of drug-drug interaction studies

The objective of enzyme inhibition studies is to replicate *in vitro* the principal metabolic pathways of the drug or probe substrate as they would occur *in vivo* and to quantify the inhibitory potency and mechanism of inhibition of a suspected inhibitor. Of the different *in vitro* models available to study enzyme inhibition, liver microsomes are usually the preferred *in vitro* model of choice for a wide range of different metabolism-based studies. The advantage of using liver microsomes is the presence of microsomal enzymes in the proportions found *in vivo*. This is particularly important where more than a single enzyme contributes to the initial or primary metabolism of a compound (i.e. Phase I) under investigation. Isolated hepatocyte preparations are often used to characterize both Phase I and II reactions to obtain information on the complete hepatic metabolic profile of a drug (Fisher *et al.* 1997). Hepatocyte preparations are also used to characterize the inhibitory potency of new drug entities where there is some suggestion that Phase II enzymes play a significant role in the metabolic clearance of the parent drug. Some of the practical aspects of enzyme inhibition studies are discussed below with respect to the use of liver microsomal protein, substrate and inhibitor concentrations, and the kinetics of enzyme inhibition that can potentially affect the interpretation of the experimental data obtained.

Determination of IC_{50} and K_i

One of the simplest approaches to obtain a measure of the inhibitory potency of a NDE utilizes a fixed concentration of the probe substrate, at or below the K_m for the reaction measured, for a particular drug-metabolizing enzyme and a range of NDE concentrations (figure 3). The decrease in the rate of a reaction with increasing drug concentration yields an estimate of the concentration of NDE required to inhibit 50% (IC_{50}) the control activity (*vo*). The advantages of determining IC_{50} is that fewer samples require analysis and therefore can be used to rapidly screen the inhibitory potential of new chemical entities in discovery. A drawback from determining IC_{50} , however, is that the mechanism of inhibition can not be determined. Providing a sufficient number of data are

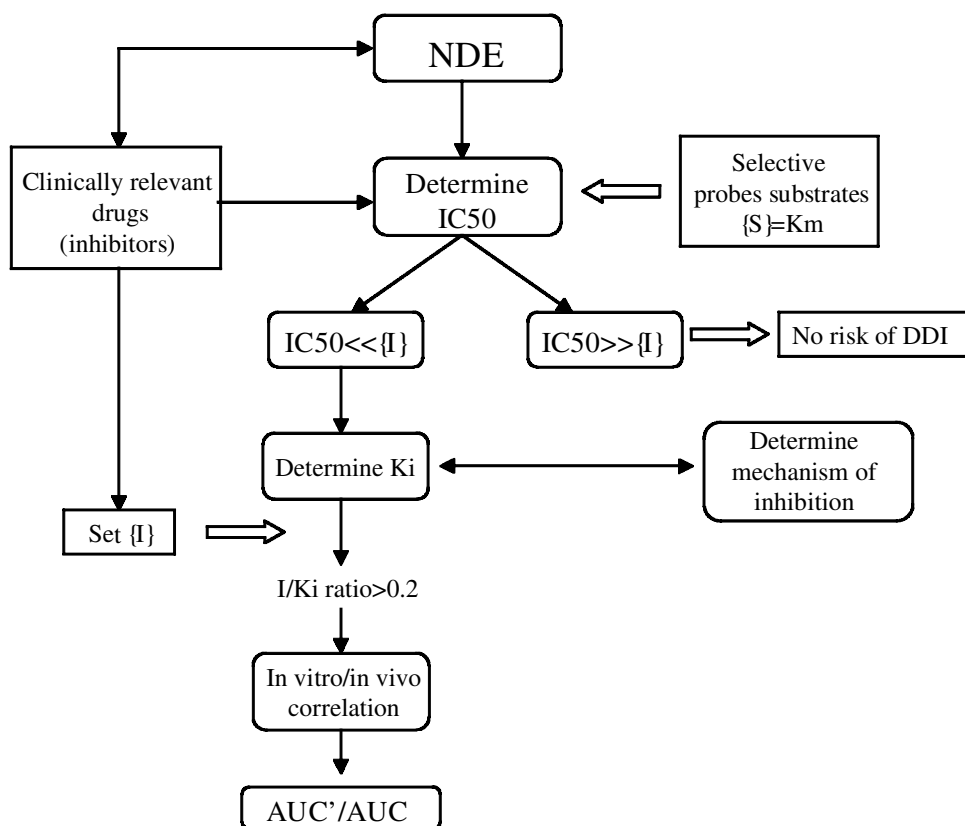


Figure 3. Scheme for the determination of IC_{50} and K_i values. NDE is incubated with CYP-probe substrates and/or clinically relevant drugs to derive IC_{50} and K_i values. Conversely, CYP-probe inhibitors and clinically relevant inhibitors of drug-metabolising enzymes are co-incubated with the NDE to assess potential inhibition of NDE metabolism.

collected, then atypical or non-Michaelis–Menten kinetics can be inferred if the IC_{50} curve shows initial positive cooperativity at low concentrations followed by inhibition at higher concentrations of inhibitor (see below). Partial inhibition of enzyme activities can also be revealed using the IC_{50} curves. Although partial inhibition is often attributed to poor solubility of a drug, several other explanations can also be offered such as a lack of substrate specificity or multiple substrate binding sites associated with the enzyme concerned. Where there is some question about the selectivity of a probe substrate, it might be appropriate to use single enzyme expression systems to confirm complete or partial inhibition of an enzyme-catalysed reaction.

IC_{50} are limited with respect to their use in *in vitro*–*in vivo* scaling, since the IC_{50} is only applicable to the substrate concentration used in the *in vitro* study. For example, if the inhibition of a reaction is competitive then the IC_{50} will be equal to twice the K_i , providing that the substrate concentration used is less than or approaching the K_m of the enzyme-catalysed reaction. However, with increasing concentrations of substrate ($[S] \gg K_m$), determination of IC_{50} will overestimate K_i . In non-competitive enzyme inhibition, $IC_{50} = K_i$ and the inhibition of the enzyme-catalysed reaction is independent of the substrate concentration used.

The K_i associated with a reversible inhibitor of CYP, with the possible exception of CYP3A4, is independent of the substrate or route of metabolism by which the substrate is metabolized. With CYP3A4-catalysed reactions, it appears that K_i may be specific for some substrate-inhibitor pairs (von Moltke *et al.* 1996).

The determination of K_i relies on the co-incubation of a range of both substrate and inhibitor concentrations. The results can be analysed by non-linear regression analysis to yield the K_i and the mechanism of inhibition can be determined using either visual inspection of Lineweaver-Burk and Dixon plots, or an assessment of the statistical goodness-of-fit from non-linear regression analysis. In using either approach to determine K_i , there remains at present no independent biochemical test to validate the apparent mechanism of inhibition. The number of data points gathered for the determination of IC_{50} or K_i is obviously important when attempting to characterize the mechanism and extent of enzyme inhibition. It may also become apparent that with additional experimental data points, hyperbolic kinetics no longer apply and with further modelling it becomes apparent there is more than one enzyme involved in the reaction measured, whether it be cooperative substrate binding (activation), or product inhibition and secondary metabolism. Goodness-of-fit criteria obtained from mathematical modelling techniques may provide an indication of the mechanisms involved. However, beyond the immediate practical requirements of obtaining intrinsic clearance and preliminary inhibition potential of a drug, further experimental data and more complex modelling studies might obscure the practical issues of just obtaining good approximations of the intrinsic clearance and potency of inhibition required to formulate a decision on the future development of a NDE.

Determining the mechanisms of enzyme inhibition

It might be assumed that inhibition is reversible without *a priori* knowledge of the actual mechanisms of inhibition involved. Assumptions like these leads to potentially erroneous estimates of K_i and consequently inaccurate *in vivo* predictions of the changes in AUC ratio. The inappropriate use of a reversible inhibition model, for example, failed to predict the *in vivo* inhibition of several CYP3A4 substrates by clarithromycin (Albani *et al.* 1993, Sketris 1996, Gorski *et al.* 1998) fluoxetine (Greenblatt *et al.* 1992) and diltiazem (Ahonen *et al.* 1996, Azie *et al.* 1998). By assuming reversible inhibition, the apparent K_i determined for clarithromycin, fluoxetine and diltiazem were 50, 10 and 60 μM respectively. Since the apparent K_i are much higher than the *in vivo* steady-state plasma concentrations for all three drugs ($I/K_i < 0.2$), no interactions *in vivo* between these three drugs and other CYP3A4 substrates would be expected to occur (Rodrigues and Wong 1997). However, the appropriate use of an irreversible inhibition model to incorporate rates of CYP3A inactivation greatly improves *in vivo* predictions of an increase in the plasma AUC of a co-administered drug with each of these three CYP3A4 inhibitors (Mayhew *et al.* 2000). Important links between K_i , k_{inact} and time-dependent inhibitory potency (IC_{50}^t) for the determination of mechanism-based inactivation of several enzyme-catalysed reactions are clearly important requirements when correctly identifying or predicting *in vivo* drug interactions (Maurer *et al.* 2000, Mayhew *et al.* 2000).

Quasi-irreversible and irreversible inhibition

A number of drugs in clinical use are metabolized by CYP to form quasi-irreversible inhibitory complexes, which render the enzyme inactive. These complexes can be reversed *in vitro* by the addition of highly lipophilic substrates that displace the inhibitory complex thereby restoring the catalytic activity of CYP. Other techniques employed to distinguish quasi-irreversible inhibitors of CYP involve the addition of potassium ferricyanide to disrupt the ferrous complex by oxidation of the haem function to form the ferric state thus restoring functional activity of the enzyme (Pessayre *et al.* 1982).

Irreversible inhibitors are metabolized by CYP to form reactive intermediates. These reactions are often referred to as mechanism-based or suicide inhibitors and involve alkylation of the haem thereby completely rendering the enzyme inactive. Alternatively, reactive intermediates react with the apoprotein of CYP with varying degrees of inhibitory potency depending on the region of the protein modified by the reactive intermediate and the active site. Pre-incubation of the suspected inhibitor with liver microsomes and cofactor prior to the addition of substrate can help distinguish between competitive and mechanism-based inhibition and can show significant differences in the measured K_m , V_{max} and K_i . For example, proadifen with no pre-incubation affects the K_m but not V_{max} , whereas in contrast, pre-incubation of proadifen with liver microsomes results in a decrease in V_{max} but with no change in the apparent K_m of the reaction (Franklin *et al.* 1977). This example, of which there are probably many more, illustrates the importance of correctly designing *in vitro* experiments to identify mechanisms of enzyme inhibition.

Inhibitor and substrate concentrations

The use of clinically relevant inhibitor and substrate concentrations is an important consideration when designing studies to investigate the intrinsic clearance and the inhibitory potential of drugs under investigation. The use of inhibitor concentrations in excess of the expected *in vivo* plasma concentration may demonstrate inhibition that is not necessarily clinically relevant. The inappropriate use of substrate concentrations might, for example, implicate less important enzymes in the metabolic clearance of a NDE and in turn, overestimate the predicted effect of inhibiting these enzymes in the clinic. For example, CYP2C-dependent *N*-demethylation of diazepam is the major metabolic route of elimination in human subjects receiving an oral dose of diazepam. CYP2C-dependent *N*-demethylation of diazepam is also the principal route of metabolic elimination catalysed by human liver microsomes at clinically relevant plasma concentrations of diazepam. However, liver microsomal incubations containing 0.2 mM diazepam would, by contrast, have identified CYP3A4-dependent *N*-demethylation of diazepam as the most important route of diazepam clearance. Furthermore, the importance of diazepam *N*-demethylation as a route of metabolic elimination changes with changing substrate concentration (figure 4). At high substrate concentrations (0.2 mM) diazepam 3-hydroxylase activity is between 3- and 4-fold higher than diazepam *N*-demethylase activity, whereas at low substrate concentrations (0.02 mM) the activities of both pathways are similar (Yasumori *et al.* 1993, Jung *et al.* 1997).

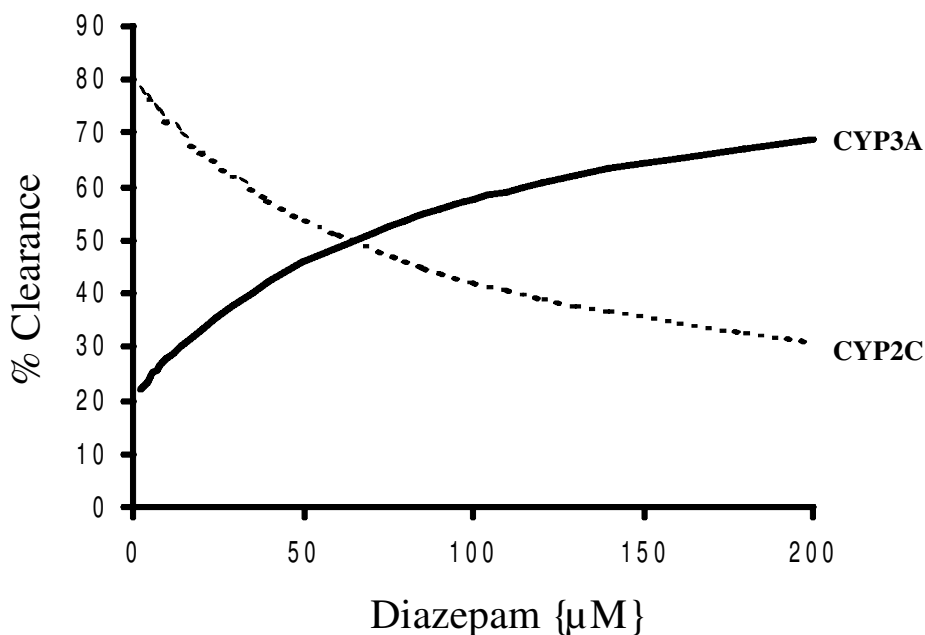


Figure 4. Effect of substrate concentration on CYP-dependent diazepam N-dealkylation. Diazepam N-dealkylation catalysed by human CYP2C and CYP3A. (—) % clearance by CYP3A, (---) % clearance by CYP2C. K_m and V_{max} values used to calculate CYP2C ($K_m = 20 \mu M$, $V_{max} = 0.27 \text{ nmol mg}^{-1} \cdot \text{min}^{-1}$) and CYP3A ($K_m = 686 \mu M$, $V_{max} = 2.4 \text{ nmol} \cdot \text{mg}^{-1} \cdot \text{min}^{-1}$) dependent clearance of diazepam were obtained from Yasamori *et al.* 1993.

Protein binding

Physiological models (well-stirred and parallel tube) used to describe the hepatic extraction of drugs are dependent on the hepatic intrinsic clearance, liver blood flow and blood protein binding. The well-stirred model is probably the most commonly used (equation 6), providing a link between intrinsic clearance for a particular drug and hepatic clearance (CL_H), where Q_H is the hepatic blood flow, fraction of drug unbound in plasma (f_u) is the unbound fraction of drug in blood, CL_{int} is the intrinsic measure of the enzyme activity (V_{max}/K_m), and E is the hepatic extraction ratio. By use of either model to describe the hepatic extraction of drugs, Obach *et al.* (1997) found that, for a large number of drug compounds, the predicted hepatic clearance was markedly lower than the observed values. Furthermore, the data revealed that the under prediction of *in vivo* clearance was closely associated with highly bound drugs, the majority of which were lipophilic amines. By removing the term (f_u) used to describe plasma protein binding for highly protein bound lipophilic compounds, predictions of *in vivo* clearance appear to improve markedly. Obach *et al.* (1997) concluded that the fractional binding of basic lipophilic compounds to plasma and microsomes was probably similar and consequently the term used to describe f_u effectively cancels:

$$CL_H = Q_H \cdot E = \left(\frac{Q_H \cdot f_u \cdot CL_{int}}{Q_H + f_u \cdot CL_{int}} \right) \quad (6)$$

Acidic drugs such as tolbutamide, phenytoin and caffeine do not bind appreciably to liver microsomes (low K_D) and the extent of this non-specific binding is

independent of the drug concentration. In contrast, non-specific binding of weak lipophilic bases to liver microsomes at concentrations equal to or greater than the binding dissociation constant (K_D) can result in complex sigmoidal effects on the kinetics of an enzyme-catalysed reaction with respect to total and unbound drug concentrations (McLure *et al.* 1999). Interestingly, it has been suggested that the sigmoidal kinetics sometimes associated with autoactivation of CYP-catalysed reactions might occur as a consequence of non-specific binding (McLure *et al.* 2000). At saturating concentrations the free fraction (f_{umic}) is assumed to vary with both substrate and microsomal protein concentrations. Therefore, in enzyme inhibition studies, knowledge of the free fraction of inhibitor ($f_{\text{umic}}I$) in an *in vitro* incubation mixture is equally as important as determining the K_m from the free fraction of drug substrate ($f_{\text{umic}}S$) present in the incubation mixture. The potency of inhibition is also likely to be underestimated if the protein concentration used markedly affects the free fraction of inhibitor present in a reaction either because the inhibitor itself is rapidly metabolized or as a result of non-specific binding to microsomal protein. Finally, the use of high microsomal protein concentrations ($> 1 \text{ mg mL}^{-1}$) is sometimes associated with futile binding and non-linear first-order kinetics – largely avoided by using lower microsomal protein concentrations ($< 1 \text{ mg mL}^{-1}$) in the reaction mixture.

Combinations of different protein, inhibitor and substrate concentrations are likely to be important contributors to the interlaboratory variation of K_i reported in the literature for a given reaction. K_i for the inhibition of human liver microsomal CYP3A-dependent midazolam hydroxylation by ketoconazole varies by up to 28-fold ($0.004\text{--}0.1 \mu\text{M}$) between different studies (Wrighton and Ring 1994, von Moltke *et al.* 1996b, Gibbs *et al.* 1999). The apparent K_i for ketoconazole-CYP3A4 interactions at 1.5 and 0.25 mg mL^{-1} protein is 8 and $0.03 \mu\text{M}$ respectively, representing a 6-fold change in protein but a > 270 -fold difference in the apparent K_i (Lampen *et al.* 1995, Bourri  *et al.* 1996, von Moltke *et al.* 1996b). Studies have also shown that the extent of CYP3A4 inhibition ($1 - V_i/V_o$) is substantially decreased with increasing protein concentration for two highly protein bound inhibitors of CYP, clotrimazole and ketoconazole, consistent with a reduction in the free fraction of these inhibitors. In contrast, CYP3A4 inhibition by fluconazole is largely unaffected by changes in protein concentration because fluconazole itself exhibits low protein binding by liver microsomes (Humphrey *et al.* 1985, Gibbs *et al.* 1999).

Estimation of inhibitor concentrations

Estimating the free fraction of substrate ($f_{\text{umic}}S$) and inhibitor ($f_{\text{umic}}I$) are potentially important parameters required for the experimental determination of K_m and K_i . Two factors likely to contribute to the inaccuracy of the measured K_m and K_i are the uncertainties in determining non-specific protein binding and from estimating the suspected *in vivo* hepatocellular concentrations of substrate and inhibitor.

In vitro metabolism reactions are typically optimized by use of inorganic buffers and non-physiological pH to achieve maximal turnover of a substrate under investigation. Furthermore, *in vitro* incubations are devoid of plasma thereby raising issues of correction factors required to adjust for the unbound fraction of drug in plasma and hepatic cytosol to reflect the *in vivo* situation. The

net effect of plasma and hepatic cytosol on substrate and inhibitor binding is likely to be mixed involving a combination of hydrophobic and ionic interactions between, for example, negatively charged phospholipids and the ionic charge associated with the substrate and inhibitor under investigation. In cases where partitioning is extensive, then the concentration of drug and inhibitor is likely to markedly differ from the concentration at the active site. The effect of this will be to obtain an apparent K_i that exceeds the true value and therefore an underestimation of the extent of *in vivo* inhibition. Extensive partitioning is well known for basic lipophilic compounds. Where tissue partitioning to the liver is high, the extent of tissue-plasma partitioning appears to correlate with phosphatidylserine concentrations in tissues. Phospholipids are used in the reconstitution of purified protein. However, the concentrations used to achieve turnover, whilst optimal for use in *in vitro* studies (Shaw *et al.* 1989) are also unlikely to reflect the role of the intact phospholipid barrier in predetermining *in vivo* concentrations of substrate and inhibitor at the active site of metabolism.

Whilst there are methods to derive non-specific microsomal binding, there are no amenable methods of directly measuring the effective *in vivo* concentration of the drug substrate or inhibitor (Miyauchi *et al.* 1993, Yamano *et al.* 1999). As a consequence, hepatocellular substrate and inhibitor concentrations are often assumed equal to the total therapeutic concentration in plasma for use in *in vitro-in vivo* correlation studies (He *et al.* 1995, Ring *et al.* 1995, Kunze *et al.* 1996, von Moltke *et al.* 1996b, Wrighton and Ring 1999). Using this approach has arguably correctly predicted a number of *in vivo* drug interactions (Ring *et al.* 1998). In other examples, however, use of plasma concentrations as a means of setting drug substrate and inhibitor concentrations have resulted in an under-prediction of several *in vivo* drug-drug interactions. The different *in vitro-in vivo* approaches adopted by investigators to compensate for the under-prediction of *in vivo* drug-drug interactions are discussed below along with examples of *in vitro-in vivo* correlation studies.

Non-CYP drug interaction studies

Although glucuronidation of drugs is considered quantitatively the most important of the Phase II reactions in drug metabolism, there are few examples describing *in vitro-in vivo* correlation of glucuronosyltransferase activity (Mistry and Houston 1987, Rance *et al.* 1987). This is in part attributable to a number of uncertainties associated with rate controlling factors such as native versus activated microsomes, co-substrate concentrations and non-specific binding within the microsomal preparations. The uncertainties in successfully predicting Phase II *in vivo* CL_{int} are clearly evident with large differences (10–30-fold) between the predicted and observed CL_{int} for several Phase II reactions, including glucuronosyltransferase (Mistry and Houston 1987, Rance *et al.* 1987, Pang and Chiba 1994).

Drugs extensively cleared by Phase II conjugation (fraction of total metabolic clearance (f_m) > 0.3), either as a result of direct conjugation or conjugation of Phase I metabolites, are subject to potential drug-drug interactions the effects of which are either to increase the free drug concentration or to affect conjugation of endogenous metabolites (see above). Lamotrigine is an antiepileptic compound metabolized mainly by direct glucuronidation in man to form *N*-glucuronides

(Sinz and Rimmel 1991). Human liver microsomes supplemented with UDPGA similarly form glucuronides of lamotrigine, a reaction that is inhibited by chlorpromazine, testosterone, ethinylestradiol and norethindrone, but not amitriptyline or imipramine (Magdalou *et al.* 1992). The selective competitive inhibition of lamotrigine *N*-glucuronidation suggests a specificity of UDPGT activity and a possible indication of Phase II drug interactions in the clinic. Zileuton, a selective 5-lipoxygenase inhibitor, is extensively metabolized by direct glucuronidation ($f_m = 0.8$), and to a much lesser extent hydroxylation and sulfoxidation by CYP1A2, CYP2C9, CYP2D6 and CYP3A4 ($f_m = 0.1$). Clinical interaction studies demonstrate a 52% decrease in the plasma clearance of theophylline *N*3-demethylation by zileuton implicating the potential inhibition of CYP1A2. Zileuton also decreases plasma clearances of propranolol (42%), phenytoin (15%) and naproxen (15%), possibly through the inhibition of CYP1A2, CYP2D6 and CYP2C9. However, the high K_m for CYP-dependent metabolism of zileuton (~ 0.35 mM) and the fraction of total clearance attributable to direct glucuronidation ($f_m = 0.8$) could infer possible changes in plasma clearance due, at least in part, to the inhibition of UDPGT activity (Sweeny *et al.* 1992, Machinist *et al.* 1995). The potential for complex patterns of enzyme inhibition involving both Phase I and II enzymes will require studies specifically designed for the purpose of assessing possible drug interactions involving Phase II enzymes.

Influence of intestinal metabolism and consequences of predicting in vivo drug interactions

Hepatic extraction has long been recognized to affect bioavailability, whilst it is only recently that intestinal drug-metabolizing enzymes have been shown to play an important role in the first-pass elimination of several important drugs (Sharp *et al.* 1992, *passim*). The contribution of intestinal enzymes to the first-pass elimination of certain drugs can be significant with overall bioavailability determined by both hepatic and intestinal extraction. The impact of intestinal metabolism will depend on a number of factors such as the residence time of the drug, the relative contribution of the various hepatic and intestinal enzymes, and kinetics governing inhibition and metabolism. The p-gp drug-efflux pump is also known to play an important part in modulating the absorption of many drugs (Wacher 1998) where it acts as an efflux transporter of drugs in the luminal membrane of gut epithelial cells (van Asperen *et al.* 1998, Yu 1999). Studies have also revealed a notably high number of instances of overlaps between substrates for and inhibitors of CYP3A4 and p-gp (Wacher *et al.* 1995), suggesting these proteins have complementary roles in the pharmacokinetics of many drugs.

It is perhaps not surprising that poor predictions of *in vivo* CL_{int} and drug interactions, based solely on *in vitro* hepatic data are obtained, where there are also extrahepatic components contributing to the overall first-pass extraction *in vivo*. For example, Azie *et al.* (1998) have shown that the co-administration of two CYP3A substrates – diltiazem and lovastatin – results in a significant increase in the oral AUC of lovastatin (3.6-fold). However, based on the *in vitro* hepatic K_i (50–100 μ M) for CYP3A4 inhibition by diltiazem and an average steady-state diltiazem serum concentration of 0.3 μ M, no pharmacokinetic interaction would be predicted; I/K_i is well below the expected threshold of 0.2, above which an interaction would be predicted. The authors attribute the lack of *in vitro-in vivo*

prediction due to a significant contribution of intestinal CYP3A4-mediated first-pass elimination as well as the potential inhibition of p-gp-mediated transport. A contributing factor leading to the under prediction of diltiazem-mediated drug interaction is the use of the reversible K_i for a reaction that is ostensibly irreversible in nature. The example of diltiazem and lovastatin illustrates the potential pitfalls of predicting 'false-negative' drug interaction scenarios from hepatic microsomal studies for a number of drugs that are important substrates of gastrointestinal proteins. Therefore, to avoid 'false-negative' predictions pharmacokinetic absorption models similar to the model proposed by Ito *et al.* (1999) is going to be required if meaningful predictions of the likelihood of pharmacokinetic drug interactions are going to be successfully predicted.

Enzyme activation

From a practical consideration of *in vitro*-*in vivo* scaling, activation is a phenomenon that requires careful consideration when attempting to interpret *in vitro* clearance data. Preliminary studies, particularly in the context of early drug discovery projects, might be to attempt the prediction of drug clearance with insufficient data to characterize fully the kinetics of a reaction. One of the consequences of this is for sigmoidal kinetics to remain unidentified and therefore *in vitro* clearance and predictive *in vivo* clearances to be underestimated (Houston and Kenworthy 2000). The impact of approximating inhibition data towards hyperbolic kinetics for the assessment of *in vivo* predictive modelling of drug interactions is difficult to fully evaluate *in view* of the many other unresolved variables used in predictive human drug interaction studies. However, use of IC_{50} measurements, particularly for high-throughput screening with a minimal number of data points, poses issues of missing atypical concentration-effects, or fractional inhibition-inhibitor concentration profiles typical of non-hyperbola kinetics. If the kinetic data are wrongly interpreted, erroneous estimates of K_i are likely to be obtained especially as inhibitor and substrate concentrations increase or decrease relative to their respective K_i and K_m . Therefore, to obtain meaningful data (i.e. IC_{50} and K_i), it is suggested that adequate data are obtained and analysed using appropriate models to describe multiple substrate binding kinetics (Leff and Dougall 1993, Houston and Kenworthy 2000).

Extrapolation of *in vitro* data to the *in vivo* situation

The use of *in vitro* studies to identify and characterize the inhibitory potential of new drug entities is an important component of the drug discovery and lead optimization process. Equally important is the ability to correctly extrapolate *in vitro* interaction data to the *in vivo* situation, often requiring a series of assumptions by which to make initial estimates of likely drug interactions *in vivo*. Most *in vitro* approaches assume the liver is the main organ of drug elimination linking hepatic intrinsic clearance, binding and hepatic blood flow to obtain a measure of hepatic clearance. The well-stirred model (equation 6) is effectively independent of the mechanism of inhibition because a change in either V_{max} (e.g. non-competitive) or K_m (e.g. competitive) results in decreased intrinsic clearance (CL_{int}) of the drug.

Hepatic clearance is directly related to CL_{int} and f_u for drugs with low hepatic extraction ($< 30\%$ hepatic blood flow) and any change in CL_{int} will result in an almost equal change in clearance. For high-clearance drugs ($> 70\%$ hepatic blood flow) hepatic clearance is limited by hepatic blood flow ($CL_H = Q_H f_u$).

For an orally administered drug, the *in vivo* CL_{int} and AUC are directly related (equation 7), where f_a is the fraction of drug absorbed and f_u is the fraction of unbound drug in blood:

$$AUC_{\text{oral}} = \frac{f_a \cdot \text{dose}}{f_u \cdot CL_{\text{int}}} \quad (7)$$

It is useful to be able to link *in vitro* CL_{int} to the *in vivo* plasma concentration of drugs in the presence and absence of an inhibitor (equation 8), but to achieve this requires a number of assumptions. First, any change in the *in vitro* CL_{int} of a probe substrate used to measure the effect of an inhibitor must similarly reflect a corresponding change in the ratio of the intrinsic clearance *in vivo*. Second, it must be assumed that the *in vivo* pharmacokinetics is linear in the presence and absence of a reversible inhibitor, and the liver is the principle organ of elimination. Third, the dose is 100% available at the active site of metabolism and there is only one metabolic route of elimination for the probe substrate:

$$\frac{AUC_{\text{oral}}}{AUC_{\text{orali}}} = \frac{CL_{\text{inti}}}{CL_{\text{int}}} = 1 + \frac{[I]}{K_i} \quad (8)$$

If the assumptions are correct then in the presence of a reversible competitive inhibitor a change in the intrinsic clearance (CL_{inti}) will reflect a change in the ratio of the area under the curve in the presence ($AUC_{\text{oral}i}$) and absence (AUC_{oral}) of the inhibitor. Furthermore, the relationship between AUC and CL_{int} suggests the inhibitory potential might be predicted based on the ratio of $[I]$ and K_i alone. Clinically relevant interactions are considered more likely if $[I]/K_i > 0.2$, where $[I]$ is the maximal expected therapeutic inhibitor concentration (Ito *et al.* 1998).

The metabolic elimination of most drugs often involves more than a single enzyme and route of metabolic elimination. Therefore, to obtain better estimates of the inhibitory potential for a particular metabolic route of elimination, the fraction of total metabolic clearance inhibited (f_m) can be obtained by use of (equation 9) (Rowland and Matin 1973):

$$\frac{AUC_{\text{oral}(i)}}{AUC_{\text{oral}}} = \frac{CL'_{\text{int}}}{CL'_{\text{int}(i)}} = \frac{1}{\frac{f_m}{1 + I/K_i} + (1 - f_m)} \quad (9)$$

Equation (9) assumes that there is adequate information on routes of metabolism for the inhibited drug under investigation – normally only available with the use of radiolabelled drug.

To minimize the likelihood of ‘false-negative’ or under predictions of $[I]$, estimates of the unbound blood concentration of inhibitor $[I_u]$ reaching the hepatic portal vein following the absorption of an oral dose has also been used as a means to setting $[I]$ (equation 10) (Ito *et al.* 1998).

$$I_u = \left(I_{\text{max}} + k_a \cdot F_a \cdot \frac{\text{dose}}{Q_h} \right) \cdot f_p, \quad (10)$$

where $I_{\max}$ is the maximum inhibitor concentration in portal blood, k_a is the first-order rate constant for gastrointestinal absorption, f_a is the fraction of the dose absorbed from the GI tract and f_p is the unbound fraction in plasma.

Approaches used to predict *in vivo* drug-drug interactions

An inspection of the current literature reveals that in most cases *in vitro-in vivo* correlation studies are at best empirical estimates of the likely outcome of a drug-drug interaction occurring in the clinic. This is in part due to a lack of appropriate models required to determine true substrate and inhibitor concentrations at the active site of metabolism (see above). It is also conceivable that with the diversity of physicochemical and pharmacokinetic characteristics of drugs, no single approach for the *in vitro-in vivo* correlation of different drug combinations can be readily achieved. The ratio of the inhibitor concentration $[I]$ and corresponding inhibition constant K_i are widely regarded as the more important parameters required to estimate the potency of enzyme inhibition (Ito *et al.* 1998, von Moltke *et al.* 1998). However, simply determining $[I]/K_i$ raises several questions with respect to the appropriate determination of $[I]$, such as estimating the concentration of the inhibitor at the site of metabolism, plasma-tissue distribution and use of either the unbound or total plasma concentrations of the inhibitor. In an attempt to predict successfully the *in vivo* drug interaction of concomitantly administered drugs, several *in vitro-in vivo* approaches have been proposed based on the total bound or unbound concentrations of inhibitor in plasma as a measure of the concentration of inhibitor in liver. Alternatively, *in vitro-in vivo* approaches have used estimates of the liver tissue:plasma or liver tissue:water partition coefficients. In each of the *in vitro-in vivo* correlation approaches described in the literature, the steady-state concentration or peak plasma concentration of the inhibitor ($I_{\max}$) following single administration are used as the basis on which to formulate *in vivo-in vitro* predictions.

Restrictive elimination

One of the controversies surrounding *in vitro-in vivo* correlation studies is the use of either the unbound or total plasma concentration of inhibitor. The basic doctrine of pharmacokinetic studies is that the unbound concentration in blood remains in equilibrium with intracellular concentrations and it is only the unbound concentration which is pharmacologically active (Houston 1994, Lin and Lu 1998). However, there is evidence to suggest that the total plasma concentration rather than the unbound concentration can be used to achieve good *in vitro-in vivo* predictions of drug interactions.

Kohl and Steinkellner (2000) described an approach to incorporate the use of total and unbound plasma concentration to achieve predictions of the CYP2C9-mediated interactions between the anti-inflammatory drugs lornoxicam and tenoxicam, and the anti-coagulant drugs *S*-warfarin, phenprocoumon and acenocoumarol. In the case of *S*-warfarin, tenoxicam and phenprocoumon, the unbound drug concentrations were used as an estimate of the concentration available at the site of metabolism as the hepatic extraction ratio for each of these compounds was less than the unbound fraction in plasma (restrictive elimination). For acenocoumarol and lornoxicam, total plasma drug concentrations were chosen as the hepatic

Table 2. *In vitro-in vivo* predictions.

Drug	Inhibitor		K_i (μM)	I/K_i	I_{umax} HPV	Liver: blood K_p	Predicted AUC ratio	Observed AUC ratio
Tolbutamide	acidic	sulfaphenazole ¹	0.3	88.9	yes	no	4.8	5.3
Tolbutamide	acidic	sulfamethizole ¹	53	1.2	yes	no	1.8	1.6
S-warfarin	acidic	lornoxica ^m . ²	4	0.9	no	no	1.58	1.32
R-acenocoumarol	acidic	lornoxica ^m . ²	10	0.3	no	no	1.10	0.96–1.20
S-phenprocoumon	acidic	lornoxica ^m . ²	12	0.3	no	no	1.13	1.24
Triazolam	basic	ketoconazole ^{3,4}	0.023	15.0	yes	no	7.8	8.9
Triazolam	basic	ketoconazole ³	0.006	4.0	no	no	9.0	8.3
			0.023	–		yes		
			0.006	–		yes		
Terfenadine		ketoconazole ⁴	0.024	3.8	yes	no	1.3	> 10
			0.24	0.4				
			34.0	0.004				
S-warfarin	acidic	tenoxicam ²	34.0	0.004	no	no	1	1
Midazolam	basic	itraconazole ⁵	0.28			yes		
Erythromycin		theophylline		0.6	yes	no	1.1	1.1
Alprazolam	basic	fluconazole	83.0	0.01	no	yes		
Midazolam	basic	fluoxetine ^{†6}	5.3	–	no	no	1.4	1.3
		clarithromycin ^{†6}	5.5	–	no	no	6	2.6

Liver: blood, ratio determined for use in *in vitro-in vivo* correlation of AUC ratios; I_{umax} HBV, maximum hepatic portal vein concentration of inhibitor determined for *in vitro-in vivo* correlation of AUC ratios;[†] K_{inact} (0.017 – 0.072 min⁻¹) was determined to predict the increase in AUC.
¹ Komatsu *et al.* (2000); ² Kohl and Steinkeller (2000); ³ von Moltke *et al.* (1996); ⁴ Ito *et al.* (1998); ⁵ von Moltke *et al.* (1998); ⁶ Mayhew *et al.* (2000).

extraction ratio for each of these drugs was greater than the unbound fraction found in plasma, suggesting that the hepatic extraction of acenocoumarol and lornoxica^m is not restricted to the unbound fraction alone. Drug interactions were predicted from the experimental determination of competitive K_i (equation 1) and with no correction for microsomal binding; non-specific binding is low for acidic drugs (Obach *et al.* 1997). By classification of low clearance drugs into restrictively and non-restrictively eliminated drugs, *in vitro-in vivo* correlation of lornoxica^m-acenocoumarol, lornoxica^m-(*S*)-warfarin and lornoxica^m (*S*)-phenprocoumon were sufficiently accurate to predict correctly the relative change in the steady-state plasma concentrations or areas under the plasma concentration–time curve (table 2).

Liver partitioning

The rationale for the construction of the liver:plasma partition model derives from data in the literature to suggest that drug concentrations in liver do not necessarily reflect free or total plasma drug concentrations. *In vivo* and *in vitro* data have consistently shown extensive partitioning for a number of drugs into liver tissue and isolated hepatocytes. Furthermore, human autopsy data show high liver to plasma drug concentration ratios for *lipophilic* drugs, irrespective of the extent of plasma protein binding (von Moltke *et al.* 1998, *passim*).

From the evidence suggesting extensive hepatic uptake and liver partitioning of drugs, von Moltke *et al.* (1996b) proposed an *in vitro-in vivo* scaling approach to account for the liver partitioning of drugs. Initial testing of the *in vitro-in vivo*

approach correctly predicted the *in vivo* inhibitory potency of ketoconazole on triazolam clearance (table 2). Triazolam is eliminated in humans mainly by CYP3A4-dependent metabolite formation of α -hydroxy- and 4-hydroxy triazolam (Eberts *et al.* 1981, Kronbach *et al.* 1989). Components necessary for the model were a K_i for ketoconazole inhibition of triazolam clearance by human liver microsomes, clinical data on *in vivo* clearance of triazolam and an estimate of liver/plasma partitioning of ketoconazole. To estimate ketoconazole concentrations in the liver, mouse liver homogenate was mixed with human plasma and ketoconazole, centrifuged and the supernatant analysed. The concentration of ketoconazole in liver was calculated from the plasma concentration and liver:plasma partition ratio to estimate $[I]$ for ketoconazole. Using K_i and the estimated $[I]$, the fractional activity could be calculated using equations (1) and (2). The *in vitro* prediction closely reflected the nine-fold increase following co-administration of an oral 0.125 mg dose of triazolam and 200 mg dose of ketoconazole (41 ± 7 versus $337 \pm 41 \text{ ml min}^{-1}$). Several studies using a similar approach to estimate liver:plasma partitioning have been reasonably well predicted for other combinations of substrates (alprazolam, desipramine, terfenadine) and inhibitors (fluoxetine, sertraline) (von Moltke *et al.* 1998, *passim*).

Prediction of $I_{in,max}$

The unbound concentration of inhibitor at the site of metabolism is required to obtain an accurate measure of the $[I]/K_i$ ratio. Unfortunately, the intracellular unbound concentration at the site of metabolism cannot be determined experimentally and for drugs transported into the liver by passive diffusion, the unbound drug concentration in liver is assumed to equal the plasma concentration. Whilst this assumption is adequate to predict some *in vivo* drug interactions, it is nevertheless found that to minimize the underpredictions of *in vivo* drug interaction, the total (unbound and bound) plasma concentration or liver:plasma partitioning of inhibitors are required as a semi-empirical approach to setting a higher $[I]$ to avoid 'false-negative' or under-predictions of *in vivo* drug interactions (see below).

Most drug combinations are administered orally. This means that following absorption from the gastrointestinal tract, drug concentrations reaching the liver via the hepatic portal vein are likely to reach the maximum unbound drug concentration in plasma. Using this assumption, Sugiyama and colleagues (Ito *et al.* 1998) proposed a pharmacokinetic model for the quantitative estimation of the likely maximum unbound concentration ($I_{in,max}$) of drug reaching the liver following oral absorption (equation 10). $I_{in,max}$ is dependent on knowing K_a and F_a for the inhibitor, but in the absence of these data estimates of the upper and lower limits expected for K_a and F_a can be used to forecast the extent of *in vivo* drug interactions. The maximum unbound concentration ($I_{in,max}f_u$) can be used to set the inhibitor concentration for the determination of $[I]/K_i$ ratio and to predict the maximum per cent inhibition accordingly.

Using $I_{in,max}$ to set $[I]$, Ito *et al.* (1998) successfully predicted the *in vitro-in vivo* correlation for a number of drug combinations using data available in the literature. Good agreement between several substrate-inhibitor combinations, including triazolam-ketoconazole, sulphafenazole-tolbutamide, erythromycin-theophylline, is obtained (table 2). The triazolam-ketoconazole *in vitro-in vivo*

prediction using the liver:plasma partitioning of drugs to set $[I]$ (von Moltke *et al.* 1996b) and the methods of calculating $I_{in,max}$ both gave good *in vitro-in vivo* predictions. However, a few substrate-inhibitor combinations were poorly correlated with observed *in vivo* interactions. Ito *et al.* (1998) attributed the poor correlation to a number of factors associated with absorption, active transport of drug substrates, metabolic inactivation with the formation of MICs and turnover of the inhibitor relative to the substrate producing a time-exposure effect. Poor predictions associated with enzyme inactivation or formation of MIC complexes are likely to be improved by incorporating the use of K_{inact} (equation 4) in addition to setting $[I]$ (Mayhew *et al.* 2000). Studies incorporating the use of $I_{in,max}$ have successfully been used to predict the inhibitory effects of several sulphonamides on tolbutamide CYP2C9-dependent metabolism (Kanamitsu *et al.* 1999, Koumatsu *et al.* 2000).

Future prospects

Drug interaction studies are an essential means of understanding, predicting and avoiding ADR. The application of *in vitro* models for studying human drug-metabolizing enzymes and identifying the likelihood of potential drug-drug interactions in the clinic is widely acknowledged among DMPK scientists. The rapid expansion and integration of *in vitro* drug metabolism and drug interaction studies in drug discovery and development has largely been fuelled by rapid advances in the identification and characterization of the drug-metabolizing enzymes. The majority of drug-drug interaction studies focus on human liver microsomal cytochromes P450 as the knowledge of these enzymes substantially exceeds those of other drug-metabolizing enzymes. As knowledge of other hepatic and extrahepatic enzymes increase, it will become important to include these enzymes as part of the overall assessment of drug biotransformation and drug-drug interactions. With our current understanding of the role of CYPs in drug biotransformation and enzyme inhibition, an increasing number of empirical and quantitative *in vitro-in vivo* approaches are now used to support the 'class-labelling' of drugs before marketing authorization (Marroum *et al.* 2000).

In the future, it will become increasingly important to develop population pharmacokinetic models to simulate drug-drug interactions that combine the present knowledge of the interindividual variability of human drug metabolism (Tooley *et al.* 1999, Nestorov *et al.* 2000) and physiologically based pharmacokinetics (Nestorov *et al.* 1998a, b) to help forecast with greater accuracy likely pharmacokinetic drug-drug interactions. By incorporating variability associated with *in vitro* K_m , V_{max} and K_i predictions using simulated population data instead of predictions based solely on mean data will provide more meaningful information on the frequency distribution of pharmacokinetic drug-drug interactions in a target population, particularly if pharmacological response is linked to pharmacodynamic models. Finally, rapid knowledge discovery using integrative databases ('data-mining') will inevitably grow with the availability of data and the application of artificial neural networks (Nestorov *et al.* 1999, Kumar and Ballantyne 2000) to help resolve pharmacokinetic issues in future drug discovery and development programmes.

Acknowledgements

The author thanks the many colleagues, past and present who have contributed to many valuable discussions on the subject of drug metabolism and drug–drug interactions.

References

- AHONEN, J., OIKKOLA, K. T., SALMENPERA, M., HYNYNEN, M. and NEUVONEN, P. J., 1996, Effect of diltiazem on midazolam and alfentanil disposition in patients undergoing coronary artery bypass grafting. *Anesthesiology*, **85**, 1246–1252.
- AZIE, N. E., BRATER, D. C., BECKER, P. A., JONES, D. R. and HALL, S. D., 1998, The interaction of diltiazem with lovastatin and pravastatin. *Clinical Pharmacology and Therapeutics*, **64**, 369–377.
- BACK, D. J., TJIA, J. F., KARBWANG, J. and COLBERT, J., 1998, *In vitro* inhibition studies of tolbutamide hydroxylase activity of human liver microsomes by azoles, sulphonamides and quinolines. *British Journal of Clinical Pharmacology*, **26**, 23–29.
- BENSOUSSAN, C., DELAFORGE, M. and MANSUY, D., 1995, Particular ability of cytochrome P450 3A to form inhibitory P450–iron–metabolite complexes upon metabolic oxidation of aminodugs. *Biochemical Pharmacology*, **49**, 591–602.
- BERNARD, S. A. and BRUERA, E., 2000, Drug interactions in palliative care. *Journal of Clinical Oncology*, **18**, 1780–1799.
- BOBERG, E., LIEM, A., MILLER, E. C. and MILLER, J. A., 1987, Inhibition by pentachlorophenol of the initiating and promoting activities of 1-hydroxysafrole for the formation of enzyme-altered foci and tumors in rat liver. *Carcinogenesis*, **8**, 531–539.
- BOCK, K. W., FORSTER, A., GSCHADMEIER, H., BRUCK, M., MUNZEL, P., SCHARECK, W., FOURNEL-GIGLEUX, S. and BURCHELL, B., 1993, Paracetamol glucuronidation by recombinant rat and human phenol UDP-glucuronosyltransferases. *Biochemical Pharmacology*, **45**, 1809–1814.
- BOURRIE, M., MEUNIER, V., BERGER, Y. and FABRE, G., 1996, Cytochrome P450 isoform inhibitors as a tool for the human liver microsomes. *Journal of Pharmacology and Experimental Therapeutics*, **277**, 321–331.
- BRIAN, W. R., SARI, M. A., IWASAKI, M., SHIMADA, T., KAMINSKY, L. S. and GUENGERICH, F. P., 1990, Catalytic activities of human liver cytochrome P-450 IIIA4 expressed in *Saccharomyces cerevisiae*. *Biochemistry*, **29**, 11280–11292.
- BURCHELL, B. and COUGHTRIE, M. W. H., 1989, UDP-glucuronosyltransferases. *Pharmacology and Therapeutics*, **43**, 261–289.
- BURCHELL, B., COUGHTRIE, M. W. H., JACKSON, M. R., SHEPHERD, S. R. P., HARDING, D. and HUME, R., 1987, Genetic deficiency of bilirubin glucuronidation in rats and humans. *Molecular Aspects of Medicine*, **9**, 429–455.
- BUTLER, M. A., LANG, N. P., YOUNG, J. F., CAPORASO, N. E., VINEIS, P., HAYES, R. B., TEITEL, C. H., MASSENGILL, J. P., LAWSON, M. F. and KADLUBAR, F. F., 1992, Determination of CYP1A2 and NAT2 phenotypes in human populations by analysis of caffeine urinary metabolites. *Pharmacogenetics*, **2**, 116–127.
- CAMPBELL, N. R. C., VAN LOON, J. A., SUNDARAM, R. S., AMES, M. M., HANSCH, C. and WEINSHILBOUM, R., 1987, Human and rat liver phenol sulfotransferase: structure–activity relationships for phenolic substrates. *Molecular Pharmacology*, **32**, 813–819.
- CARLILE, D. J., HABOOZ, N., BAYLISS, M. K. and HOUSTON, J. B., 1999, Microsomal prediction of *in vivo* clearance of CYP2C9 substrates in humans. *British Journal of Clinical Pharmacology*, **47**, 625–635.
- CDER, 1997, Guidance for industry: drug metabolism/drug interactions in the drug development process: studies *in vitro* [<http://www.fda.gov/cder>].
- CDER, 1998, Guidance for industry: *in vivo* drug metabolism/drug interaction studies: study design, data analysis and recommendations for dosing and labelling [<http://www.fda.gov/cder>].
- CHANG, T. K. H., GONZALEZ, F. J. and WAXMAN, D. J., 1994, Evaluation of triacetyloleandomycin, alpha-naphthoflavone and diethyldithiocarbamate as selective chemical probes for inhibition of human cytochromes P450. *Archives of Biochemistry and Biophysics*, **311**, 437–442.
- CHIU, L. S. H. and HUSKEY, S. E. W., 1998, Aspect *n*-glucuronidation of xenobiotics symposium. Species differences in *n*-glucuronidation. *Drug Metabolism and Disposition*, **26**, 838–847.
- CLARK, S. E., 1998, *In vitro* assessment of human cytochrome P450. *Xenobiotica*, **28**, 1167–1202.
- CONNEY, A., 1982, Induction of microsomal enzymes by foreign chemicals and carcinogenesis by polycyclic aromatic hydrocarbons: G. H. A. Clowes Memorial Lecture. *Cancer Research*, **42**, 4875–4917.
- CPMP, 1998, Note for guidance on the investigation of drug interactions, EWP/560/95 [<http://www.eudra.org/emea.html>].

- DIAZ, D., FABRE, I., DAUJAT, M., SAINTAUBERT, B., BORIES, P., MICHEL, H. and MAUREL, P., 1990, Omeprazole is an aryl hydrocarbon-like inducer of human hepatic cytochrome P450. *Gastroenterology*, **99**, 737–747.
- DICKINS, M., ELCOMBE, C. R., MALONEY, S. J., NETTER, K. J. and BRIDGES J. W., 1979, Further studies on the dissociation of the isosafrole metabolite–cytochrome P450 complex. *Biochemical Pharmacology*, **28**, 231–238.
- DONATO, M. T., CASTELL, J. V. and GOMEZ-LECHON, M. J., 1995, Effect of model inducers on cytochrome P450 activities of human hepatocytes in primary culture. *Drug Metabolism Disposition*, **23**, 553–558.
- EAGLING, V. A., TJIA, J. F. and BACK, D. J., 1998, Differential selectivity of cytochrome P450 inhibitors against probe substrates in human and rat liver microsomes. *British Journal of Clinical Pharmacology*, **45**, 107–114.
- EBERTS, F. S., PHILOPOULOS, Y., REINEKE, L. M. and VLIEK, R. W., 1981, Triazolam disposition. *Clinical Pharmacology and Therapeutics*, **29**, 81–93.
- EDWARDS, R. J., 1998, Targeting anti-peptide antibodies towards P450 enzymes. In I. R. Phillips and E. A. Shepard (eds), *Cytochrome P450 Protocols* (Humana Press Inc.).
- EL-SANKARY, W., PLANT, N. J., GIBSON, G. G. and MOORE, D. J., 2000, Regulation of the CYP3A4 gene by hydrocortisone and xenobiotics: role of the glucocorticoid and pregnane X receptors. *Drug Metabolism and Disposition*, **28**, 493–496.
- FISCHER, U., ROHDE, B., WACKE, R., STANGE, J., NITSCHKE, F. P., ADAM, U. and DREWELOW, B., 1997, Prediction of *in vivo* drug interaction from *in vitro* systems exemplified by interaction between verapamil and cimetidine using human liver microsomes and primary hepatocytes. *Journal of Clinical Pharmacology*, **37**, 1150–1159.
- FISHER, M. B., VANDENBRANDEN, M., FINDLAY, K., BURCHELL, B., THUMMEL, K. E., HALL, S. D. and WRIGHTON, S. A., 2000, Tissue distribution and interindividual variation in human UDP-glucuronosyltransferase activity: relationship between UGT1A1 promoter genotype and variability in a liver bank. *Pharmacogenetics*, **10**, 727–739.
- FRYE, R. F., MATZKE, G. R., ADEDOYIN, A., PORTER, J. A. and BRANCH, R. A., 1997, Validation of the five-drug 'Pittsburgh cocktail' approach for assessment of selective regulation of drug-metabolizing enzymes. *Pharmacokinetics and Drug Disposition*, **62**, 365–376.
- FURLONG, B. B., WEAVER, R. P. and GOLDSTEIN, J. A., 1987, Covalent binding to DNA mutagenicity of 2,4-diaminotoluene metabolites produced by isolated hepatocytes and 9000g supernatant from Fischer 344 rats. *Carcinogenesis*, **8**, 247–251.
- GIBBS, M. A., KUNZE, K. L., HOWALD, W. N. and THUMMEL, K. E., 1999, Effect of inhibitor depletion on inhibitory potency: tight binding inhibition of CYP3A by clotrimazole. *Drug Metabolism and Disposition*, **27**, 596–599.
- GIDDINGS, S. J., CLARKE, S. E. and GIBSON, G. G., 1997, CYP4A1 gene transfection studies and the peroxisome proliferator-activated receptor: development of a high-throughput assay to detect peroxisome proliferators. *European Journal of Drug Metabolism and Pharmacokinetics*, **22**, 315–319.
- GOLDSTEIN, J. A., FALETTO, M. B., ROMKES-SPARKS, M., SULLIVAN, T., KITAREEWAN, S., RAUCY, J. L., LASKER, J. M. and GHANAYEM, B. I., 1994a, Evidence that CYP2C19 is the major (S)-mephenytoin 4'-hydroxylase in humans. *Biochemistry*, **33**, 1743–1752.
- GOLOVINSKY, E., NAYDENOVA, Z. and GRANCHAROV, K., 1998, UDP-glucuronosyltransferase inhibitors. *European Journal of Drug Metabolism and Pharmacokinetics*, **23**, 453–456.
- GORSKI, J. C., JONES, D. R., HAECHNER-DANIELS, B. D., HAMMAN, M. A., O'MARA, JR, E. M. and HALL, S. D., 1998, The contribution of intestinal and hepatic CYP3A to the interaction between midazolam and clarithromycin. *Clinical Pharmacology and Therapeutics*, **64**, 133–143.
- GRANNEMAN, G. R., BRAECKMAN, R. A., LOCKE, C. S., CAVANAUGH, J. H., DUBE, L. M. and AWNI, W. M., 1995, Effect of zileuton on theophylline pharmacokinetics. *Clinical Pharmacokinetics*, **29**, 77–83.
- GRANT, D. M., BLUM, M., BEER, M. and MEYER, U. A., 1991, Monomorphic and polymorphic human arylamine N-acetyltransferases: a comparison of liver isozymes and expressed products of two cloned genes. *Molecular Pharmacology*, **39**, 184–191.
- GREEN, M. D., KING, C. D., MOJARRABI, B., MACKENZIE, P. I. and TEPHLY, T. R., 1998, Glucuronidation of amines and other xenobiotics catalyzed by expressed human UDP-glucuronosyltransferase 1A3. *Drug Metabolism and Disposition*, **26**, 507–512.
- GREENBLATT, D. J., VON MOLTE, L. L., HARMATZ, J. S. and SHADER, R. I., 1999, Human cytochromes and some newer antidepressants: kinetics, metabolism, and drug interactions. *Journal of Clinical Psychopharmacology*, **19**, 23s–35s.
- GROTHUSEN, A., HARDT, J., BRAUTIGAM, L., LANG, D. and BROKER, R., 1996, A convenient method to discriminate between cytochrome P450 enzymes and flavin-containing monooxygenases in human liver microsomes. *Archives of Toxicology*, **71**, 64–71.
- GUENGERICH, F. P., 1988, Oxidation of 17 alpha-ethynylestradiol by human liver cytochrome P-450. *Molecular Pharmacology*, **33**, 500–508.

- GUENGERICH, F. P., 1990, Mechanism-based inactivation of human liver microsomal cytochrome P450 IIIA4 by gestodene. *Chemical Research and Toxicology*, **3**, 363-371.
- GUENGERICH, F. P., 1996, *In vitro* techniques for studying drug metabolism. *Journal of Pharmacokinetics and Biopharmaceutics*, **24**, 521-533.
- GUENGERICH, F. P., KIM, D. H. and IWASAKI, M., 1991, Role of human cytochrome P-450 IIE1 in the oxidation of many low molecular weight cancer suspects. *Chemical Research and Toxicology*, **4**, 168-179.
- HALPERT, P., 1981, Covalent modification of lysine during the suicide inactivation of rat liver cytochrome P-450 by chloramphenicol. *Biochemical Pharmacology*, **30**, 875-881.
- HEATH, E. C. and DINGELL, J. V., 1974, The interaction of foreign chemical compounds with the glucuronidation of estrogens *in vitro*. *Drug Metabolism and Disposition*, **2**, 556-565.
- HEIN, D. W., 1988, Acetylator genotype and arylamine-induced carcinogenesis. *Biochimica et Biophysica Acta*, **948**, 37-66.
- HICKMAN, D., WANG, J.P., WANG, Y. and UNADKAT, J. D., 1998, Evaluation of the selectivity of *in vitro* probes and suitability of organic solvents for the measurement of human cytochrome P450 monooxygenase activities. *Drug Metabolism and Disposition*, **26**, 207-215.
- HONIG, P. K., WORTHAM, D. C., ZAMANI, K., CONNER, D. P., MULLIN, J. C. and CANTILENA, L. R., 1993, Terfenadine-ketoconazole interaction: pharmacokinetics and electrocardiographic consequences. *Journal of the American Medical Association*, **269**, 1513-1518.
- HOUSTON, J. B., 1994, Utility of *in vitro* drug metabolism data in predicting *in vivo* metabolic clearance. *Biochemical Pharmacology*, **47**, 1469-1474.
- HOUSTON, J. B. and KENWORTHY, K. E., 2000, *In vitro-in vivo* scaling of CYP kinetic data not consistent with the classical Michaelis-Menten model. *Drug Metabolism and Disposition*, **28**, 246-254.
- HUMPHREY, M. J., JEVONS, S. and TARBIT, M. H., 1985, Pharmacokinetic evaluation of UK-49,858, a metabolically stable triazole antifungal drug, in animals and humans. *Antimicrobial Agents and Chemotherapy*, **28**, 648-653.
- HUNT, C. M., WATKINS, P. G., SAENGER, P., STAVE, G. M., BARLASCINI, N., 1992, Heterogeneity of CYP3A isoforms metabolising erythromycin and cortisol. *Clinical Pharmacology and Therapeutics*, **51**, 18-23.
- HURWITZ, A., VACEK, J. L., BOTTERON, G. W., SZTERN, M. I., HUGHES, E. M. and JAYARAJ, A., 1991, Mexiletine effects on theophylline disposition. *Clinical Pharmacology and Therapeutics*, **50**, 299-307.
- ITO, K., IWATSUBO, T., KANAMITSU, S., NAKAJIMA, Y. and SUGIYAMA, Y., 1998, Quantitative prediction of *in vivo* drug clearance and drug interactions from *in vitro* data on metabolism, together with binding and transport. *Annual Review of Pharmacology and Toxicology*, **38**, 461-499.
- ITO, K., KUSUHARA, H. and SUGIYAMA, Y., 1999, Effects of intestinal CYP3A4 and p-glycoprotein on oral drug absorption-theoretical approach. *Pharmaceutical Research*, **16**, 225-231.
- IWATSUBO, T., HIROTA, N., OOIE, T., SUZUKI, H., SHIMANDA, N., CHIBA, K., ISHIZAKI, T., GREEN, C. E., TYSON, C. A. and SUGIYAMA, Y., 1997, Prediction of *in vivo* drug metabolism in the human liver from *in vitro* metabolism data. *Pharmacology and Therapeutics*, **73**, 147-171.
- JANKEL, C. A. and FITTERMAN, L. K., 1993, Epidemiology of drug-drug interactions as a cause of hospital admissions. *Drug Safety*, **9**, 51-59.
- JOHNSON, M. D., NEWKIRK, G. and WHITE, J. R., 1999, Clinically significant drug interactions. *Postgraduate Medicine, Drug Interactions*, **105**, 193-222.
- JONEN, H. G., HUTHWOHL, B., KAHL, R. and KAHL, G. F., 1974, Influences of pyridine and some pyridine derivatives on spectral properties of reduced microsomes and on microsomal drug metabolizing activity. *Biochemical Pharmacology*, **23**, 1319-1329.
- JUNG, F., RICHARDSON, T. H., RAUCY, J. L. and JOHNSON, E. F., 1997, Diazepam metabolism by cDNA-expressed human 2C P450s identification of P4502C18 and P4502C19 as low K_m diazepam N-demethylases. *Drug Metabolism and Disposition*, **25**, 133-139.
- JURIMA-ROMET, M., HUANG, H. S., BECK, D. J. and LI, A. P., 1996, Evaluation of drug interactions in intact hepatocytes: inhibitors of terfenadine metabolism. *Toxicology In Vitro*, **10**, 655-663.
- KANAMITSU, S., ITO, K. and SUGIYAMA, Y., 2000, Quantitative prediction of *in vivo* drug-drug interactions from *in vitro* data based on physiological pharmacokinetics: Use of maximum unbound concentrations of inhibitor at the inlet to the liver. *Pharmaceutical Research*, **17**, 2000.
- KENWORTHY, K. E., BLOOMER, J. C., CLARKE, S. E. and HOUSTON, J. B., 1999, CYP3A4 Drug interactions: correlation of 10 *in vitro* probe substrates. *British Journal of Clinical Pharmacology*, **48**, 716-727.
- KINIRONS, M. T., O'SHEA, D., DOWNING, T. E., FITZWILLIAM, A. T., JOELLENBECK, L., GROOPMAN, J. D., WILKINSON, G. R. and WOOD, A. J. J., 1993, Absence of correlations among three putative *in vivo* probes of human cytochrome P4503A activity in young healthy men. *Clinical Pharmacology and Therapeutics*, **54**, 621-629.
- KINIRONS, M. T., O'SHEA, D., GROOPMAN, J. D., THUMMEL, K. E., WOOD, A. J. and WILKINSON, J. R., 1994, Route of administration does not explain the lack of correlation between putative *in vivo* probes of cytochrome P4503A. *British Journal of Clinical Pharmacology*, **37**, 501P.

- KOHL, C. and STEINKELLNER, M., 2000, Prediction of pharmacokinetic drug/drug interactions from *in vitro* data: interactions of the nonsteroidal anti-inflammatory drug lornoxicam with oral anticoagulants. *Drug Metabolism and Disposition*, **28**, 161–168.
- KOLARS, J. C., SCHMIEDLIN-REN, P., SCHUETZ, J. D., FANG, C. and WATKINS, P. B., 1992, Identification of rifampin-inducible P45011A4 (CYP3A4) in human bowel enterocytes. *Journal of Clinical Investigations*, **90**, 1871–1878.
- KOMATSU, K., ITO, K., NAKAJIMA, Y., KANAMITSU, S.I., IMAOKA, S., FUNAE, Y., GREEN, C. E., TYSON, C. A., SHIMADA, N. and SUGIYAMA, Y., Prediction of *in vivo* drug–drug interactions between tolbutamide and various sulfonamides in human based on *in vitro* experiments. *Drug Metabolism and Disposition*, **28**, 475–481.
- KORZEKWA, K. R., KRISHNAMACHARY, N., SHOU, M., OGAI, A., PARISE, R. A., RETTIE, A. E., GONZALEZ, F. J. and TRACY, T. S., 1998, Evaluation of atypical cytochrome P450 kinetics with two-substrate models: evidence that multiple substrates can simultaneously bind to cytochrome P450 active sites. *Biochemistry*, **37**, 4137–4147.
- KOSTER, H., HALSEMA, I., SCHOLTENS, E., MEERMAN, J. H. N., PANG, K. S. and MULDER, G. J., 1982, Selective inhibition of sulfate conjugation in the rat: pharmacokinetics and characterization of the inhibitory effect of 2,6-chloro-4-nitrophenol. *Biochemical Pharmacology*, **31**, 1919–1924.
- KRONBACH, T., MATHYS, D., UMEMO, M., GONZALEZ, F. J. and MEYER, U. A., 1989, Oxidation of midazolam and triazolam by human liver cytochrome P450 3A4. *Molecular Pharmacology*, **36**, 89–96.
- KUMAR, K. and BALLANTYNE, J., 2000, Use of general regression neural networks for determination of inhibitor/inducer activity of coadministered agents. *AAPS*, abstr 3549.
- KUPFER, A., SCHMID, B., PREISIG, R. and PFA, G., 1984b, Dextromethorphan as a safe probe for debrisoquine hydroxylation polymorphism. *Lancet*, **ii**, 517–518.
- LANG, D. H., YEUNG, C. K., IBARRA, PETER, R. M., GASSER, R., ITAGAKI, K., PHILPOT, R. M. and RETTIE, A. E., 1998, Isoform specificity of trimethylamine *N*-oxygenation by human flavin-containing monooxygenase (FMO) and P450 enzymes: selective catalysis by FMO3. *Biochemical Pharmacology*, **56**, 1005–1012.
- LASKER, J. M., WESTER, M. R., ARAMSOBATDEE, E. and RAUCY, J. L., 1998, Characterization of CYP2C9 from human liver: respective roles in microsomal tolbutamide, *s*-mephenytoin, and omeprazole hydroxylations. *Archives of Biochemistry and Biophysics*, **353**, 16–28.
- LAWTON, M. P., CASHMAN, J. R., CRESTEIL, T., DOLPHIN, C. T., ELFARRA, A. A., HINES, R. N., HODGSON, E., KIMURA, T., OZOLS, J., PHILLIPS, I. R., PHILPOT, R. M., POULSEN, L. L., RETTIE, A. E., SHEPARD, E. A., WILLIAMS, D. E. and ZIELGER, D. M., 1994, A nomenclature for the mammalian flavin-containing monooxygenase gene family based on amino acid sequence identities. *Archives of Biochemistry and Biophysics*, **308**, 254–257.
- LE BIGOT, J. F., CRESTEIL, T., KIECHEL, J. R. and BEAUNE, P., 1983, Metabolism of ketotifen by human liver microsomes. *In vitro* characterization of a tertiary ammonium glucuronide. *Drug Metabolism and Disposition*, **11**, 585–589.
- LE, J. H. and DOUGALL, I. G., 1993, Further concerns over Cheng–Prusoff analysis. *Trends in Pharmacological Sciences*, **14**, 110–112.
- LEHMAN, J. P., FENSELAU, C. and DEPAULO, J. R., 1983, Quaternary ammonium-linked glucuronides of amitriptyline, imipramine and chlorpromazine. *Drug Metabolism and Disposition*, **11**, 221–225.
- LESICA, P., RADINARIVO, E., LESCOINTE, P. and MANSUY, D., 1979, A class of strong inhibitors of microsomal monooxygenases: the ellipticines. *Chemico-Biological Interactions*, **24**, 189–197.
- LEWIS, D. F. V., 1996a, P450 substrate specificity and metabolism. In *Cytochromes P450: Structure, Function and Mechanism* (London: Taylor & Francis), pp. 115–167.
- LEWIS, D. F. V., 1996b, Induction, regulation and inhibition. In *Cytochromes P450: Structure, Function and Mechanism* (London: Taylor & Francis), pp. 169–208.
- LI, L. I., MAUREL, P., GOMEZ-LECHON, M. J., CHENG, L. C. and JURIMA-ROMET, M., 1997, Preclinical evaluation of drug–drug interaction potential: present status of the implication of primary human hepatocytes in the evaluation of cytochrome P450 induction. *Chemico-Biological Interactions*, **107**, 5–16.
- LIN, J. H. and LU, A. Y. H., 1998, Inhibition and induction of cytochrome P450 and the clinical implications. *Clinical Pharmacokinetic*, **35**, 361–390.
- LOWN, K. S., THUMMEL, K. E., BENEDICT, P. E., SHEN, D. D., TURGEON, D. K., BERENT, S. and WATKINS, P. B., 1995, The erythromycin breath test predicts the clearance of midazolam. *Clinical Pharmacology and Therapeutics*, **57**, 16–24.
- LU, A. Y., 1998, Drug-metabolism research challenges in the new millennium. Individual variability in drug therapy and drug safety. *Drug Metabolism and Disposition*, **26**, 1217–1222.
- MACHINIST, J. M., MAYER, M. D., SHET, M. S., FERRERO, J. L. and RODRIGUES, A. D., 1995, Identification of the human liver cytochrome P450 enzymes involved in the metabolism of zileuton (ABT-077) and its *n*-dehydroxylated metabolite, abbott-66193. *Drug Metabolism and Disposition*, **23**, 1163–1174.

- MACKENZIE, P. I., OWENS, I. S., BURCHELL, B., BOCK, K. W., BAIROCH, A., BELANGER, A., FURNEL-GIGLEUX, S., GREEN, M., HUM, D. W., IYANAGI, T., LANCET, D., LOUISOT, P., MAGDALOU, J., CHOWDHURY, J. R., RITTER, J. K., SCHACHTER, H., TEPHLY, T. R., TIPTON, K. F. and NEBERT, D. W., 1997, The UDP-glycosyltransferase gene super family: recommended nomenclature update based on evolutionary divergence. *Pharmacogenetics*, **7**, 255-269.
- MADANI, S., PAINE, M. F., LEWIS, L., THUMMEL, K. E. and SHEN, D. D., 1999, Comparison of CYP2D6 content and metoprolol oxidation between microsomes isolated from human livers and small intestines. *Pharmaceutical Research*, **16**, 1199-1205.
- MAGDALOU, J., HERBER, R., BIDAULT, R. and SIEST, G., 1992, *In vitro* *n*-glucuronidation of a novel antiepileptic drug, lamotrigine, by human liver microsomes. *Journal of Pharmacology and Experimental Therapeutics*, **260**, 1166-1173.
- MALATY, L. I. and KUPER, J. J., 1999, Drug Interactions of HIV protease inhibitors. *Drug Safety*, **20**, 147-169.
- MARROUM, P. L., UPPOOR, R. S., PARMELLE, T., AJAYI, F., BURNETT, A., YUAN, R., SVADJIAN, R., LESKO, L. J. and BALIAN, J. D., 2000, *In vivo* drug-drug interaction studies - a survey of all new molecular entities approved from 1987 to 1997. *Clinical Pharmacology and Therapeutics*, **68**, 280-285.
- MATSUI, M. and HOMMA, H., 1994, Biochemistry and molecular biology of drug-metabolizing sulfotransferase. *International Journal of Biochemistry*, **26**, 1237-1247.
- MAURER, T. S., TABRIZI-FARD, M. A. and FUNG, H., 2000, Impact of mechanism-based enzyme inactivation on inhibitor potency: implications for rational drug discovery. *Journal of Pharmaceutical Sciences*, **89**, 1404-1414.
- MAURICE, M., PICHARD, L., DAUJAT, M., FABRE, I., JOYEUX, H., DOMERGUE, J. and MAUREL, P., 1992, Effects of imidazole derivatives on cytochromes P450 from human hepatocytes in primary culture. *FASAB Journal*, **6**, 752-758.
- MAYHEW, B. S., JONES, D. R. and HALL, S. D., 2000, An *in vitro* model for predicting *in vivo* inhibition of cytochrome P450 3A4 by metabolic intermediate complex formation. *Drug Metabolism and Disposition*, **28**, 1031-1037.
- MCDONNELL, W. M., HITOMI, E. and ASKARI, F. K., 1996, Identification of bilirubin UDP-GTs in the human alimentary tract in accordance with the gut as a putative metabolic organ. *Biochemical Pharmacology*, **51**, 483-488.
- MCGINNITY, D. F., PARKER, A. J., SOARS, M. and RILEY, R. J., 2000, Automated definition of the enzymology of drug oxidation by the major human drug metabolizing cytochrome P450s. *Drug Metabolism and Disposition*, **28**, 1327-1334.
- McLURE, J. A., MINERS, J. O. and BIRKETT, D. J., 2000, Nonspecific binding of drugs to human liver microsomes. *British Journal of Clinical Pharmacology*, **49**, 453-461.
- MEERMAN, J. H. N., STERENBORG, H. M. J. and MULDER, G. J., 1983, Use of pentachlorophenol as long-term inhibitor of sulfation of phenols and hydroxamic acids in the rat *in vivo*. *Biochemical Pharmacology*, **32**, 1587-1593.
- MEIJER, D. K. F., HOOVELD, G. J. E. J., SCHINKEL, A. H., VAN MONTFOORT, J. E., HAAS, M., DE ZEEUW, D., MOOLENAAR, F., SMIT, J. W. and MEIER, P. J., 1999, Transport mechanisms for cationic drugs and proteins in kidney, liver and intestine: implication for drug interactions and cell-specific drug delivery. *Nephrology Dialysis Transplantation*, **14** (suppl. 4), 1-3.
- MEREDITH, C. G., MALDONADO, A. L. and SPEEG, K. V., 1985, The effect of ketoconazole on hepatic oxidative drug metabolism in the rat *in vivo* and *in vitro*. *Drug Metabolism and Disposition*, **13**, 156-162.
- MERRY, C., MULCHAHY, F., BARRY, M., GIBBONS, S. and BACK, D., 1997, Saquinavir interaction with midazolam: pharmacokinetic considerations when prescribing protease-inhibitors for patients with HIV-disease. *AIDS*, **11**, 268-269.
- MINERS, J. O. and BIRKETT, D. J., 1996, The use of caffeine as a metabolic probe for human drug metabolizing enzymes. *General Pharmacology*, **2**, 245-249.
- MINERS, J. O. and BIRKETT, D. J., 1998, Cytochrome P4502C9: an enzyme of major importance in human drug metabolism. *British Journal of Clinical Pharmacology*, **45**, 525-538.
- MISTRY, M. and HOUSTON, J. B., 1987, Glucuronidation *in vitro* and *in vivo*: comparison of intestinal and hepatic conjugation of morphine, naloxone, and buprenorphine. *Drug Metabolism and Disposition*, **15**, 710-717.
- MIYAUCHI, S., SAWADA, Y., IGA, T., HANANO, M. and SUGIYAMA, Y., 1993, Comparison of the hepatic uptake clearances of fifteen drugs with a wide range of membrane permeabilities in isolated rat hepatocytes and perfused rat livers. *Pharmaceutical Research*, **10**, 434-440.
- MOLDEUS, P., ANDERSON, B. and GERGELY, V., 1979, Regulation of glucuronidation and sulphate conjugation in isolated hepatocytes. *Drug Metabolism and Disposition*, **7**, 416-419.
- MORTIMER, O., LINDSTROM, B., LAURELL, H., BERGMAN, U. and RANE, A., Dextromethorphan: polymorphic serum patterns of the *O*-demethylated and didemethylated metabolites in man. *British Journal of Clinical Pharmacology*, **27**, 223-227.

- MULDER, G. J., 1990, Competition between Conjugations for the Same Substrate. In G. J. Mulder (ed.), *Conjugation reactions in drug metabolism* (London: Taylor & Francis), pp. 41–49.
- MULDER, G. J., COUGHTRIE, M. W. H. and BURCHELL, B., 1990, Glucuronidation. In G. J. Mulder (ed.), *Conjugation Reactions in Drug Metabolism* (London: Taylor & Francis), pp. 51–105.
- MULDER, G. J. and JAKOBY, W. B., 1990, Sulfation. In G. J. Mulder (ed.), *Competition Between Conjugations for the Same Substrate* (London: Taylor & Francis), pp. 107–161.
- MURRAY, M., 1988, Complexation of cytochrome P450 isozymes in hepatic microsomes from SKF 525-A-induced rats. *Archives of Biochemistry and Biophysics*, **262**, 381–388.
- MURRAY, M., 1992, P450 enzymes: inhibition mechanisms, genetic regulation and effects of liver disease. *Clinical Pharmacokinetic Concepts*, **23**, 132–146.
- NAKAMURA, H., SAMO, H., YAMAZAKI, M. and SUGIYAMA, Y., 1994, Carrier-mediated active transport of histamine H₂ receptor antagonists, cimetidine and nizatidine, into isolated rat hepatocytes: contribution of type I system. *Journal of Pharmacology and Experimental Therapeutics*, **269**, 1220–1226.
- NARIMATSU, S., YAMAMOTO, S., KATO, R., MASUBUCHI, Y. and HORI, T., 1999, Contribution of flavin-containing monooxygenase and cytochrome P450 to imipramine N-oxidation in rat hepatic microsomes. *Biological and Pharmaceutical Bulletin*, **22**, 567–571.
- NELSON, D. R., KAMATAKI, T., WAXMAN, D. J., GUENGERICH, F. P., ESTABROOK, R. W., FEYEREISEN, R., GONZALEZ, F. J., COON, F. J., GUNSALUS, I. C., GOTOH, O., OKUDA, K. and NEBERT, D. W., 1993, The P450 superfamily: update on new sequences, gene mapping, accession numbers, early trivial names of enzymes and nomenclature. *DNA and Cell Biology*, **12**, 1–51.
- NESTOROV, I., AARONS, L. J. and ROWLAND, M., 1998a, Lumping of whole body physiologically based pharmacokinetic models. *Journal of Pharmacokinetics and Biopharmaceutics*, **26**, 21–46.
- NESTOROV, I., AARONS, L. J. and ROWLAND, M., 1998b, Quantitative structure–pharmacokinetic relationships. II. Mechanistically based model for the relationship between the tissue distribution parameters and the lipophilicity of the compounds. *Journal of Pharmacokinetics and Biopharmaceutics*, **26**, 521–545.
- NESTOROV, I., CARLILE, D., JONES, H., HOUSTON, J. B. and ROWLAND, M., 2000, Incorporating variability and uncertainty into the *in vitro*–*in vivo* prediction of hepatic clearance. In *IFAC Symposium: Modelling and Control in Biomedical Systems*, Greifswald, Germany, pp. 109–114.
- NESTOROV, I., PETROV, I., HADJITODOROV, S. and ROWLAND, M., 1999, Empirical versus mechanistic modelling: comparison of an artificial neural network to a mechanistically based model for quantitative structure pharmacokinetics relationship. *Pharmaceutical Sciences*, **1**, article 17.
- NEWTON, D. J., WANG, R. W. and LU, A. Y. H., 1995, Evaluation of specificities in the *in vitro* metabolism of therapeutic agents by human liver microsomes. *Drug Metabolism and Disposition*, **23**, 154–158.
- NGUI, J. S., TANG, W., STEARNS, R. A., SHOU, M., MILLER, R. R., ZHANG, Y., LIN, J. H. and BAILLIE, T. A., 2000, Cytochrome P450 3A4-mediated interaction of diclofenac and quinidine. *Drug Metabolism and Disposition*, **28**, 1043–1050.
- OBACH, R. S., 1997, Nonspecific binding to microsomes: impact on scale-up of *in vitro* intrinsic clearance to hepatic clearance as assessed through examination of warfarin, imipramine, and propranolol. *Drug Metabolism and Disposition*, **25**, 1359–1369.
- OBACH, R. S., BAXTER, J. G., LISTON, T. E., SILBER, B. A., JONES, B. C., MACINTYRE, F., RANCE, D. J. and WASTALL, P., 1997, The prediction of human pharmacokinetics parameters from preclinical and *in vitro* metabolism data. *Journal of Pharmacology and Experimental Therapeutics*, **283**, 46–58.
- OGG, M. S., GRAY, T. J. B. and GIBSON, G. G., 1997, Development of an *in vitro* reporter gene assay to assess xenobiotic induction of the human CYP3A4 gene. *European Journal of Drug Metabolism and Pharmacokinetics*, **22**, 311–313.
- OGG, M. S., WILLIAMS, J. M., TARBITT, M., GOLDFARB, P. S., GRAY, T. J. B. and GIBSON, G. G., 1999, A reporter gene assay to assess the molecular mechanisms of xenobiotic-dependent induction of the human CYP3A4 gene *in vitro*. *Xenobiotica*, **29**, 269–279.
- OIKKOLA, K. T., ARANKO, K., LUURILA, H., HILLER, A., SAARNIVAARA, L., HIMBERG, J. J. and NEUVONEN, P. J., 1993, A potentially hazardous interaction between erythromycin and midazolam. *Clinical Pharmacology and Therapeutics*, **53**, 298–305.
- ONG, C. E., COULTER, S., BIRKETT, D. J., BHASKER, C. R. and MINERS, J. O., 2000, The xenobiotic inhibitor profile of cytochrome P4502C8. *British Journal of Clinical Pharmacology*, **50**, 573–580.
- ONO, S., HATANAKA, T., HOTTA, H., SATOH, T. and GONZALEZ, F. J., 1996, Specificity of substrate and inhibitor probes for cytochrome P450s: evaluation of *in vitro* metabolism using cDNA-expressed human P450s and human liver microsomes. *Xenobiotica*, **26**, 681–693.
- ORTIZ DE MONTELLANO, O., 1988, Suicide inhibitors for drug metabolising enzymes: mechanisms and biological consequences. In *Progress in Drug Metabolism*, vol. 11 (New York: Taylor & Francis), pp. 99–148.

- PAINE, M. F., SCHMIEDLIN-REN, P. and WATKINS, P. B., 1999, Cytochrome P-450 1A1 expression in human small bowel: interindividual variation and inhibition by ketoconazole. *Drug Metabolism and Disposition*, **27**, 360–364.
- PANG, K. S., 1990, Kinetics of conjugation reactions in eliminating organs. In G. J. Mulder (ed.), *Competition between Conjugations for the Same Substrate* (London: Taylor & Francis), pp. 5–39.
- PANG, K. S. and CHIBA, M., 1994, Metabolism: scaling-up from *in vitro* to organ and whole body. In P. G. Welling and L. P. Balaut (eds), *Handbook of Experimental Pharmacology* (New York: Springer-Verlag), pp. 101–187.
- PARK, B. K., KITTINGHAM, N. R., PIRMOHAMED, M. and TUCKER, G. T., 1996, Relevance of induction of human drug-metabolising enzymes: pharmacological and toxicological implications. *British Journal of Clinical Pharmacology*, **41**, 477–491.
- PARK, C.S., CHUNG, W.G., KANG, J.H., ROH, H.K., LEE, K.H. and CHA, Y.N., 1999, Phenotyping of flavin-containing monooxygenase using caffeine metabolism and genotyping of FMO3 gene in a Korean population. *Pharmacogenetics*, **9**, 155–164.
- PARKINSON, A., 1996a, An overview of current cytochrome P450 technology for assessing the safety and efficacy of new materials. *Toxicology Pathology*, **24**, 45–57.
- PARKINSON, A., 1996b, Biotransformation of xenobiotics. In C. D. Klassen, M. O. Amdur and J. Doull (eds), *Casarett & Doull's Toxicology: The Basic Science of Poisons* (New York: McGraw-Hill), pp. 113–186.
- PASCUSSI, J.M., GERBAL-CHALOIN, S., FABRE, J.-M., MAUREL, P. and VILAREM, M.-J., 2000, Dexamethazone enhances constitutive androstane receptor expression in human hepatocytes: consequences on cytochrome P450 gene regulation. *Molecular Pharmacology*, **58**, 1441–1450.
- PESSAYRE, D., DESCATOIRE, V., TINEL, M. and LARREY, D., 1982, Self-induction by oleandomycin of its own transformation into a metabolite forming an inactive complex with reduced cytochrome P450. Comparison with troleandomycin. *Journal of Pharmacology and Experimental Therapeutics*, **221**, 215–221.
- PITCHARD, L., FABRE, I., FABRE, G., DOMERGUE, J., SAINT-AUBERT, B., MOURAD, G. and MAUREL, P., 1990, Cyclosporin A drug interactions: screening for inducers and inhibitors of cytochrome P450 (cyclosporin A oxidase) in primary cultures of human hepatocytes and in liver microsomes. *Drug Metabolism and Disposition*, **18**, 595–606.
- PORTER, S. J., SOMOGYI, A. A. and WHITE, J. M., 2000, Kinetics and inhibition of the formation of 6(-naltrexol from naltrexone in human liver cytosol. *British Journal of Clinical Pharmacology*, **50**, 465–471.
- RANE, A., SAWE, J., LINDBERG, B., VENSSON, J. O., GARLE, M., ERWALD, R. and JORULF, H., 1984, Morphine glucuronidation in the rhesus monkey: a comparative *in vivo* and *in vitro* study. *Journal of Pharmacology and Therapeutics*, **229**, 571–576.
- RENDIC, S. and DI CARLO, F. J., 1997, Human cytochrome P450 enzymes: a status report summarizing their reactions, substrates, inducers, and inhibitors. *Drug Metabolism Reviews*, **29**, 413–580.
- RETTIE, A. E. and LANG, D. H., 2000, Can caffeine metabolism be used as an *in-vivo* probe for human flavin-containing monooxygenase activity. *Pharmacogenetics*, **10**, 275–277.
- RODRIGUES A. D. and WONG, S. L., 1997, Application of human liver microsomes in metabolism-based drug–drug interactions: *in vitro-in vivo* correlations and the Abbott Laboratories experience. *Advances in Pharmacology*, **43**, 65–101.
- ROGERSON, T. D., WILKINSON, C. F. and HETARSKI, K., 1977, Steric factors in the inhibitory interaction of imidazoles with microsomal enzymes. *Biochemical Pharmacology*, **26**, 1039–1042.
- ROOS, T. C. and MERK, H. F., 2000, Important drug interactions in dermatology. *Drugs*, **59**, 181–192.
- ROWLAND, M. and MATIN, S. B., 1973, Kinetics of drug interactions. *Journal of Pharmacokinetics and Biopharmaceutics*, **1**, 553–567.
- SADEQUE, A. J., EDDY, A. C., NEIER, G. P. and RETTIE, A. E., 1992, Stereoselective sulfoxidation by human flavin-containing monooxygenase: evidence for catalytic diversity between hepatic, renal and fetal forms. *Drug Metabolism and Disposition*, **20**, 832–839.
- SATO, T., MATSUI, M. and TAMURA, H.O., 2000, Sulfotransferases in a human colon carcinoma cell, caco-2. *Biological and Pharmaceutical Bulletin*, **27**, 810–814.
- SCHMIDER, J., GREENBLATT, D. J., HARMATZ, J. S. and SHADER, R. I., 1996, Enzyme kinetic modelling as a tool to analyse the behaviour of cytochrome P450 catalysed reactions: application to amitriptyline N-demethylation. *British Journal of Clinical Pharmacology*, **41**, 593–604.
- SEGAL, I. H., 1993, *Enzyme Kinetics, Behaviour and Analysis of Rapid Equilibrium and Steady-State Enzyme Systems* (New York: Wiley).
- SHARP, S., MAK, L. Y., SMITH, D. J. and COUGHTRIE, M. W. H., 1992, Inhibition of human and rabbit liver steroid and xenobiotic UDP-glucuronosyltransferases by tertiary amine drugs – implications for adverse drug reactions. *Xenobiotica*, **22**, 13–25.
- SHAW, P. M., BARNES, T. S., CAMERON, D., ENGEST, J., MELVIN, W. T., OMAR, G., PETRIE, J. C., RUSH, W. R., SNYDER, C. P., WHITING, P. H., WOLF, C. R. and BURKE, M. D., Purification and characterization of an anticonvulsant-induced human cytochrome P-450 catalysing cyclosporin metabolism. *Biochemical Journal*, **263**, 653–663.

- SHIMADA, T., YAMAZAKI, H., MIMURA, M., INUI, Y. and GUENGERICH, P., 1994, Interindividual variations in human liver cytochrome P450 enzymes involved in the oxidation of drugs, carcinogens and toxic chemicals: studies with liver microsomes of 30 Japanese and 30 Caucasians. *Journal of Pharmacology and Experimental Therapeutics*, **270**, 414–423.
- SILVERMAN R. B., 1988, Criteria for mechanism-based enzyme inactivation. In *Mechanism-based Enzyme Inactivation: Chemistry and Enzymology* (Boca Raton: CRC Press), pp. 3–27.
- SINZ, M. W. and REMMEL, R. P., 1991, Isolation and characterization of a novel quaternary ammonium-linked glucuronide of lamotrigine. *Drug Metabolism and Disposition*, **19**, 149–153.
- SPIELBERG, S. P., 1996, N-acetyltransferases: pharmacogenetics and clinical consequences of polymorphic drug metabolism. *Journal of Pharmacokinetics and Biopharmaceutics*, **24**, 509–519.
- SPINA, E., GITTO, C., AVENOSO, A., CAMPO, G. M., CAPUTTI, A. P. and PERUCCA, E., 1997, Relationship between plasma desipramine levels, CYP2D6 phenotype and clinical response to desipramine: a prospective study. *European Journal of Clinical Pharmacology*, **51**, 395–398.
- STEVENS, G. J., PAYTON, M., SIM, E. and MCQUEEN, C. A., 1999, N-acetylation of the heterocyclic amine batracylin by human liver. *Drug Metabolism and Disposition*, **27**, 966–971.
- STÖRMER, E., ROOTS, I. and BROCKMÖLLER, J., 2000, Benzydamine *n*-oxidation as an index reaction reflecting FMO activity in human liver microsomes and impact of FMO3 polymorphisms on enzyme activity. *British Journal of Clinical Pharmacology*, **50**, 553–561.
- STREETMAN, D. S., BERTINO, J. S., JR. and NAFZIGER, A. N., 2000, Phenotyping of drug-metabolizing enzymes in adults: a review of *in-vivo* cytochrome P450 phenotyping probes. *Pharmacogenetics*, **10**, 187–216.
- STREETMAN, D. S., BLEAKLEY, J. F., KIM, J. S., NAFZIGER, A. N., LEEDER, J. S., GAEDIGK, A., GOTSCHALL, R., KEARNS, G. L. and BERTINO, J. S., 2000, Combined phenotypic assessment of CYP1A2, CYP2C19, CYP2D6, CYP3A, N-acetyltransferase-2, and xanthine oxidase with the 'Cooperstown' cocktail. *Clinical Pharmacology and Therapeutics*, **68**, 375–383.
- SUZUKI, H., KNELLER, B., RAO, S., JONES, J., TRAGER, W. F. and RETTIE, A. E., 2000, Hydantoin and barbiturate inhibitors of CYP2C19 and CYP2C9, ISSX, Indianapolis, USA. *Drug Metabolism Reviews*, **32**, suppl. 2.
- SWEENEY, D. J., BOUSKA, J., MACHINIST, J., BELL, R., CARTER, G., CEPÄ, S. and NELLANS, H. N., 1992, Glucuronidation of zileuton (A-64077) by human hepatic microsomes. *Drug Metabolism and Disposition*, **20**, 328–329.
- TANAKO M., HASEGAWA, R., FUKUDA, T., YUMOTO, R., NAGAI, J. and MURAKAMI, T., 1998, Interaction with p-glycoprotein and transport of erythromycin, midazolam and ketoconazole in Caco-2 cells. *European Journal of Pharmacology*, **358**, 289–294.
- TASSANEYAKUL, M. E., VERNONESE, M. E., BIRKETT, D. J., GONZALEZ, F. J. and MINERS, J. O., 1993, Validation of 4-nitrophenol as an *in vitro* substrate probe for human liver CYP2E1 using cDNA expression and microsomal kinetic techniques. *Biochemical Pharmacology*, **46**, 1975–1981.
- TASSANEYAKUL, W., BIRKETT, D. J. and MINERS, J. O., 1998, Inhibition of human hepatic cytochrome P4502E1 by azole antifungals, CNS-active drugs and non-steroidal anti-inflammatory agents. *Xenobiotica*, **28**, 293–301.
- TASSANEYAKUL, W., GUO, L. Q., FUKUDA, K., OHTA, T. and YAMAZOE, Y., 2000, Inhibition selectivity of grapefruit juice components on human cytochromes P450. *Archives of Biochemistry and Biophysics*, **378**, 356–363.
- TESTA, B. and JENNER, P., 1981, Inhibitors of cytochrome P450s and their mechanism of action. *Drug Metabolism Reviews*, **12**, 1–117.
- THUMMEL, K. E., O'SHEA, D., PAINE, M. F., SHEN, D. D., KUNZE, K. L., PERKINS, J. D. and WILKINSON, G. R., 1996, Oral first-pass elimination of midazolam involves both gastrointestinal and hepatic CYP3A-mediated metabolism. *Clinical Pharmacology and Therapeutics*, **59**, 491–503.
- THUMMEL, K. E., SHEN, D. D., CARITHERS, R. L., HARTWELL, P., PODOLL, T. D., TRAGER, W. F. and KUNZE, K. L., 1993, Prediction of *in vivo* midazolam clearance from hepatic CYP3A content and midazolam 1-hydroxylation activity in liver transplant patients. *ISSX Proceedings*, **4**, 235–230.
- THUMMEL, K. E., SHEN, D. D., PODOLL, T. D., KUNZE, K. L., TRAGER, W. F., HARTWELL, P. S., RAISYS, V. A., MARSH, C. L., MCVICAR, J. P., BARR, D. M., PERKINS, J. D. and CARITHERS, JR, R. L., 1994a, Use of midazolam as a human cytochrome P450 3A probe: I. *In vitro* in correlations in liver transplant patients. *Journal of Pharmacology and Experimental Therapeutics*, **271**, 549–556.
- THUMMEL, K. E., SHEN, D. D., PODOLL, T. D., KUNZE, K. L., TRAGER, W. F., HARTWELL, P. S., RAISYS, V. A., MARSH, C. L., MCVICAR, J. P., BARR, D. M., PERKINS, J. D. and CARITHERS, JR, R. L., 1994b, Use of midazolam as a human cytochrome P450 3A probe: II. Characterization of inter- and intraindividual hepatic CYP3A variability after liver transplantation. *Journal of Pharmacology and Experimental Therapeutics*, **271**, 557–566.
- TOOLEY, A., ROSTAMI-HODJEGAN, A., LENNARD, M. S. and TUCKER, G. T., 1999, Acute inhibition of methadone metabolism by ritonavir: projection of interindividual variability from *in vitro* data. *British Journal of Clinical Pharmacology*, **48**, 883p–884p.
- TUKEY, R. H. and STRASSBURG, C. P., 2000, Human UDP-glucuronosyltransferases: metabolism, expression and disease. *Annual Reviews of Pharmacology and Toxicology*, **40**, 581–616.

- VAN ASPEREN, J., VAN TELLINGEN, O. and BEIJNEN, J. H., 1998, The pharmacological role of P-glycoprotein in the intestinal epithelium. *Pharmacological Research*, **37**, 432-435.
- VERSCHRAAGEN, M., KOKS, C. H. W., SCHELLENS, J. H. M. and BEIJNEN, J. H., 1999, P-glycoprotein system as a determinant of drug interactions: the case of digoxin-verapamil. *Pharmacological Research*, **40**, 301-306.
- VON MOLTKE, L. L., GREENBLATT, D. J., COTREAU-BIBBO, M. M., HARMATZ, J. S. and SHADER, R. I., 1994, Inhibitors of alprazolam metabolism *in vitro*: effect of serotonin-reuptake-inhibitor antidepressants, ketoconazole and quinidine. *British Journal of Clinical Pharmacology*, **38**, 23-31.
- VON MOLTKE, L. L., GREENBLATT, D. J., HARMATZ, J. S., DUAN, S. X., HARREL, L. M., COTREAU-BIBBO, M. M., PRITCHARD, G. A., WRIGHT and SHADER, R. I., 1996b, Triazolam biotransformation by human liver microsomes *in vitro*: effects of metabolic inhibitors and clinical confirmation of a predicted interaction with ketoconazole. *Journal of Pharmacology and Experimental Therapeutics*, **276**, 370-379.
- VON MOLTKE, L. L., GREENBLATT, D. J., SCHMIDER, J., DUAN, S. X., WRIGHT, C. E., HARMATZ, J. S. and SHADER, R. I., 1996a, Midazolam hydroxylation by human liver microsomes *in vitro*: inhibition by fluoxetine, norfluoxetine, and by azole antifungal agents. *Journal of Clinical Pharmacology*, **36**, 783-791.
- WACHER, V. J., WU, C. and BENET, L. Z., 1995, Overlapping substrate specificities and tissue distribution of cytochrome P450 3A and p-glycoprotein: implications for drug delivery and activity in cancer chemotherapy. *Molecular Carcinogenesis*, **13**, 135-145.
- WANG, R. W., NEWTON, D. J., LUI, N., ATKINS, W. M. and LU, A. Y. H., 2000, Human cytochrome P-450 3A4: *in vitro* drug-drug interaction patterns are substrate-dependent. *Drug Metabolism and Disposition*, **28**, 360-366.
- WARD, S. and BACK, D. J., 1993, Metabolism of gestodene in human liver cytosol and microsomes *in vitro*. *Journal of Steroid Biochemical Molecular Biology*, **46**, 235-243.
- WATKINS, P. B., MURRAY, S. A., WINKLEMAN, L. G., HEUMAN, D. M., WRIGHTON, S. A. and GUZALIAN, P. S., 1989, Erythromycin breath-test as an assay of glucocorticoid-inducible liver cytochromes P-450: studies in rats and patients. *Journal of Clinical Investigations*, **83**, 688-697.
- WATKINS, P. B., TURGEON, D. K., SAENGER, P., LOWN, K. S., KOLARS, J. C., HAMILTON, T., FISHMAN, K., GUZELIAN, P. S. and VOORHEES, J. J., 1992, Comparison of urinary 6 β -cortisol and the erythromycin breath test as measures of hepatic P450III_A (CYP3A) activity. *Clinical Pharmacology and Therapeutics*, **52**, 265-273.
- WATKINS, P. B., WRIGHTON, S. A., MAUREL, P., SCHEUTZ, E. G., MENDEZ-PICON, G., PARKER, G. A. and GUZILIAN, P. S., 1985, Identification of an inducible form of cytochrome P-450 in human liver. *Proceedings of the National Academy of Sciences, USA*, **82**, 6310-6314.
- WATKINS, P. B., WRIGHTON, S. A., SCHEUTZ, E. G., MOLOWA, D. T. and GUZELIAN, P. S., 1987, Identification of glucocorticoid-inducible cytochrome P-450 in the intestinal mucosa in rats and man. *Journal of Clinical Investigation*, **80**, 1029-1036.
- WEAVER, R. J., DICKINS, M. and BURKE, M. D., 1993, Cytochrome P450 2C9 is responsible for hydroxylation of the naphthoquinone antimalarial drug 58C80 in human liver. *Biochemical Pharmacology*, **46**, 1183-1197.
- WEAVER, R. J., DICKINS, M. and BURKE, M. D., 1995, Hydroxylation of the antimalarial drug 58C80 by CYP2C9 in human liver microsomes: comparison with mephenytoin and tolbutamide hydroxylations. *Biochemical Pharmacology*, **49**, 997-1004.
- WEAVER, R. J., DICKINS, M. and BURKE, M. D., 1999, A comparison of basal and induced hepatic microsomal cytochrome P450 monooxygenase activities in the cynomolgous monkey (*Macaca fascicularis*) and man. *Xenobiotica*, **29**, 467-482.
- WEBER, W. W., LEVY, G. N. and HEIN, D. W., 1990, Acetylation. In G. J. Mulder (ed.), *Conjugation Reactions in Drug Metabolism* (London: Taylor & Francis), pp. 163-191.
- WILKINSON, C. F., HETNARSKI, K., CANTWELL, G. P. and DI CARLO, F. J., 1974, Structure-activity relationships in the effects of 1-alkylimidazoles on microsomal oxidation *in vitro* and *in vivo*. *Biochemical Pharmacology*, **23**, 2377-2386.
- WOOD, M., 1991, Pharmacokinetic drug interactions in anaesthetic practice. *Clinical Pharmacokinetics*, **21**, 285-307.
- WRIGHTON, S. A. and RING, B. J., 1994, Inhibition of human CYP3A catalyzed 1-hydroxy midazolam formation by ketoconazole, nifedipine, erythromycin, cimetidine and nizatidine. *Pharmaceutical Research*, **11**, 921-923.
- WRIGHTON, S. A., RING, B. J. and VANDENBRANDEN, M., 1995, The use of *in vitro* metabolism techniques in the planning and interpretation of drug safety studies. *Toxicology Pathology*, **23**, 199-208.
- YAMANO, K., YAMAMOTO, K., KOTAKI, H., SAWADA, Y. and IGA, T., 1999, Quantitative prediction of metabolic inhibition of midazolam by itraconazole and ketoconazole in rats: implication of concentrative uptake of inhibitors into liver. *Drug Metabolism and Disposition*, **27**, 395-402.
- YAMAZAKI, M., SUZUKI, H., HANANO, M., TOKUI, T., KOMAI, T. and SUGIYAMA, Y., 1993, Na⁺-independent multispecific anion transporter mediates active transport of pravastatin into rat liver. *American Journal of Physiology*, **264**, G36-G44.

- YASUMORI, T., NAGATA, K., YANG, S. K., CHEN, L. S., MURAYAMA, N., YAMAZOE, Y. and KATO, R., 1993, Cytochrome P450 mediated metabolism of diazepam in human and rat: involvement of human CYP2C in *N*-demethylation in the substrate concentration-dependent manner. *Pharmacogenetics*, **3**, 291–301.
- YEATES, R. A., LAUTEN, H. and ZIMMERMAN, T., 1996, Interaction between midazolam and clarithromycin: comparison with azithromycin. *International Journal of Clinical Pharmacology and Therapeutics*, **34**, 400–405.
- YEUNG, C. K., LANG, D. H., THUMMEL, K. E. and RETTIE, A. E., 2000, Immunoquantitation of FMO1 in human liver, kidney, and intestine. *Drug Metabolism and Disposition*, **28**, 1107–1111.
- YU, D. K., 1999, The contribution of p-glycoprotein to pharmacokinetics drug–drug interactions. *British Journal of Clinical Pharmacology*, **39**, 1203–1211.
- ZALESKI, J., BANSAL, S. K. and GESSNER, T., 1983, Formation of glucuronide, sulphate and glutathione conjugates of benzo[*a*]pyrene metabolites in hepatocytes isolated from inbred strains of mice. *Carcinogenesis*, **4**, 1359–1366.
- ZAND, R., SIDNEY, N., SLATTERY, J., THUMMEL, K., KALHORN, T., ADAMS, S. and WRIGHT, J., 1993, Inhibition and induction of cytochrome P4502E1-catalyzed oxidation by isoniazid in humans. *Clinical Pharmacology and Therapeutics*, **54**, 142–149.
- ZHANG, Q., DUNBAR, D., OSTROWSKA, A., ZEISLOFT, S., YANG, J. and KAMINSKY, L. S., 1999, Characterization of human small intestinal cytochromes P-450. *Drug Metabolism and Disposition*, **27**, 804–809.
- ZHANG, Y., BLOUIN, R. A., MCNAMARA, P. J., STEINMETZ, J. and WEDLUND, P. J., 1991, Limitation to the use of the urinary *S*-/*R*-mephenytoin ratio in pharmacogenetic studies. *British Journal of Clinical Pharmacology*, **31**, 350–352.
- ZIELGER, D. M., 1991, Mechanisms, multiple forms and substrate specificities of flavin-containing mono-oxygenases. In *N-oxidation of drugs*, P. Hlavica and L. A. Damani (eds) (London: Chapman & Hall), pp. 59–70.

Implications and consequences of enzyme induction on preclinical and clinical drug development

P. D. WORBOYS[†] and D. J. CARLILE^{‡*}

[†] Merck Sharp & Dohme, Terlings Park, Neuroscience Research Centre, Harlow CM20 2QR, UK

[‡] Celltech, Granta Park, Great Abington, Cambridge CB1 6GS, UK

Received 10 March 2001

1. Enzyme induction has traditionally been studied during drug development to assess the potential of drug entities to interact with concomitant medications and alter their pharmacological effects, and clearly it is an unwanted phenomenon. However, another hurdle caused by induction occurs during preclinical development via the attainment of safety data, obtained by dosing high quantities of compound to species used in toxicology assessment. This review considers the techniques that can now be utilized in drug discovery, their relevance, the pharmacokinetic aspects of this phenomenon, and it discusses the consequences and implications of induction during preclinical and clinical development.

2. It is becoming increasingly routine to employ hepatocyte cultures and novel techniques such as quantitative real-time reverse transcriptase PCR to identify enzyme inducers *in vitro*. The major challenge is to utilize these *in vitro* data to predict the consequences of induction *in vivo*. From an understanding of pharmacokinetic principles and low clinical doses relative to preclinical studies, there is limited potential for induction by a development candidate to significantly alter the pharmacological efficacy of a co-administered drug.

3. The most comprehensive approach when considering induction involves integrating quantitative *in vitro* data, information on the pharmacokinetic behaviour of the compound and the PK/PD relationship in order to predict its consequences. The generation of this holistic strategy would enable more detailed and informed decision-making about both the suitability of molecules for development and the development strategy itself.

Introduction

With advances in *in vitro* methodology during the last decade, some issues in drug metabolism and pharmacokinetics (DMPK) within the pharmaceutical industry that have traditionally caused the failure of new chemical entities (NCEs) in drug development (e.g. poor absorption, inappropriate clearance, drug-drug interactions) are now routinely studied in early drug discovery (Houston and Carlile 1997, Yee 1997, Crespi *et al.* 1998, Favreau *et al.* 1999, Stenberg *et al.* 2000). The huge costs involved in drug development have led to DMPK becoming a routine part of the drug discovery process, alongside both medicinal chemistry and pharmacology, in order to reduce the failure rate in development. There is a need, at least partially, to address the DMPK issues that tend to cause the failure of a NCE during drug development prior to the nomination of a potential development candidate. The latest of these issues

* Author for correspondence; e-mail: david.carlile@celltechgroup.com

that is increasingly becoming part of the drug discovery process is enzyme induction.

The potential for some drug molecules to induce particular cytochrome P450 (CYP) levels is a well known phenomenon (Bock *et al.* 1990, Okey 1990). The recent advances in real-time reverse transcriptase PCR (RT-PCR) have enabled the mRNA levels of individual drug-metabolizing enzymes to be accurately quantified after exposure to drug molecules (Bowen *et al.* 2000, Pan *et al.* 2000). Such technology, coupled with the availability of fresh human liver, has increased the potential for the study of induction *in vitro* prior to first dosing in man. Consequently, there has been renewed interest in the pharmaceutical and biopharmaceutical industries to quantify the effects of NCEs on the important CYPs involved in xenobiotic metabolism. The aim of this review is to consider the consequences of enzyme induction for both preclinical and clinical development and the subsequent implications for drug discovery.

This review will detail the *in vitro* techniques available to the pharmaceutical and biotechnology industries to investigate enzyme induction coupled with *in vitro:in vivo* correlations, together with a consideration of the pharmacokinetics involved. In addition the consequences of induction for drug development, both preclinical and clinical, will be discussed.

Pharmacokinetic aspects

For the majority of drugs, their efficacy is a function of the total exposure of the individual to drug, measured by the area under the plasma concentration–time curve (*AUC*). *AUC* is related to both the clearance of the drug (*CL*) and the dose administered (*D*):

$$AUC = \frac{D}{CL}. \quad (1)$$

Assuming that clearance is entirely hepatic, then the venous equilibration model (well-stirred model) describes hepatic clearance (CL_H) in terms of hepatic blood flow (Q_H), drug binding within the blood matrix (fu) and intrinsic clearance (CL_{int} , which is defined as the enzyme activity towards a drug) (Pang and Rowland 1977):

$$CL_H = \frac{Q_H \cdot fu \cdot CL_{int}}{Q_H + fu \cdot CL_{int}}. \quad (2)$$

CL_{int} can also be defined in enzyme kinetic terms, from the Michaelis–Menten parameters, where V_{max} is the maximal reaction velocity and K_m is the substrate concentration at 50% of V_{max} :

$$CL_{int} = \frac{V_{max}}{K_m}. \quad (3)$$

As enzyme induction leads to an increase in the amount of enzyme in the body there should be no effect on the affinity of the enzyme for the drug (K_m) and the only enzyme kinetic parameter which should change is V_{max} (Park *et al.* 1996). Moreover, increases in enzyme concentration should have no effect on either hepatic blood flow or protein binding of drug. In addition, as the majority of drugs are administered orally, changes in clearance may change the hepatic bioavailability (F_H) by altering the first-pass effect:

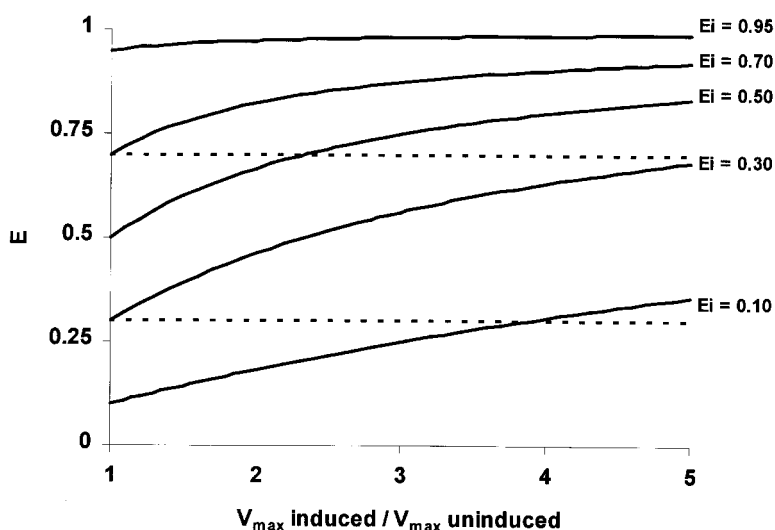


Figure 1. Simulation of the effect of an induction in enzyme activity on hepatic extraction ratio where the extraction ratio in the uninduced state (E_i) ranges from 0.10 to 0.90. The dotted lines represent the lower limit for high extraction classification (0.7) and the upper limit for low extraction classification (0.3).

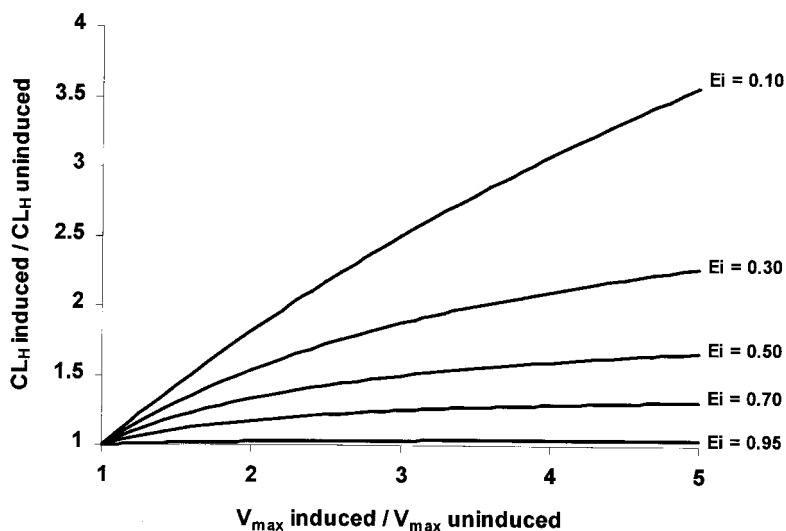


Figure 2. Simulation of the effect of enzyme induction on hepatic clearance for a range of scenarios where the initial extraction ratio (E_i) was between 0.10 and 0.90.

$$F_H = \frac{Q_H}{Q_H + f_u \cdot CL_{int}} \quad (4)$$

Figures 1–3 present a series of simulations undertaken to assess the impact of enzyme induction on clearance and bioavailability. For these simulations, $V_{\max}$ was increased up to 5-fold, and expressed as the $V_{\max}$ induced/ $V_{\max}$ uninduced ratio, K_m remained unaltered, and was fixed to 20 mg l^{-1} (which corresponds to a $K_m = 50 \mu\text{M}$ for a drug with a molecular weight of 400 – this is significantly higher

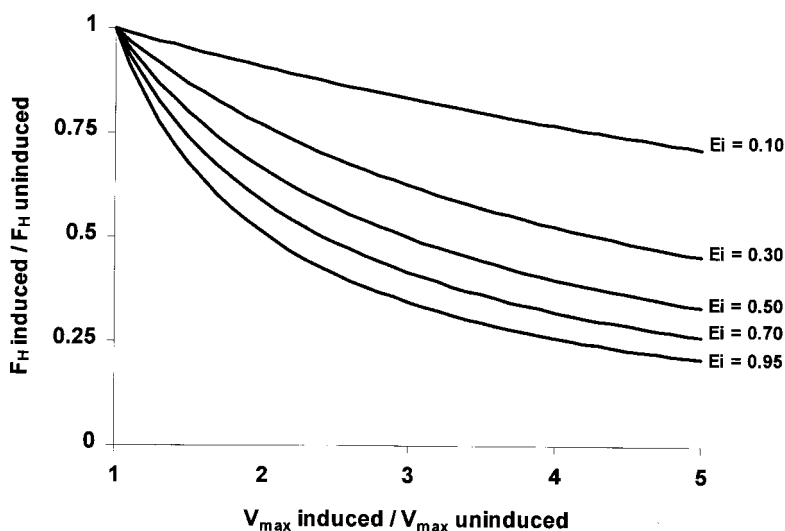


Figure 3. Simulation describing the reduction in hepatic bioavailability, compared with the uninduced state caused by an induction of hepatic metabolic activity for a range of initial extraction ratios (E_i).

than circulating plasma concentrations for the vast majority of marketed drugs), hepatic blood flow was fixed at 1.5 l min^{-1} , blood binding was assumed to be negligible and hence f_u was assigned as 1. Simulations were performed to assess the impact of increases in $V_{\max}$ on hepatic clearance and hepatic bioavailability for situations where the hepatic extraction ratio (E) of drug in the uninduced state was very low (0.10), low (0.30), intermediate (0.50), high (0.70) or very high (0.95).

Figure 1 presents the effects of an induction in $V_{\max}$ on E . Clearly there is very little effect when E is either high or very high in the uninduced state, whereas there are marked effects on this parameter for the intermediate and low E groups. For example, a 5-fold increase in $V_{\max}$ results in E changing from low (0.30) to the lower boundary for high extraction classification (0.68). Figure 2 presents the effect of increasing $V_{\max}$ on CL_H . Again the most significant effects are observed with situations where the initial E is low; thus a 5-fold increase in $V_{\max}$ causes almost a 3.5-fold increase in CL_H when E was initially 0.10 and 2-fold when E was 0.30. For drugs that have a high E changes in metabolic clearance have negligible effects upon CL_H ; as clearances approach blood flow, the rate-limiting step is the presentation of drug to the enzymes and hence Q_H assumes prominence. Therefore in these instances substantial increases in enzyme levels and hence $V_{\max}$ have only minor effects on total-body clearance. In contrast, for low clearance drugs, where the rate-limiting step in hepatic extraction is metabolic activity (essentially CL_{int}), then substantial changes in enzyme activity have a direct impact on body clearance. Figures 1 and 2 represent the effects on clearance of enzyme induction, regardless of the route of administration.

However, the vast majority of drug treatments are via the oral route. Figure 3 presents the effects of induction on the bioavailability of drug. The most marked reductions in F_H occur for drugs which have a high extraction classification, with less marked alterations for low extraction compounds. Therefore marked reductions in F_H will result in large decreases in the systemic exposure. This is almost

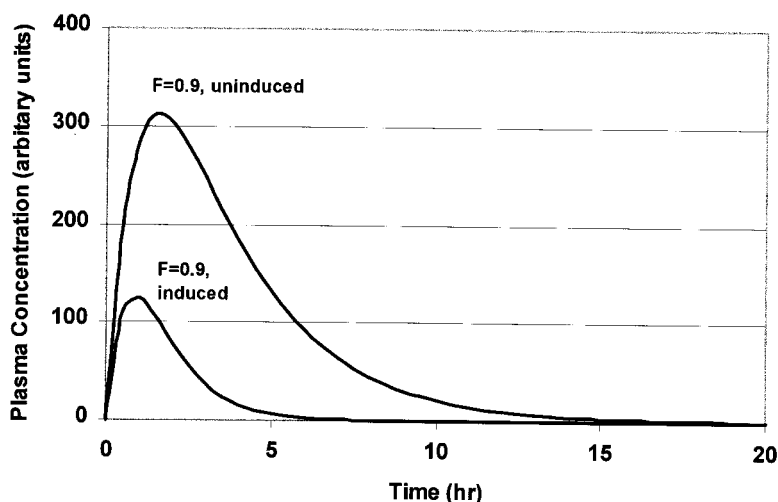


Figure 4. Simulations of plasma concentration–time profiles following oral administration comparing the uninduced state with a 5-fold increase in $V_{\max}$ where the hepatic bioavailability is high (0.90).

the exact opposite situation to the systemic effects, where the groups most affected were of low extraction. In the present instance the most dramatic alterations occur for compounds which already have low bioavailability. The AUC for these cases, following an oral dose, will be disproportionately lower after enzyme induction than for compounds that have a much higher bioavailability. A series of simulations was performed using Winnonlin (Pharsight Corporation, CA, USA) to represent this phenomenon by showing the changes in plasma concentration–time profiles caused by a 5-fold increase in $V_{\max}$ for two scenarios, one where F_H was high (0.9) and the other where F_H was low (0.3). It was assumed for these simulations that the plasma concentration–time profile after oral administration was best described by a one-compartment pharmacokinetic model (equation 5), comprising V (volume of distribution), K_a (absorption rate constant) and K_{el} (elimination rate constant):

$$C(t) = \left(\frac{D \cdot K_a}{V(K_a - K_{el})} \right) \cdot [e^{(-K_{el} \cdot t)} - e^{(-K_a \cdot t)}]. \quad (5)$$

The volume of distribution was assumed to be 40 litres (after i.v. administration, approximating to total body water), K_a was assigned as 1.0 min^{-1} and hence K_{el} was altered to reflect a change in $V_{\max}$ from the uninduced state to a 5-fold induction. Figures 4 and 5 present these simulations for two cases, first where F_H is high (0.9), and second where it is low (0.3). It is clear from both figures that a 5-fold induction in metabolic activity can alter the plasma profile of a NCE, however the most significant effects occur when the initial bioavailability is low (figure 5). When initial F_H is high (0.9), AUC after a 5-fold increase in $V_{\max}$ is 20% of the uninduced value, however when the initial F_H was only 0.3, AUC after a 5-fold induction is only 6% of the uninduced value. It is noteworthy that most pharmaceutical and biopharmaceutical companies strive to achieve orally active molecules which have good, reproducible bioavailability coupled with low clearance to achieve good systemic exposure.

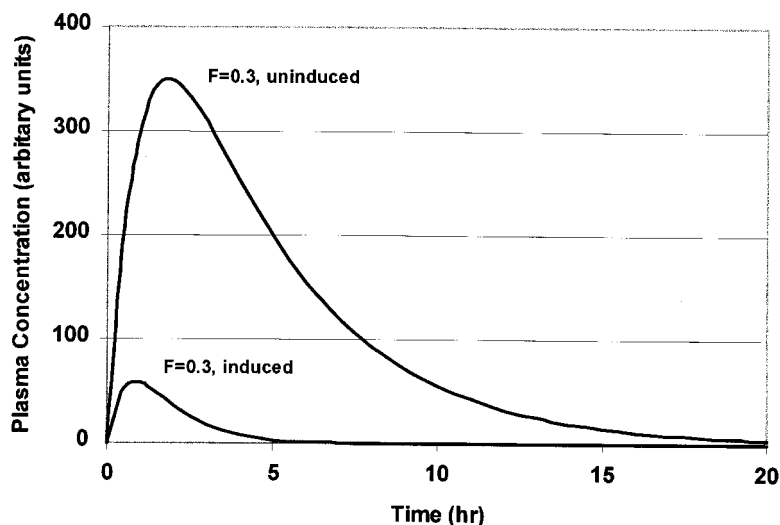


Figure 5. Simulations of plasma concentration–time profiles following oral administration comparing the uninduced state with a 5-fold increase in $V_{\max}$ where the hepatic bioavailability is low (0.30).

As a consequence of the above, dosage alterations may be required for drugs, as discussed by Park *et al.* (1996). However, it should be emphasized that for the above simulations it was assumed that body clearance is purely hepatic and that metabolism occurs via one pathway only (i.e. there is one route of metabolism that accounts for its clearance). The vast majority of clinically used drugs involve multiple pathways of metabolism with the involvement of several enzymes, and moreover, for some drugs renal elimination or non-hepatic metabolism can contribute significantly to their clearance. Therefore, as the importance of individual metabolic pathways on the overall clearance of drug decreases, the effects of the induction of a particular enzyme on the overall body clearance of the drug will decrease concomitantly. These simulations can be regarded as ‘worst case’ scenarios and for the vast majority of compounds enzyme induction has relatively minor consequences (Smith 2000), and hence there is little requirement for clinical intervention and dosage adjustment. For drugs with a narrow therapeutic index that undergo therapeutic drug monitoring, some adjustment in dose may be necessary in order to maintain efficacy. For example, co-administration of rifampin and warfarin results in decreases in anti-coagulation efficacy and higher warfarin doses are required (Romankiewicz and Ehrman 1975), warfarin and phenobarbital co-administration may also necessitate dosage adjustment of warfarin (Patsalos and Duncan 1993).

***In vitro* technologies**

The assessment of enzyme induction has traditionally been carried out through the dosing of a test compound to an animal, followed by removal of the liver and the preparation of microsomes. These are then probed for CYP activity using substrates specific for particular CYPs with the extent of induction being determined by comparison with microsomes prepared from vehicle-treated animals (Gorski *et al.* 1994, Ekins *et al.* 1995, Sharer *et al.* 1995). In addition to this activity

analysis, increases in apoprotein can be assessed with antibodies specific for individual CYPs using Western blotting or ELISA detection methods (Bars *et al.* 1989, Wortelboear *et al.* 1991, Wright *et al.* 1994). However, there are several drawbacks to this method of assessing enzyme induction. As a result of the high doses typically administered the compound requirement is high, approximately several hundred milligrams for a rat study. These quantities are rarely available in a drug discovery environment and synthesizing such amounts will have serious implications with respect to the number of NCEs being synthesized. Furthermore dosing will typically take 1 week with another week for analysis. In addition to the analytical resource required the compound throughput will be low, maybe one to five compounds per experiment. These factors render this approach unsuitable for modern drug discovery given the increasing productivity of medicinal chemistry groups.

Similar techniques involving probe substrates and immunoblotting have also been applied to *in vitro* systems. Although there is still considerable interest in the use of liver slices (Price *et al.* 1998), cultured hepatocytes are the preferred system. A large body of data is available demonstrating the inducibility of CYP1A, CYP2B, CYP3A and CYP4A in cultured hepatocytes (Paine *et al.* 1990, LeCluyse *et al.* 1996). In addition to the CYPs, other phase I oxidative enzymes and phase II conjugating enzymes, transcriptional factors, response elements and receptors found *in vivo* are present in hepatocytes (Strom *et al.* 1996). Furthermore, perhaps the major advantage with hepatocytes is their amenability to miniaturization and the 96-well plate technology increasingly utilized in drug discovery.

In a study by Nicoll-Griffith *et al.* (2001), a cyclooxygenase-2 inhibitor was incubated with rat hepatocytes and the levels of CYP3A apoprotein determined by Western blotting and activity by measuring rates of testosterone metabolism. CYP3A apoprotein levels in these hepatocytes were found to exceed those detected when 10 μM dexamethasone was employed as the positive control inducing agent (a concentration giving a maximal response for this inducer). The induction of enzyme activity was assessed by probing the cells with testosterone at a substrate concentration of 250 μM . Perhaps not surprisingly the extent of induction as determined by the two methods differed, as Western blotting probes for immunoreactive protein and is therefore capable of detecting inactive as well as active protein.

There are issues to be considered when assessing enzyme induction using the probe substrate method. As discussed, induction should not affect the affinity of the enzyme for substrate (K_m), rather it increases activity (V_{max}). Therefore, the probe substrate concentration used typically approximates V_{max} and induction is assessed by comparing the rates of turnover in control and treated cultures. However, in *in vitro* experiments this may be complicated by the issue of cooperativity between substrates giving rise to non Michaelis–Menten kinetics, particularly with CYP3A. This subject is reviewed in detail by Houston and Kenworthy (2000). The issue for induction studies arises from the possibility of the test compound also being a substrate for the enzyme it is inducing and thereby altering the kinetic properties of the probe substrate via either positive or negative cooperativity. Cells are typically cultured in media containing the test compound and at the end of the incubation period enzyme activity is determined by replacing the media with fresh media containing the probe substrate of choice. Although replacing the media will remove test compound dissolved in it, the extent of drug

distribution and non-specific binding within hepatocytes can, for some molecules, be considerable; these phenomena are highly compound-dependent (Worboys *et al.* 1997). Thus hepatocytes may contain considerable amounts of test compound, even when media are replaced. Therefore, there is a possibility that a NCE may exert some cooperativity (either activation or inhibition) with the enzyme which influences the metabolism of the probe substrate.

The increasing interest in determining the ability of test compounds to induce enzymes at the discovery phase has partly arisen through recent advances in determining mRNA levels. In the vast majority of instances enzyme induction is mediated through increases in mRNA levels, either through increased transcription or message stabilization. Measuring mRNA levels has traditionally utilised Northern blotting (Schuetz *et al.* 1994, Ryu *et al.* 1996), however this technique is low throughput and often does not give the quantitative quality required.

Ribonuclease protection assays have been employed for some time in detecting CYP mRNA levels (Gonzalez *et al.* 1992, Czerwinski *et al.* 1994). Recent advances have seen this technique applied to cultured rat hepatocytes in a high throughput manner (Surry *et al.* 1999). In this assay, radiolabelled riboprobes are employed with 96-well plates to give a rapid measurement of the mRNA level. Further refinements to this assay have employed radiolabelled oligonucleotides which hybridize to CYP-specific mRNA giving a signal detectable again using 96-well plates (Surry *et al.* 2000). Both assays can provide the throughput of compounds for induction to be studied in early drug discovery.

However, these techniques are being superseded by advances in quantitative RT-PCR (Bowen *et al.* 2000, Pan *et al.* 2000). Although compound throughput with this technique is not dramatically increased, sensitivity is. This has increased the utility of human hepatocytes for induction studies and reduced the number of cells that are needed to assess each compound.

From 1 million rat hepatocytes (an approximate yield from one liver being 50–100 million hepatocytes) typically 50 µg mRNA can be isolated. A single RT-PCR analysis uses ~2 µg mRNA and results are typically standardized to a control 'house-keeping' gene such as albumin or β -actin. Multiplexing allows the levels of the test and control mRNA to be determined in an individual reaction vessel. Furthermore, with the ability to run multiple tests on different wells of a 96-well plate, many mRNAs can be assessed on a single plate. A typical plate taking ~1 h to analyse.

Allowing for three replicates, the levels of 32 separate mRNAs could be determined by RT-PCR on a single 96-well plate. However, the use of DNA array techniques offers the ability to probe many more mRNAs simultaneously. This technology is being extensively used in the areas of basic biology, disease diagnosis, target validation, and increasingly, safety assessment (van Hal *et al.* 2000, Young 2000). Recently Nguyen *et al.* (2000) utilized this technology for the study of CYP mRNA in blood mononuclear cells. By carefully choosing the genes to be placed on a chip this technology has the ability to screen mRNA samples for the induction of many drug-metabolizing genes simultaneously and, for example, to link these to markers of toxicity. Investigations regarding the sensitivity of various types of chips and their utility with *in vitro* systems are currently underway in many laboratories.

Another system of investigating induction employs gene assays (Ogg *et al.* 1999). As with the direct detection of mRNA these systems monitor transcription.

However, rather than monitoring for mRNA levels they involve transfecting a transformed cell line with a plasmid containing the promoter sequence from a CYP coupled to a gene giving rise to an easily detected product, e.g. alkaline phosphatase. These systems are extremely useful tools for the investigation of transcriptional regulators of gene induction and being a transformed cell line are particularly suited to a screening environment. However, being derived from a transformed cell line there are certain limitations. It has been demonstrated that the extent of induction by dexamethasone of the human CYP3A4 promoter in a HepG2 cell line could be doubled by co-transfecting with a second plasmid containing the glucocorticoid receptor (Ogg *et al.* 1999). This and other studies comparing reporter sequences from various species transfected into cells from other species have shown that inducibility is markedly affected by the cellular environment (Barwick *et al.* 1996 and Xie *et al.* 2000). This could include the level and type of activator and repressor proteins present in a cell relative to those found *in vivo*. Additionally in transfected cell lines the level of expression of the transfected promoter may be markedly different to that found physiologically resulting in different responses. Finally, the distribution and availability of the inducer within a transformed or non-native cell could be also be significantly different. All these factors are highly compound dependent and have implications for the use of transfected cell lines as quantitative tools for the assessment of new chemical entities as inducers. Hence, for the present these systems remain valuable qualitative tools with their quantitative value being unproven.

At the present time the cultured hepatocyte is perhaps the most favoured method to detect enzyme induction *in vitro* as it is thought most closely to resemble the *in vivo* situation. However, during isolation and subsequent culture, hepatocytes de-differentiate and their phenotypical resemblance, and ability to respond to inducing agents, can markedly differ to hepatocytes *in vivo*. This de-differentiation process includes rapid changes in the levels of various mRNAs, including those of CYPs (Padgham and Paine 1993, Padgham *et al.* 1993). Various investigators are continuing to work on an understanding of the molecular basis of this process (Varley and Dickson 1999), and to develop culture conditions to minimize it (Gomez-Lechon *et al.* 1998).

***In vitro:in vivo* correlations**

At the simplest level there is a need to establish whether a NCE has the potential to cause enzyme induction. A number of molecules have been observed to induce individual CYP levels in cultured human hepatocytes, and *in vivo* studies have confirmed that induction determined *in vitro* frequently can be observed *in vivo* as measured by drug interaction studies, where there are alterations in pharmacokinetic parameters. Table 1 presents some typical clinical enzyme inducers, together with an indication of whether any *in vitro* studies have also demonstrated induction.

Even this relatively simple, qualitative technique can be powerful, for example in a recent study, Pichard-Garcia *et al.* (2000) used human hepatocyte culture to predict a lack of induction of eliotriptan *in vivo*. Such a study provides confidence that enzyme induction is unlikely to occur *in vivo*. This can be particularly useful if it is known from previous experience that some classes of compounds for particular disease targets frequently give rise to enzyme induction *in vivo*, e.g. anti-epileptics.

Table 1. Typical examples of clinical enzyme inducers *in vivo*, together with their daily dosage regimens and an indication of any induction *in vitro*.

Compound	<i>In vivo</i> induction	Daily dosage regimen (mg)	<i>In vitro</i> induction
Rifampicin	Heimark <i>et al.</i> (1987)	600–1200	Li <i>et al.</i> (1997a)
Rifabutin	Barditch-Crovo <i>et al.</i> (1999)	150–450	Li <i>et al.</i> (1997a)
Dexamethasone	Villikka <i>et al.</i> (1998)	0.5–10	Donato <i>et al.</i> (1995)
Omeprazole	Andersson <i>et al.</i> (1990)	10–40	Curi-Perdoso <i>et al.</i> (1994)
Lansoprazole	Kokufu <i>et al.</i> (1995)	15–30	Curi-Perdoso <i>et al.</i> (1994)
Felbamate	Glue <i>et al.</i> (1997)	1200–3600	–
Carbamazepine	Lucas <i>et al.</i> (1998)	200–1200	Ogg <i>et al.</i> (1999)
Troglitazone	Loi <i>et al.</i> (1999)	200–600	Sahi <i>et al.</i> (2000)
Phenobarbitone	Somogyi <i>et al.</i> (1981)	60–180	Meunier <i>et al.</i> (2000)
Phenytoin	Backman <i>et al.</i> (1996)	150–500	Ogg <i>et al.</i> (1999)

However, there is a drive to consider the quantitative importance of enzyme induction, especially in drug discovery in order to obtain rank order comparisons of a number of molecules to select those with the most favourable properties for further investigation, and this can now be achieved in several different ways.

Induction *in vivo* is a dynamic process with several variables including the dose of inducing agent administered, the dosing interval and frequency and the length of treatment. In order to study the effects on induction *in vitro*, for example with cultured hepatocytes, then the variables are similar, the concentration of compound in the culture media and the length of exposure will influence the extent of enzyme induction. There are several simple methods that can be adopted to attempt to predict *in vivo* behaviour from the *in vitro* data. Perhaps the simplest is to investigate a concentration–effect relationship in hepatocytes. This has been explored with rifampicin (Smith 2000), the concentrations of this drug required to achieve significant increases in lignocaine metabolism in cultured human hepatocytes (10–100 μM) were compared with the unbound plasma concentration of this drug *in vivo* (4 μM) in order to predict a relatively minor in man. Clearly this approach can work well providing there is sufficient information available on the *in vivo* plasma concentration of the NCE. However, in the pharmaceutical industry it is unlikely that at the late discovery/early preclinical phase, when most *in vitro* induction studies are undertaken, there will be any clinical data on the NCE under investigation. Nonetheless, with the use of allometric modelling techniques, alongside human *in vitro* data such as CL_{int} (Lave *et al.* 1997) it should be possible to predict the pharmacokinetic behaviour of the drug in man. Coupled with this, a good knowledge of the pharmacokinetic/pharmacodynamic (PK/PD) relationship in the *in vivo* pharmacology model should enable a broad estimation of the likely therapeutic dose and effective plasma concentration required for efficacy in man. Therefore, it should be possible to compare the hepatocyte induction–concentration data with the predicted *in vivo* doses and plasma levels in order to elucidate whether there will be the likelihood of significant *in vivo* induction.

It is possible to extend this approach even further for particular drug candidates by exploiting the methodologies available to predict pharmacokinetic behaviour (Houston and Carlile 1997). *In vitro* the CL_{int} of a compound is defined as the $V_{\text{max}}/K_{\text{m}}$ ratio. Measuring this ratio for probe substrates via enzyme kinetic studies in untreated hepatocytes and comparing this ratio in hepatocytes cultured

with compound of interest allows quantitative estimates of the induction potential to be determined. Moreover the use of RT-PCR enables quantification of the mRNA levels for drug-metabolizing enzymes, either from pretreated animals, or from cultured animal and human hepatocytes. At an early stage in drug development these data should be sufficient to provide information which allows rank order comparisons of a number of molecules, and enables an assessment on the suitability of a NCE for development. In addition, if induction may be expected *in vivo* such data would assist in the clinical development plan, for example designing and implementing a drug interaction study at a particular stage.

Preclinical consequences

One of the main purposes of conducting preclinical animal toxicokinetic studies is to ensure adequate exposure, in terms of AUC and/or C_{max} , in short and long-term safety studies. The consequence of inadequate exposure in safety species could limit the maximum exposure permitted in early clinical trials until a safety database in humans is established. Safety studies are initially conducted over 2–14 weeks in rodent (typically rat) and non-rodent (typically dog or monkey) species. By design safety studies involve doses much higher than those anticipated in the clinic thereby increasing the likelihood of enzyme induction. Clearly induction by the test compound could severely limit the obtainable AUC if the enzymes induced are those responsible for metabolizing the compound. Herein lies an important distinction when considering this phenomenon. In terms of safety cover induction of a CYP which is not involved in the clearance of a test compound can be considered irrelevant, and is unlikely to be detected, unless it is involved in toxicity issues. Many other changes within the liver are associated with enzyme induction including liver weight increases, histological changes and elevation of serum enzymes such as alanine aminotransferase (Amacher *et al.* 1998). Hepatic CYP4A induction seen with compounds such as clofibrate and nafenopin is closely associated with peroxisome proliferation and hepatocarcinogenesis in rodents. However, in the clinic this class of peroxisome-proliferating compounds are not carcinogenic (Cattley *et al.* 1998) although alterations in CYP4A activity can result in tissue homeostasis being affected.

In order to interpret *in vitro* induction data, knowledge about the contribution of the various CYP isoforms to the metabolism of a compound is required. Isoform contribution studies are routinely performed early in the development of a compound. Traditionally this was done through the use of correlation analysis with a phenotyped human liver bank although the advent of singly expressed isoforms and inhibitory antibodies has greatly simplified these studies (Madan *et al.* 1998, Wang *et al.* 1999, Venkatakrishnan *et al.* 2000). Until the recent availability of singly expressed rat CYPs, these tools were predominantly human based and it is still rare for the isoforms contributing to the metabolism of a NCE to be determined in any species other than human.

Preclinical *in vitro* induction studies are typically carried out using rat hepatocytes and extrapolation of human isoform contribution data clearly raises questions of cross species comparisons. Furthermore, induction studies are rarely carried out in dog or monkey hepatocytes due to the problems of tissue availability, and are only likely to be performed if autoinduction *in vivo* is observed during preclinical toxicokinetic studies. This discussion reinforces the fact that any *in*

vitro data from drug discovery cannot be considered in isolation, but needs amalgamating with other DMPK data. These data include the contribution of CYPs, CL_{int} , E , F_H , the PK/PD relationship and the likely therapeutic treatment regimens so that sensible and coherent decisions are made about the suitability of compounds for drug development.

Clinical consequences

Conceptually the consequences of induction in man are relatively simple to specify in terms of an unwanted drug interaction. Enzyme induction could increase the metabolism and clearance of a pharmacologically active drug (either the inducing molecule itself leading to autoinduction or that of a co-administered agent) leading to a reduction in pharmacological activity. Conversely if a metabolite was the pharmacologically active agent then induction could cause an increase in pharmacological activity. However, if the parent compound is completely metabolized during hepatic first pass to the active moiety (i.e. a prodrug) then induction would not have this effect. Moreover if there were a toxic metabolite formed, and the formation pathway was induced then increased toxicity may result from enzyme induction. However, as discussed above detailed consideration of pharmacokinetics and clinical concepts are required in order to predict whether induction leads to a quantifiable change in pharmacodynamics. Moreover deciding whether such an interaction should be avoided clinically also requires a consideration of the therapeutic index of the effected drug. As Smith (2000) described, there are relatively few failures in clinical development due to induction, <2%. This perhaps reflects the differences between preclinical toxicology studies, where high doses of drug are administered chronically to define toxicological safety and to identify exaggerated pharmacological effects for humans, and clinical studies where much lower doses are typically used to provide the desired pharmacological efficacy.

Clearly the potential for drug interactions in a clinical setting is relatively low, however there are some enzyme inducers currently prescribed (table 1) and there will be situations where this is observed for NCEs in development. Should this occur then wider considerations are required in order to decide whether to progress the NCE. Several issues have to be addressed including the condition being treated, is it a 'serious' or life-threatening disease (e.g. AIDS)? If so then progression should probably ensue. However, if the condition is perhaps less 'serious' (e.g. adolescent acne) then further considerations, such as ethical and moral, are required. For example, is it appropriate to allow patients of 'non-serious' conditions potentially to undergo an interaction? It is evident though that there is no consensus of what constitutes a 'serious' and 'non-serious' condition. Commercial and regulatory considerations may also be required, for example if the disease has a similar therapy already available then it may not be commercially viable to proceed. Moreover it may be difficult to convince regulatory authorities to licence a molecule for such conditions, or to prevent severe restrictions in the labelling, particularly if an existing therapy avoids any such interactions.

If there is an unmet clinical need, and/or the disease target is 'serious' or life threatening then it may be appropriate to introduce therapeutic drug monitoring. For example phenytoin is still a frontline therapy for epilepsy, despite both its induction of CYP2C9 and CYP3A4 and its non-linear pharmacokinetics (Riva *et*

al. 1996). Through such therapeutic drug monitoring it is possible to monitor the effects of the drug and concomitant medications and hence alter dosage requirements to prevent a decrease in efficacy caused by enzyme induction.

Conclusions

It is desirable that all clinical drug interactions are avoided wherever possible, however it is widely accepted that interactions which result in impaired, rather than enhanced, elimination are potentially more serious as these increase systemic plasma concentrations and hence exposure of drugs. The presence of an enzyme induction event in humans during clinical development is unlikely to cause the termination of a programme, unless there is another currently available therapy which offers the same efficacy without the presence of induction. The major difficulty with enzyme induction in the pharmaceutical industry occurs during the preclinical repeat dose toxicology studies where safety data are generated to support clinical trials, typically by chronic administration of the maximum tolerated dose which is substantially higher than the predicted human dose. Autoinduction at this stage may result in a poor drug exposure, hence limiting the maximum dose which can initially be administered to humans, until some supportive data are generated in the clinical programme. Moreover it may even cause a complete absence of safety cover. Therefore it is sensible to identify in drug discovery whether autoinduction could occur during this preclinical phase before an expensive development project is undertaken. For programmes where autoinduction is a major issue, the drug discovery strategy may include enzyme induction screening as a critical step. Currently however there is no clear structure activity relationship with induction which could assist in this process (Smith 2000). Hence this work would involve induction screening and medicinal chemistry synthesis in an iterative loop in order to produce non-inducing compounds. As the major pitfalls with induction occur in animals, induction data in several species may assist in the selection of toxicology species. For example, if human hepatocytes indicated a complete lack of enzyme induction by a NCE, whereas studies with preclinical species (either *in vitro* and/or *in vivo*) demonstrated substantial induction it may be appropriate to choose an alternate animal species for the toxicology studies, providing the usual requirement for a similar metabolic profile between human and the toxicology species is satisfied.

Preclinical *in vivo* induction studies are costly in terms of compound requirement and limited in throughput, consequently an increasing emphasis is placed upon *in vitro* assessment. The various *in vitro* systems and methods available for assessing induction have been reviewed herewithin. As mentioned, induction studies are routinely conducted in rodent hepatocytes where tissue supply is plentiful, whereas they are performed less frequently in human hepatocytes. The ability to use cryopreserved cells would greatly increase the number of compounds for human induction and potentially allow the addition of dog and/or monkey to the preclinical species currently considered. The main issue with the use of cryopreserved cells is their poor attachment to culture medium which is currently required for conducting induction studies with cells, unattached cells rapidly perish in culture (Li *et al.* 1999). It is possible that advances in sandwich culture techniques, and increasingly sensitive methods such as RT-PCR for the

detection of mRNA (thereby resulting in shorter incubation times) may in the future overcome this limitation.

It is also worth noting that as with any *in vitro* experiment the utility of the data is greatly influenced by the quality of the protocol employed. For induction studies this includes knowledge of the metabolic stability of the test compound within the *in vitro* system and how this relates to the *in vivo* pharmacokinetics. For example, a compound which is extensively protein bound *in vivo* with a half life of several hours may be rapidly metabolized *in vitro*. In this instance adding media containing compound and culturing for 24 h could produce a false-negative result in terms of induction due to the rapid metabolism and CL_{int} of the compound. Additionally, a concentration range of the test compound should be employed rather than a single, and typically high, concentration. This allows the generation of a dose response curve and an estimate of Ind_{50} (concentration of inducer eliciting a 50% induction). This avoids false-negatives due to cytotoxicity occurring at a single, high concentration. Finally the competency of the cells at the end of the experiment should be checked, for example through a simple assay such as MTT turnover by mitochondrial dehydrogenases (Surry *et al.* 1999).

From an *in vitro* assessment of the ability of a compound to induce CYPs and knowledge of its pharmacokinetic properties a judgement of likely problems in safety assessment can be attempted. However, in addition to the issues previously described these predictions pose additional considerations. All *in vitro* predictions rely upon correlating the concentration of compound added to an incubation with that available *in vivo* (the first assumption being that cellular distribution and hence availability to induce are comparable *in vitro* and *in vivo*). Currently an estimate of the concentration of drug available to induce *in vivo* is gained from knowledge of plasma concentrations, protein binding and tissue distribution. However, these values are typically those circulating after the absorption, first pass metabolism and distribution phases have occurred. After an oral dose the concentrations in the hepatic portal vein and hence liver can be substantially higher than those found systemically. Furthermore, it is unclear how intestinal CYPs respond to an enzyme inducer after oral dosing. Induction of these enzymes could limit the hepatic first pass and systemic exposure of the compound thereby reducing the amount of induction in the liver. Conversely, the high doses employed in safety assessment studies could saturate the intestinal CYPs resulting in higher than anticipated hepatic portal vein concentrations thereby enhancing induction. Currently there are no intestinal mathematical models relating drug absorption, enzyme activity and drug availability similar to those used for the liver. This prevents an estimate of the importance of intestinal metabolic extraction from being made.

Perhaps one of the most exciting prospects is coupling human *in vitro* induction data with advances in *in silico* modelling. Tucker (2000) described the use of his SIMCYP modelling software, which incorporates a huge quantity of data including *in vitro* enzyme kinetic data, *in vitro* scaling factors, data on the composition of the liver in terms of CYP complement, plasma protein binding data, blood:plasma distribution data and their respective variabilities, in order to predict pharmacokinetic profiles *in vivo*. Moreover, by incorporating some PD models (e.g. Hill, E_{max}) Tucker demonstrated the utility of SIMCYP to simulate PD profiles and extended this to predict the likelihood of significant alterations in PD, for example if a polymorphic enzyme was involved in metabolism. It should be possible to

adopt this approach and incorporate quantitative *in vitro* induction data into the model and simulate the effects of induction on the PD of a drug, in order to estimate the likelihood of a clinically significant interaction.

This integrated approach to predict pharmacological outcomes caused by induction, rather than specific pharmacokinetic changes, should be the route by which all potential interaction studies are considered. The *in vivo* effects of a drug are mediated by a combination of both pharmacokinetic behaviour and pharmacodynamics. It is too simplistic an approach to examine individual issues, such as enzyme induction, without a consideration of the other multifactorial influences which determine whether a drug interaction is clinically relevant. With the exponential growth in computing power, and the powerful tools now available to the pharmaceutical industry, it is now time to adopt a holistic strategy to the investigation of phenomena such as enzyme induction.

Acknowledgements

The views expressed here are those of the authors and do not reflect the official views of Merck Sharp & Dohme or Celltech.

References

- AMACHER, D. E., SCHOMAKER, S. J. and BURKHARDT, J. E., 1998, The relationship among microsomal enzyme induction, liver weight and histological change in rat toxicology studies. *Food and Chemical Toxicology*, **36**, 831–839.
- ANDERSSON, T., CEDERBERG, C., EDVARDSSON, G., HEGGELUND, A. and LUNDBORG, P., 1990, Effect of omeprazole treatment on diazepam plasma levels in slow versus normal rapid metabolizers of omeprazole. *Clinical Pharmacology and Therapeutics*, **47**, 79–85.
- BACKMAN, J. T., OLKKOLA, K. T., LAAKSOVIRTA, H. and NEUVONEN, P. J., 1996, Concentrations and effects of oral midazolam are greatly reduced in patients treated with carbamazepine or phenytoin. *Epilepsia*, **37**, 253–257.
- BARDITCH-CROVO, P., TRAPNELL, C. B., ETTÉ, E., ZACUR, H. A., CORESH, J., ROCCO, L. E., HENDRIX, C. W. and FLEXNER, C., 1999, The effects of rifampicin and rifabutin on the pharmacokinetics and pharmacodynamics of a combination oral contraceptive. *Clinical Pharmacology and Therapeutics*, **65**, 428–438.
- BARS, R. G., MITCHELL, A. M., WOLF, C. R. and ELCOMBE, C. R., 1989, Induction of cytochrome P-450 in cultured rat hepatocytes. The heterogeneous localization of specific isoenzymes using immunocytochemistry. *Biochemical Journal*, **262**, 151–158.
- BARWICK, J. L., QUATTROCHI, L. C., MILLS, A. S., POTENZA, C., TUKEY, R. H. and GUZELIAN, P. S., 1996, Trans-species gene transfer for analysis of glucocorticoid-inducible transcriptional activation of transiently expressed human CYP3A4 and rabbit CYP3A6 in primary cultures of adult rat and rabbit hepatocytes. *Molecular Pharmacology*, **50**, 10–16.
- BOCK, K. W., LIPP, H. P. and BOCK-HENNIG, B. S., 1990, Induction of drug-metabolizing enzymes by xenobiotics. *Xenobiotica*, **20**, 1101–1111.
- BOWEN, W. P., CAREY, J. E., MIAH, A., McMURRAY, H. F., MUNDAY, P. W., JAMES, R. S., COLEMAN, R. A. and BROWN, A. M., 2000, Measurement of cytochrome P450 gene induction in human hepatocytes using quantitative real-time reverse transcriptase-polymerase chain reaction. *Drug Metabolism and Disposition*, **28**, 781–788.
- CATTLEY, R. C., DELUCA, J., ELCOMBE, C., FENNER-CRISP, P., LAKE, B. G., MARSMAN, D. S., PASTOOR, T. A., POPP, J. A., ROBINSON, D. E., SCHWETZ, B., TUGWOOD, J. and WAHLI, W., 1998, Do peroxisome proliferating compounds pose a hepatocarcinogenic hazard to humans? *Regulatory Toxicology and Pharmacology*, **27**, 47–60.
- CRESPI, C. L., MILLER, V. P. and PENMAN, B. W., 1998, High throughput screening for inhibition of cytochrome P450 metabolism. *Medicinal Chemistry Research*, **8**, 457–471.
- CURI-PEDROSA, R., DAUJAT, M., PICHARD, L., OURLIN, J. C., CLAIR, P., GERVOT, L., LESCA, P., DOMERGUE, J., JOYEUX, H., FOURTANIER, G. and MAUREL, P., 1994, Omeprazole and lansoprazole are mixed inducers of CYP1A and CYP3A in human hepatocytes in primary culture. *Journal of Pharmacology and Experimental Therapeutics*, **269**, 384–392.

- CZERWINSKI, M., MCLEMORE, T. L., GELBOIN, H. V. and GONZALEZ, F. J., 1994, Quantification of CYP2B7, CYP4B1, and CYPOR messenger RNAs in normal human lung and lung tumors. *Cancer Research*, **54**, 1085–1091.
- DONATO, M. T., CASTELL, J. V. and GOMEZ-LECHON, M. J., 1995, Effect of model inducers on cytochrome P450 activities of human hepatocytes in primary culture. *Drug Metabolism and Disposition*, **23**, 553–558.
- EKINS, S., MURRAY, G. I., BURKE, M. D., WILLIAMS, J. A., MARCHANT, N. C. and HAWKSWORTH, G. M., 1995, Quantitative differences in phase I and II metabolism between rat precision-cut liver slices and isolated hepatocytes. *Drug Metabolism and Disposition*, **23**, 1274–1279.
- FAVREAU, L. V., PALAMANDA, J. R., LIN, C.-C. and NOMEIR, A. A., 1999, Improved reliability of the rapid microtitre plate assay using recombinant enzyme in predicting CYP2D6 inhibition in human liver microsomes. *Drug Metabolism and Disposition*, **27**, 436–439.
- GLUE, P., BANELD, C. R., PERHACH, J. L., MATHER, G. G., RACHA, J. K. and LEVY, R. H., 1997, Pharmacokinetic interactions with felbamate. *In vitro-in vivo* correlation. *Clinical Pharmacokinetics*, **33**, 214–224.
- GOMEZ-LECHON, M. J., JOVER, R., DONATO, T., PONSODA, X., RODRIGUEZ, C., STENZEL, K. G., KLOCKE, R., PAUL, D., GUILLEN, I., BORT, R. and CASTELL, J. V., 1998, Long-term expression of differentiated functions in hepatocytes cultured in three-dimensional collagen matrix. *Journal of Cell Physiology*, **177**, 553–562.
- GONZALEZ, F. J., CRESPI, C. L., CZERWINSKI, M. and GELBOIN, H. V., 1992, Analysis of human cytochrome P450 catalytic activities and expression. *Tohoku Journal of Experimental Medicine*, **168**, 67–72.
- GORSKI, J. C., HALL, S. D., JONES, D. R., VANDEN-BRANDEN, M. and WRIGHTON, S. A., 1994, Regioselective biotransformation of midazolam by members of the human cytochrome P450 3A (CYP3A) subfamily. *Biochemical Pharmacology*, **47**, 1643–1653.
- HEIMARK, L. D., GIBALDI, M., TRAGER, W. F., O'REILLY, R. A. and GOULART, D. A., 1987, The mechanism of the warfarin-rifampin drug interaction in humans. *Clinical Pharmacology and Therapeutics*, **42**, 388–394.
- HOUSTON, J. B. and CARLILE, D. J., 1997, Prediction of hepatic clearance from microsomes, hepatocytes and liver slices. *Drug Metabolism Reviews*, **29**, 891–922.
- HOUSTON, J. B. and KENWORTHY, K. E., 2000, *In vitro-in vivo* scaling of CYP kinetic data not consistent with the classical Michaelis-Menten model. *Drug Metabolism and Disposition*, **28**, 246–254.
- KOKUFU, T., IHARA, N., SUGIOKA, N., KOYAMA, H., OHTA, T., MORI, S. and NAKAJIMA, K., 1995, Effects of lansoprazole on pharmacokinetics and metabolism of theophylline. *European Journal of Clinical Pharmacology*, **48**, 391–395.
- LAVE, T., DUPIN, S., SCHMITT, C., CHOU, R. C., JAECK, D. and COASSOLO, P., 1997, Integration of *in vitro* data into allometric scaling to predict hepatic metabolic clearance in man: application to 10 extensively metabolized drugs. *Journal of Pharmaceutical Sciences*, **86**, 246–254.
- LECLUYSE, E. L., BULLOCK, P. L. and PARKINSON, A., 1996, Strategies for restoration and maintenance of normal hepatic structure and function in long-term cultures of rat hepatocytes. *Advances in Drug Delivery Reviews*, **22**, 133–186.
- LI, A. P., GORYCKI, P. D., HENGSTLER, J. G., KEDDERIS, G. L., KOEBE, H. G., RAHMANI, R., DE SOUSAS, G., SILVA, J. M. and SKETT, P., 1999, Present status of the application of cryopreserved hepatocytes in the evaluation of xenobiotics: consensus of an international expert panel. *Chemico-Biological Interactions*, **121**, 117–123.
- LI, A. P., MAUREL, P., GOMEZ-LECHON, M. J., CHENG, L. C. and JURIMA-ROMET, M., 1997b, Preclinical evaluation of drug-drug interaction potential: present status of the application of primary human hepatocytes in the evaluation of cytochrome P450 induction. *Chemico-Biological Interactions*, **107**, 5–16.
- LI, A. P., REITH, M. K., RASMUSSEN, A., GORSKI, J. C., HALL, S., D., XU, L., KAMINSKI, D. L. and CHENG, L. K., 1997a, Primary human hepatocytes as a tool for the evaluation of structure-activity relationship in cytochrome P450 induction potential of xenobiotics: evaluation of rifampin, rifapentine and rifabutin. *Chemico-Biological Interactions*, **107**, 17–30.
- LOI, C. M., STERN, R., KOUP, J. R., VASSOS, A. B., KNOWLTON, P. and SEDMAN, A. J., 1999, Effect of troglitazone on the pharmacokinetics of an oral contraceptive agent. *Journal of Clinical Pharmacology*, **39**, 410–417.
- LUCAS, R. A., GILLAN, D. J. and BERGSTROM, R. F., 1998, A pharmacokinetic interaction between carbamazepine and olanzapine: observations on possible mechanism. *European Journal of Clinical Pharmacology*, **54**, 639–643.
- MADAN, A., PARKINSON, A. and FAIMAN, M. D., 1998, Identification of the human P-450 enzymes responsible for the sulfoxidation and thiono-oxidation of diethyldithiocarbamate methyl ester: role of P-450 enzymes in disulfiram bioactivation. *Alcohol: Clinical and Experimental Research*, **22**, 1212–1219.

- MEUNIER, V., BOURRIE, M., JULIAN, B., MARTI, E., GUILLLOU, F., BERGER, Y. and FABRE, G., 2000, Expression and induction of CYP1A1/1A2, CYP2A6 and CYP3A4 in primary cultures of human hepatocytes: a 10-year follow-up. *Xenobiotica*, **30**, 589–607.
- NGUYEN, L. T., RAMANATHAN, M., WEINSTOCK-GUTTMAN, B., DOLE, K., MILLER, C., PLANTER, M., PATRICK, K., BROWNSCHIEDLE, C. and JACOBS, L. D., 2000, Detection of cytochrome P450 and other drug-metabolizing enzyme mRNAs in peripheral blood mononuclear cells using DNA arrays. *Drug Metabolism and Disposition*, **28**, 987–993.
- NICOLL-GRITH, D. A., SILVA, J. M., CHAURET, N., DAY, S., LEBLANC, Y., ROY, P., YERGEY, J. A., DIXIT, R. and PATRICK, D., 2001, Application of rat hepatocyte culture to predict *in vivo* metabolic auto-induction: studies with DFP, a cyclooxygenase-2 inhibitor. *Drug Metabolism and Disposition*, **29**, 159–165.
- OGG, M. S., WILLIAMS, J. M., TARBIT, M., GOLDFARB, P. S., GRAY, T. J. B. and GIBSON, G. G., 1999, A reporter gene assay to assess the molecular mechanisms of xenobiotic-dependent induction of the human CYP3A4 gene *in vitro*. *Xenobiotica*, **29**, 269–279.
- OKEY, A. B., 1990, Enzyme induction in the cytochrome P-450 system. *Pharmacology and Therapeutics*, **45**, 241–298.
- PADGHAM, C. R., BOYLE, C. C., WANG, X. J., RALEIGH, S. M., WRIGHT, M. C. and PAINE, A. J., 1993, Alteration of transcription factor mRNAs during the isolation and culture of rat hepatocytes suggests the activation of a proliferative mode underlies their de-differentiation. *Biochemical and Biophysical Research*, **197**, 599–605.
- PADGHAM, C. R. W. and PAINE, A. J., 1993, Altered expression of cytochrome P-450 mRNAs, and potentially of other transcripts encoding key hepatic functions, are triggered during the isolation of rat hepatocytes. *Biochemical Journal*, **289**, 621–624.
- PAINE, A. J., 1990, The maintenance of cytochrome P-450 in rat hepatocyte culture: some applications of liver cell cultures to the study of drug metabolism, toxicity and the induction of the P-450 system. *Chemico-Biological Interactions*, **74**, 1–31.
- PAN, J., XIANG, Q. and BALL, S., 2000, Use of a novel real-time quantitative reverse transcription-polymerase chain reaction method to study the effects of cytokines on cytochrome P450 mRNA expression in mouse liver. *Drug Metabolism and Disposition*, **28**, 709–713.
- PANG, K. S. and ROWLAND, M., 1977, Hepatic clearance of drugs. I. Theoretical considerations of a 'well stirred' and a 'parallel tube' model: influence of hepatic blood flow, plasma and blood cell binding, and the hepatocellular enzymatic activity on hepatic drug clearance. *Journal of Pharmacokinetics and Biopharmaceutics*, **5**, 625–653.
- PARK, B. K., KITTINGHAM, N. R., PIRMOHAMED, M. and TUCKER, G. T., 1996, Relevance of induction of human drug-metabolizing enzymes: pharmacological and toxicological implications. *British Journal of Clinical Pharmacology*, **41**, 477–491.
- PATSALOS, P. N. and DUNCAN, J. S., 1993, Antiepileptic drugs. A review of clinically significant drug interactions. *Drug Safety*, **9**, 156–184.
- PICHARD-GARCIA, L., HYLAND, R., BAULIEU, J., FABRE, J. M., MILTON, A. and MAUREL, P., 2000, Human hepatocytes in primary culture predict lack of cytochrome P-450 3A4 induction by eliotriptan *in vivo*. *Drug Metabolism and Disposition*, **28**, 51–57.
- PRICE, R. J., BALL, S. E., RENWICK, A. B., BARTON, P. T., BEAMAND, A. and LAKE, B. G., 1998, Use of precision-cut rat liver slices for studies of xenobiotic metabolism and toxicity: comparison of the Krumdieck and Brendel tissue slicers. *Xenobiotica*, **28**, 361–371.
- RIVA, R., ALBANI, F., CONTIN, M. and BARUZZI, A., 1996, Pharmacokinetic interactions between antiepileptic drugs. Clinical considerations. *Clinical Pharmacokinetics*, **31**, 470–493.
- ROMANKIEWICZ, J. A. and EHRLMAN, M., 1975, Rifampin and warfarin: a drug interaction. *Annals of Internal Medicine*, **82**, 224–225.
- RYU, D. Y., LEVI, P. E., FERNANDEZ-SALGUERO, P., GONZALEZ, F. J. and HODGSON, E., 1996, Piperonyl butoxide and acenaphthylene induce cytochrome P450 1A2 and 1B1 mRNA in aromatic hydrocarbon-responsive receptor knock-out mouse liver. *Molecular Pharmacology*, **50**, 443–446.
- SAHI, J., HAMILTON, G., SINZ, M., BARROS, S., HUANG, S. M., LESKO, L. J. and LeCLUYSE, E. L., 2000, Effect of troglitazone on cytochrome P450 enzymes in primary cultures of human and rat hepatocytes. *Drug Metabolism and Disposition*, **30**, 273–284.
- SCHUETZ, J. D., BEACH, D. L. and GUZELIAN, P. S., 1994, Selective expression of cytochrome P450 CYP3A mRNAs in embryonic and adult human liver. *Pharmacogenetics*, **4**, 11–20.
- SHARER, J. E., SHIPLEY, L. A., VANDENBRANDEN, M. R., BINKLEY, S. N. and WRIGHTON, S. A., 1995, Comparisons of phase I and phase II *in vitro* hepatic enzyme activities of human, dog, rhesus monkey, and cynomolgus monkey. *Drug Metabolism and Disposition*, **23**, 1231–1241.
- SMITH, D. A., 2000, Induction and drug development. *European Journal of Pharmaceutical Sciences*, **11**, 185–189.
- SOMOGYI, A., THIELSCHER, S. and GUGLER, R., 1981, Influence of phenobarbital treatment on cimetidine kinetics. *European Journal of Clinical Pharmacology*, **19**, 343–347.
- STENBERG, P., LUTHMAN, K. and ARTURSSON, P., 2000, Virtual screening of intestinal drug permeability. *Journal of Controlled Release*, **65**, 231–243.

- STROM, S. C., PISAROV, L. A., DORKO, K., THOMPSON, M. T., SCHUETZ, J. D., SCHUETZ, E. G., 1996, Use of human hepatocytes to study P450 gene induction. *Methods in Enzymology*, **272**, 388–401.
- SURRY, D. D., McALLISTER, G., MENESES-LORENTE, G. and EVANS, D. C., 1999, High throughput ribonuclease protection assay for the determination of CYP3A mRNA induction in cultured rat hepatocytes. *Xenobiotica*, **29**, 827–838.
- SURRY, D. D., MENESES-LORENTE, G., HEAVENS, R., JACK, A. and EVANS, D. C., 2000, Rapid determination of rat hepatocyte mRNA induction potential using oligonucleotide probes for CYP1A1, 1A2, 3A and 4A1. *Xenobiotica*, **30**, 441–456.
- TUCKER, G. T., 2000, Pharmacokinetic and pharmacodynamic implications of pharmacogenetics. *Drug Metabolism Reviews*, **32** (suppl. 1), 8.
- VAN HAL, N. L., VORST, O., VAN HOUWELINGEN, A. M., KOK, E. J., PEIJNENBURG, A., AHARONI, A., VAN TUNEN, A. J. and KEIJER, J., 2000, The application of DNA microarrays in gene expression analysis. *Journal of Biotechnology*, **78**, 271–280.
- VARLEY, C. L. and DICKSON, A. J., 1999, Hepatocyte isolation stimulates formation of interferon stimulatory response element DNA–protein complexes. *Biochemical and Biophysical Research Communications*, **263**, 627–631.
- VENKATAKRISHNAN, K., VON MOLTKE, L. L., COURT, M. H., HARMATZ, J. S., CRESPI, C. L. and GREENBLATT, D. J., 2000, Comparison between cytochrome P450 (CYP) content and relative activity approaches to scaling from cDNA-expressed CYPs to human liver microsomes: ratios of accessory proteins as sources of discrepancies between the approaches. *Drug Metabolism and Disposition*, **28**, 1493–1504.
- VILLIKKA, K., KIVISTO, K. T. and NEUVONEN, P. J., 1998, The effect of dexamethasone on the pharmacokinetics of triazolam. *Pharmacology and Toxicology*, **83**, 135–138.
- WANG, R. W., NEWTON, D. J., LIU, N. Y., SHOU, M., RUSHMORE, T. and LU, A. Y. H., 1999, Inhibitory anti-CYP3A4 peptide antibody: mapping of inhibitory epitope and specificity toward other CYP3A isoforms. *Drug Metabolism and Disposition*, **27**, 167–172.
- WORBOYS, P. D., BRADBURY, A. and HOUSTON, J. B., 1997, Kinetics of drug metabolism in rat liver slices. III. Relationship between metabolic clearance and slice uptake rate. *Drug Metabolism and Disposition*, **25**, 460–467.
- WORTELBOER, H. M., DE KRUIF, C. A., VAN IERSEL, A. A. J., FALKE, H. E., NOORDHOEK, J., BLAAUBOER, B. J., 1991, Comparison of cytochrome P450 isoenzyme profiles in rat liver and hepatocyte cultures. The effects of model inducers on apoproteins and biotransformation activities. *Biochemical Pharmacology*, **42**, 381–390.
- WRIGHT, M. C., PAINE, A. J., SKETT, P. and AULD, R., 1994, Induction of rat hepatic glucocorticoid-inducible cytochrome P450 3A by metyrapone. *Journal of Steroid Biochemistry and Molecular Biology*, **48**, 271–276.
- XIE, W., BARWICK, J. L., DOWNES, M., BLUMBERG, B., SIMON, C. M., NELSON, M. C., NEUSCHWANDER-TETRI, B. A., BRUNT, E. M., GUZELIAN, P. S. and EVANS, R. M., 2000, Humanized xenobiotic response in mice expressing nuclear receptor SXR. *Nature*, **406**, 435–439.
- YEE, S., 1997, *In vitro* permeability across Caco-2 cells (colonic) can predict *in vivo* (small intestine) absorption in man – fact or myth. *Pharmaceutical Research*, **14**, 763–766.
- YOUNG, R. A., 2000, Biomedical discovery with DNA arrays. *Cell*, **102**, 9–15.

High-throughput screening approaches for investigating drug metabolism and pharmacokinetics

S. A. ROBERTS*

Drug Metabolism and Pharmacokinetics, Celltech Research and Development Ltd,
Granta Park, Great Abington, Cambridge CB1 6GS, UK

Received 10 March 2001

1. High-throughput screening approaches have been adopted throughout the pharmaceutical industry to aid in the rapid discovery of new chemical entities. Because it is now well recognized that the selection of a robust candidate requires a balance of potency, safety and pharmacokinetics, the role of drug metabolism departments has widened from their traditional one of supporting drug development to include the screening of compounds during the discovery process. To put drug metabolism and pharmacokinetic (DMPK) studies in context, the evolving role of DMPK screening in the drug discovery strategy of pharmaceutical companies will be discussed and a generalized approach will be presented.

2. With the increasing numbers of compounds requiring screening, DMPK optimization methods have had to be adapted for high throughput. There have been many developments in this field over the past decade and this review will focus on the high-throughput DMPK screening methodologies used today and in the recent past.

3. *In vitro* and *in silico* (computer-based) methods have proven most amenable to high-throughput approaches and these will form the bulk of the review, but some advances with *in vivo* methods will also be discussed. As there has been a vast increase in published material on the topic of high-throughput DMPK methodologies in the past 10 years, it would be impossible to cover every method in detail, so this review will concentrate on the key areas and refer the reader to other, more detailed reviews wherever possible.

4. Most high-throughput methods would not be possible without the enabling technologies of computing, automation, new sample preparation technologies, and highly sensitive and selective detection systems, and these will also be reviewed.

5. The advantages and disadvantages of the screening methods will be presented, in particular the issue of handling the false-positives and -negatives that arise.

6. In concluding the review, future developments in this field will be discussed along with key issues that will need to be addressed.

Introduction

During the last 10 years, there has been great pressure for drug metabolism departments to reorganise themselves for the rapid evaluation of increasing numbers of compounds. This change has been driven both by developments in chemistry, molecular biology, automation and analytical technologies, and because of the concern of the industry that too few compounds are entering development. It has been assumed that if more compounds are screened then more will 'make the grade' and companies have been busy throughout the 1990s putting in place

* Author for correspondence; e-mail: simon.roberts@celltechgroup.com

systems to cope with larger numbers of compounds. Combinatorial chemistry synthesis has been important in many companies for producing greater numbers of compounds while molecular biology has helped in the elucidation of many new target proteins that can be expressed *in vitro* and used in high-throughput screening assays. The development of robots and new analytical technologies has also been a very important factor in enabling us to test more compounds in ever-shorter times.

When trying to identify a good orally active candidate drug for development, it is important that it has a suitable oral bioavailability, metabolic stability and duration of action but, historically, screening methods usually concentrated on maximizing potency against a biological target and compounds were selected mainly on this basis. Limited drug metabolism and pharmacokinetic (DMPK) studies were conducted before safety evaluation and subsequently attrition rates were high often because of poor pharmacokinetics (Prentis *et al.* 1988). In addition, much additional time and resources were spent investigating and trying to remedy the problems of less than ideal candidates once they had entered development. It is now well recognized that the selection of a robust candidate requires a balance of potency, safety and pharmacokinetics, and that these characteristics should be screened for early in the discovery process. Initially DMPK studies were used in a low-throughput manner, but now with the increasing numbers of compounds requiring screening, physicochemical, DMPK and toxicity optimization methods have had to be adapted for high-throughput. *In vitro* and *in silico* (computer-based) methods are most suited to high-throughput approaches but some advances have also been made with *in vivo* methods.

Although the term 'high-throughput screening' will be used throughout this review in respect of DMPK methodologies, it is perhaps a little misleading at present since truly high-throughput biology activity screens may achieve rates of up to 100 000 compounds per week while methods currently in use in DMPK have considerably lower throughputs (Caldwell 2000). There have been many developments in this field over the past decade and this review will focus on the high-throughput DMPK screening methodologies used today and in the recent past to help generate and select robust compounds suitable for entering development. In addition, the evolving role of high-throughput DMPK screening in the drug discovery strategy of pharmaceutical companies will be discussed, as will the likely future developments in this field.

Drug discovery strategies

In the last decade, the traditional focus of drug metabolism departments to support drug development widened to include the support of drug discovery as it became clear that the selection of candidates with suitable DMPK characteristics was critical to their success. Since the average cost to discover and develop a new drug runs into hundreds of millions of dollars, any process that might lead to the rapid selection of more robust candidates was welcome. Many investigational new drug (IND) applications fail during preclinical and clinical development and recently Kennedy (1997) estimated that 46% of compounds entering clinical development were dropped due to unacceptable efficacy and 40% due to safety reasons. Caldwell (2000) believed that understanding human PK, e.g. oral bio-

availability, half-life, metabolites, drug plasma protein binding, etc., at the drug-discovery phase would be most valuable in reducing new chemical entity (NCE) failures in preclinical and clinical development since efficacy and safety issues are related in part to PK problems. With larger numbers of compounds now requiring testing it is the aim of most companies to screen molecules wherever possible in a high-throughput manner using meaningful, validated screens. Automated strategies have thus been developed to obtain DMPK parameters rapidly on a large number of compounds.

The process of drug discovery can be divided into a number of distinct phases: 'hit generation', 'lead generation' and 'lead optimization'. The goal of the hit generation step is to screen large compound libraries to find molecules with a specific biological response, usually an *in vitro* potency. Ideally a hit should have reproducible activity in a relevant bioassay, a confirmed structure and high purity, specificity data should have been generated and it should have a chemically tractable structure and a potential for novelty (Michne 1996). However, it is often debated whether the generation of *in vitro* DMPK data at this phase is particularly useful or cost efficient. At the lead generation step, the main aim of medicinal chemistry is to apply structure-activity relationships (SAR) to improve upon these hits. Typically, screening paradigms will include a DMPK evaluation of compounds in the lead optimization phase, where some degree of potency has already been achieved. Although for some projects this will mean that there are fewer compounds to evaluate and high-throughput methods will not be required, the potential for high-throughput DMPK screens will still exist for the most productive of projects. Figure 1 shows the phases in drug discovery and the iterative nature of the process with biology and DMPK information being fed back to the medicinal chemists to guide each new round of synthesis. It is very much a generalization and does not attempt to show all the types of study performed at each phase; however, it is useful in illustrating where the *in silico*, *in vitro* and *in vivo* DMPK methods tend to have the greatest impact.

DMPK, pharmacology and toxicology selection criteria can be included in the hit generation phase but ultrahigh-throughput *in vitro* methods would be required for assaying large compound libraries. Tarbit and Berman (1998) argued that radical thinking is still needed to derive a truly high-throughput screening process that will provide the metabolic information discovery chemists need in the design of their libraries. A possible disadvantage of mass DMPK screening at the hit generation stage is that compounds demonstrating good potency may be abandoned in favour of structures less amenable to later optimization. Similarly, although high-throughput DMPK screening methods can also be used to aid in the selection of leads, pharmacological activity and selectivity are paramount and care should be taken not to reject promising compounds purely on the basis of poor DMPK. It should be remembered that it is unlikely that a hit or lead compound will have all the required characteristics and it is during the lead optimization phase that these issues are usually best addressed. Typically, the highest throughput methods applied by most companies at the hit and lead generation phases are *in silico* methods.

The starting lead template is most important for effective optimization and these compounds should be studied in greatest detail to decide what characteristics require improvement. Guidance to the future direction of synthesis can then be given and compounds arising from this, screened *in vitro* and *in vivo* to optimize

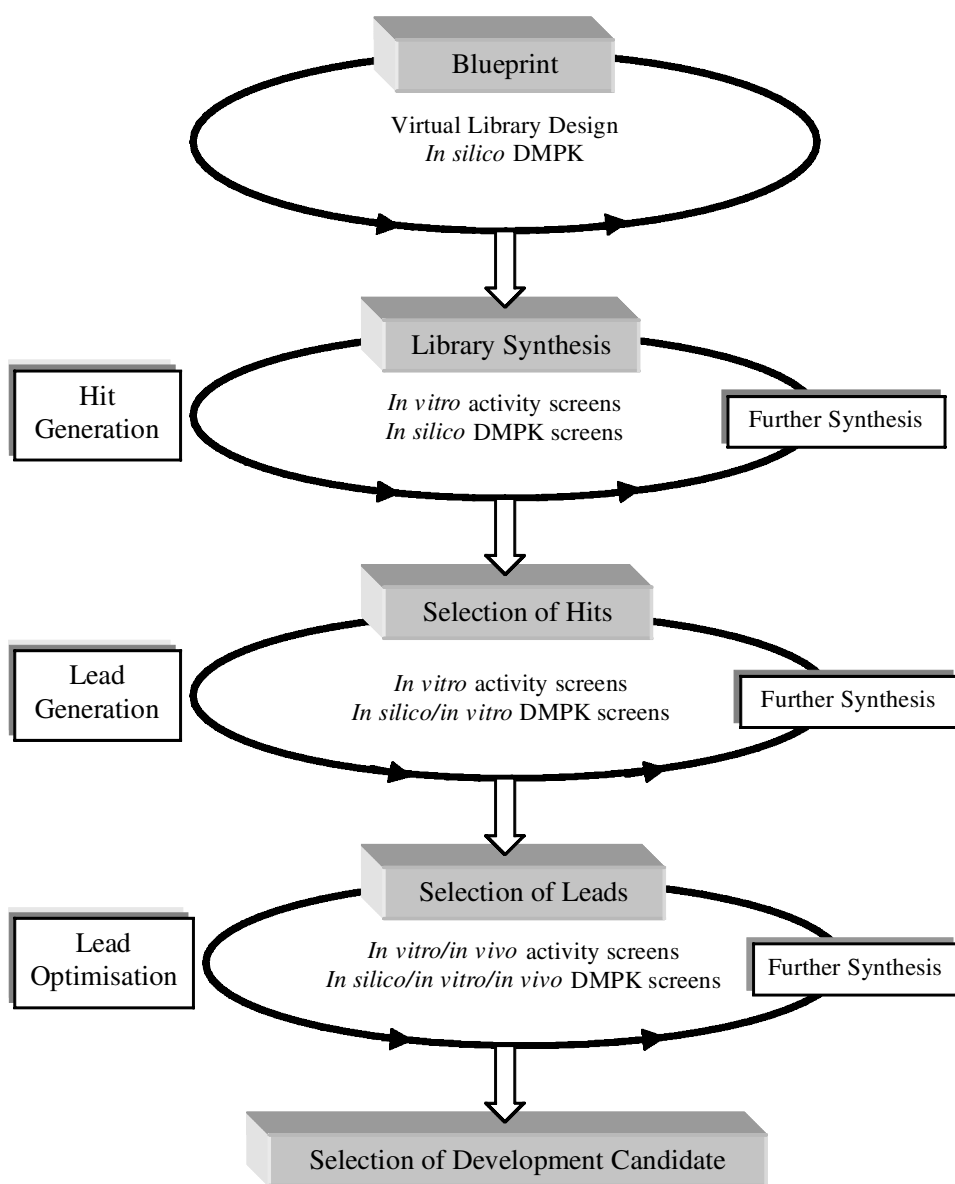


Figure 1. Simplified schematic showing the phases and iterative steps in the drug discovery process.

DMPK, efficacy and safety characteristics. The best compounds arising from each cycle of this optimization process can then be used to advise further on the next round of synthesis and so on until a suitable candidate is selected. Typically, in the lead optimization phase there are issues concerning systemic exposure and the potential for drug–drug interactions. *In vitro* assays are often used as a first-line screen followed by low-throughput *in vivo* models. Usually, *in vivo* PK studies are conducted before *in vivo* efficacy testing. Figure 2 shows the iterative nature of the DMPK activities involved in the ‘lead optimization’ process. Again, as with figure 1, it is very much a generalization of the process and does not attempt to show all

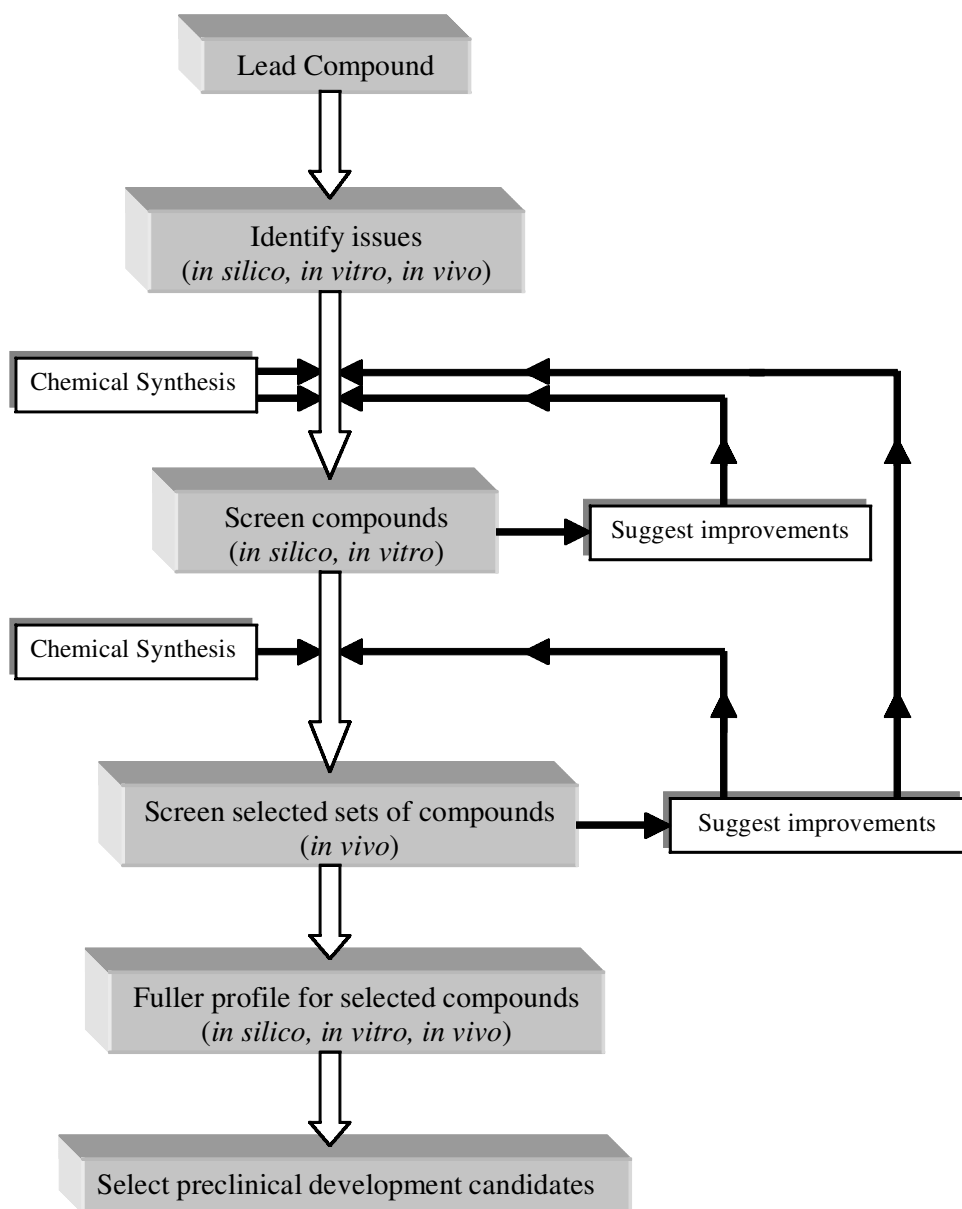


Figure 2. Simplified schematic showing the stages and iterative steps in the lead optimization process from a DMPK perspective.

the types of study performed at each phase. Clearly, parallel investigations of biological activity and other screening will occur alongside the DMPK studies but figure 2 serves to illustrate how *in silico*, *in vitro* and *in vivo* DMPK screens are used to guide the chemical synthesis at each stage.

The number of compounds requiring testing at any particular stage in the discovery process will be one of the main deciding factors as to when a high-throughput screening process is best applied. Low-throughput methods are still

likely in many cases to provide data in the required timeframe on issues relevant to the specific project. As Eddershaw *et al.* (2000) stated, the deployment of available *in vivo* and *in vitro* methods must be based on a sound understanding of the relevant absorption, distribution, metabolism and excretion (ADME)/PK issues, as they affect the desired product profile and not simply on the capacity to process large numbers of compounds.

As the time it takes for a compound to reach Phase II human trials from the initiation of a typical project is usually more than 5 years it is evident that anything that can be done to speed up the discovery process could help selected compounds reach the market sooner and thus give an earlier return on a company's investment. In the discovery process, the lead optimization phase often takes most time (typically 2–3 years) and the application of rational high-throughput screening during this phase certainly has the potential to speed up the process. However, it can be argued that applying good *in silico* models at the hit and lead generation phases should ultimately result in the selection of better quality leads and mean a reduction in the time required for optimization.

The importance of rational high-throughput screening as opposed to random screening is highlighted by Lipinski *et al.* (1997), i.e. that if pharmacokinetic properties are not incorporated into the design of molecules then the screening will lead to the largest and most lipophilic analogues being selected if activity alone is used as a selection criteria. The design of compound libraries at the outset with suitable properties should lead to a higher probability that compounds screened will have good PK. Lipinski *et al.* (1997) proposed rules now employed in one form or another by most companies, namely that to design compounds with good intestinal absorption compounds should be excluded with a molecular weight > 500, $\log P > 5$ and more than five H-bond donors and 10 H-bond acceptors. However, as there are many good marketed drugs available that do not satisfy the Lipinski rules, it would seem apparent that there are other useful 'drug-like' features that could be designed into libraries to maximize the chance of selecting good candidates. Designing libraries with favourable DMPK characteristics help improve the chances of having a good compound to select but once screening begins one needs to apply the most appropriate screens at the right times to identify what characteristics need modifying and when. Navia and Chaturvedi (1996) proposed that the clearance of new chemical entities should be examined first and then once an analogue with reasonable clearance is obtained, subsequent modifications in the physicochemical determinants of drug absorption can be manipulated. Of course, as Smith (1998) pointed out, one of the possible consequences of designing libraries with favourable PK parameters in advance of screening is that this may ultimately reduce the need for such screens to be high-throughput.

It is important to remember that companies often vary their screening approach depending on the therapeutic area of interest, productivity of their medicinal chemistry departments and the accessibility to compound libraries. Those with access to smaller libraries and less medicinal chemistry support often concentrate more on small-scale rational drug design and screening, while those with larger libraries usually utilize high-throughput screening wherever appropriate. It is the companies that have produced the larger numbers of compounds and screened many of them over the last few years that are likely to have the fullest and most useful databases. On this point, Eddershaw *et al.* (2000)

believed that ADME/PK screening had dramatically increased the size and quality of the database from which to gain a perspective on function such as metabolic stability, absorption and enzymology, and stated that advances made in understanding the principles underlying these processes through the development of *in silico* methods were enhancing the conceptual nature of ADME/PK and might lead ultimately to a truly rational *in cerebro* approach to drug design.

How a drug metabolism department applies high-throughput screening methodologies during its discovery process is therefore as important if not more so than the methods themselves. In the most productive of projects, assuming that the compounds made have been designed to have suitable DMPK, potency and safety characteristics, it is imperative that high-throughput screening methodologies are in place to keep pace with demand so that DMPK does not become a 'bottleneck'. Nevertheless, it should not be viewed as a 'numbers game' and such techniques should only be used if they can truly aid in the drug-selection process. In many instances, low-throughput methodologies may still be sufficient. Even when high-throughput assays are available, one should always question how useful the screens are and apply them appropriately. For instance, hepatic microsomes may not always be appropriate for metabolism screening if cytosolic or other non-microsomal enzymes are important in the clearance of the compounds being tested. Also, as some cytochrome P450s (CYPs) are more clinically relevant than others such as CYP3A4 (the most abundant form in human liver), CYP1A2, CYP2C9 and the polymorphically expressed CYP2D6 and CYP2C19 it would be logical to have screens for these available in advance of screens for other CYPs. Sometimes screens may not give you much extra useful information as in the case of P-glycoprotein (P-gp) and CYP3A4 screens which because of overlapping substrate and inhibitor specificities can lead to the same 'problem' compounds being highlighted.

In summary, high-throughput DMPK screening can have a very important role to play in a company's drug discovery strategy and when applied within a rational framework should prevent DMPK becoming a bottleneck. *In vitro* and *in vivo* DMPK methods continue to be of great use during the lead optimization phase, but *in silico* methods are becoming increasingly important both in the lead optimization and earlier hit and lead generation phases. High-throughput *in silico* and *in vitro* approaches should be validated wherever possible against *in vivo* data and applied when and where they will have the greatest impact. However, it should be remembered that high-throughput approaches do not always need to be applied particularly when low-throughput *in vitro* and *in vivo* methods are considered more predictive or where DMPK is not the rate-limiting step.

***In vivo* screening methodologies**

Despite the utility of *in vitro* models, *in vivo* DMPK studies are still an intrinsic part of the lead optimization process in drug discovery. The approaches selected will depend very much on the issues arising but common to many projects is the problem of achieving high enough systemic levels for sufficient periods to achieve appropriate efficacy. Poor bioavailability following an oral delivery can be due to poor absorption across the intestinal mucosa, gut first-pass metabolism and/or P-gp-mediated efflux or hepatic first-pass metabolism and/or biliary secretion. In addition to oral bioavailability, plasma elimination half-life, clearance and

volume of distribution are all useful parameters that can be generated from PK profiles following oral and intravenous dosing. Shortcomings of a lead identified in this way can help steer optimization in the right direction. However, *in vivo* methods are generally low-throughput because of the numbers of compounds that can be dosed in unit time and the time required for analytical method development. Developments in analytical instrumentation particularly mass spectrometry and software can improve the throughput as can the use of 'cassette' or 'n-in-one' dosing protocols (Berman *et al.* 1997). Cassette dosing in particular, where multiple compounds (usually about 10) are simultaneously administered to single animals, can be used to generate PK profiles more rapidly from fewer animals. Although such methods run the risk of interactions between compounds and saturation and inhibition effects, it has been found that keeping the individual doses low minimizes such possibilities. Bayliss and Frick (1999) described a strategy for cassette design that aimed to minimize the chance of obtaining false data and included keeping doses low and adding a reference compound to the cassettes. A variation on this theme is where compounds are dosed individually but combined in advance of analysis (sample pooling) thus saving on analytical time assuming the appropriate analytical methods and software are available (Olah *et al.* 1997, Hop *et al.* 1998). Another method (Cox *et al.* 1999) involves pooling of plasma samples over a 6-h time-course for single compounds, so that only one sample/compound need be analysed to give an approximation of the total AUC. The advantage of this method is that there are no concerns of drug interactions or method development.

Another important development in the last decade has been the increased use of venous and artery cannulated animals which has provided for more consistent data than was obtained from multi-animal studies and in the process led to a reduction in the number of animals used. Using systemic and portal vein sampling, additional information on absorption and hepatic extraction can be obtained from the same animals (Sawai *et al.* 1998). Some researchers have attempted to semi-automate the sampling aspects of such studies. The Culex system (Bioanalytical Systems, Cheshire, UK) is a series of instruments that works together to remove blood via indwelling cannulae, dispense it into sealed refrigerated vials and replace blood removed with an equal volume of saline. Simultaneously, animal activity is monitored to check the health of the animal. Such a system frees the workers from having to remove samples manually. There are also a number of *in situ* models that have been developed for screening compounds (Stewart *et al.* 1997) and in fact Rosenberg *et al.* (1993) details a higher throughput *in situ* model that utilizes intraduodenal administration in rat.

The use of transgenic animals to investigate DMPK and toxicological issues, although not likely to be amenable to high-throughput screening, does offer potential for speeding up the drug discovery process. Using this approach human gene sequences responsible for human drug-metabolizing enzymes can be used to replace endogenous genes in test animals. This is a relatively new science but models have been detailed (Wolf and Henderson 1998), although the introduction of specific human CYPs sometimes has no effect on metabolic disposition of a drug or toxin because of background activity in the animal. Also a number of gene 'knockout' animals exist that can be used for drug screening albeit low-throughput, in particular, ones without the genes encoding P-gp (mouse

lacking *mdr1a*) and these have been used to investigate the role of the transporter in limiting oral absorption (Kim *et al.* 1998).

In vivo PK screening is also a good adjunct to *in vitro* assays when they appear to be poorly predictive of *in vivo* kinetics as, for instance, when the compound is eliminated predominantly by renal or biliary mechanisms (Bajpai and Adkison 2000). In addition, *in vivo* models can be used to help develop and validate predictive models that utilize *in vitro* data and involve *in silico* modelling (Tarbit and Berman 1998, Bayliss and Frick 1999). No lead optimization strategy can ignore the utility of *in vivo* models and it is anticipated that however elaborate or predictive the *in vitro/in silico* approach becomes, *in vivo* data will be required for some time to confirm the findings or validate the approach.

***In vitro* screening methodologies**

In vivo studies are not always ideal for pinpointing what the main issue is since PK parameters such as bioavailability are themselves based on a number of variables. Some of the key *in vitro* models thus attempt to predict aspects of drug absorption and metabolism.

Absorption models

There are many *in vitro* methods that have been developed for screening compounds (Lee *et al.* 1997, Stewart *et al.* 1997) but it is the cell-based methods that are most amenable to high-throughput screening (Sinko 1999). The most commonly used absorption model is the 21-day Caco-2 human intestinal cell line that has proven useful in compound ranking and has been shown to be a good predictor of *in vivo* human intestinal absorption (Artursson and Karlsson 1991). It should be noted though that while a generally reliable indicator of well-absorbed compounds the interpretation of low and intermediate permeability is not as clear. Some low molecular weight water-soluble compounds often show low permeability *in vitro* yet are well absorbed *in vivo* because paracellular passage and certain active transport mechanisms are not modelled well (Delie and Rubas 1997).

Studies with Caco-2 cells can be time consuming and even with some degree of automation are not truly high throughput. Some improvements on the throughput have been gained by using the 3-day Caco-2 culture model (Chong *et al.* 1997) although these monolayers have been shown to be ~4–6-fold leakier than the traditional 21-day cultures. An alternative cell culture used by a number of companies is the 3-day Madin–Darby canine kidney (MDCK) cell line, which has shown little difference in compound ranking and correlates well with absorption *in vivo* and *in vitro* in Caco-2 cultures (Irvine *et al.* 1999). Other cell lines used to study absorption include the HT29 colon carcinoma cell line and for blood–brain barrier penetration, the bovine brain microvessel endothelial cell (Chikhale *et al.* 1994). Another limitation to throughput has been the fact that such cell culture-based screens utilize plates containing small numbers of well membrane devices (6, 12 or 24), but a recent development of a cells-on-sheets (COS) device by Affymax Research Institute (Santa Clara, CA, USA) means that monolayers can be prepared and used simultaneously in a larger number of wells (Takahashi 2000). A number of other approaches are being looked at by companies to improve throughput further including increased automation and the pooling of samples

into cassettes (McCarthy *et al.* 1996). One novel approach has been to use a genetically engineered Caco-2 variant that expresses high levels of CYP3A4 to allow the simultaneous screening of gut permeability and metabolism (Crespi *et al.* 1996).

Transporter models

In recent years there has been a growth of interest in the role of transporters in drug absorption and in particular P-gp. Yamazaki *et al.* (1996), Kim (2000) and Morgan (2001) have provided informative reviews of the more important transporters and their contribution to the disposition of drugs. The heterologous expression of transporter molecules *in vitro* is likely to lead to the proliferation of useful screens in the future. Already, cell systems exist (Caco-2, L-MDR1) that express P-gp and are suitable for the high-throughput identification of drugs that are likely to have reduced or variable oral bioavailability (Doppenschmitt *et al.* 1999, Kim 2000). Also available are assays utilizing baculovirus infected insect cells expressing human P-gp (MDR1) in a 96-well plate format (Gentest Corporation, Woburn, MA, USA). Analytical methodologies are also important for improving throughput and will be reviewed in more detail in the section on 'enabling technologies', but ones worth mentioning here are those of Doppenschmitt *et al.* (1999), who used a radioligand-binding assay, and Sharom *et al.* (1999), who described a method for measuring the intracellular accumulation of a fluorescent substrate in intact cells, reconstituted systems and isolated membranes. Stable cell lines expressing high levels of other important uptake transporters such as organic anion transporter proteins (OATP) are now being prepared using cloning and cell transfection techniques. Methods utilizing membrane vesicles (Kobayashi *et al.* 1990) and isolated hepatocytes (Hollenberg and Scarpelli 1988) have been available for some years. More recent applications of these techniques that could lend themselves to high-throughput screening include those of Niinuma *et al.* (1999) and Sugiyama and Suzuki (2000) who have employed isolated bile cannicular membranes to investigate organic anion transporters, and Shitara *et al.* (2000) who have performed isolated hepatocyte studies in the presence and absence of specific inhibitors of OATP. Another recent development has been the design of sandwich-cultured hepatocytes to maintain functioning bile canaliculi for the investigation of P-gp-mediated biliary excretion (Annaert *et al.* 2000). Efflux systems are of course present in many tissues of the body including the intestine, liver, kidney and brain (Kim 2000). For CNS drugs in particular, it is very important to know if a compound is a substrate for transporters present in the blood-brain barrier. For a review of this topic and available models for investigating blood-brain transporters, see Kasuhara and Sugiyami (2001).

Artificial membrane models

Artificial membrane systems that endeavour to mimic mucosal lipid membranes also exist. Immobilized artificial membrane (IAM) chromatography has been shown to give a fair approximation of both Caco-2 permeability and *in vivo* intestinal absorption (Pidgion *et al.* 1995, Camenisch *et al.* 1997), although throughput for well-absorbed compounds is generally low because of the longer retention times. A method that utilizes artificial phospholipid bilayers on filters in

96-well plates (the parallel artificial membrane permeation assay) and relies on UV absorbance for quantification is capable of a far higher throughput (Kansy *et al.* 1998) and is currently available commercially as the PSR4pTM through Sirius Analytical Instruments Ltd (Forest Row, UK). However, like Caco-2 cells, these methods may give misleading estimates of the absorption of some types of compounds that undergo active or paracellular transport.

Determination of physicochemical parameters

A number of models also exist for predicting absorption based on the utilization of physicochemical data such as $\log P$, pK_a and solubility calculated from molecular structure, determined experimentally or combined with physiological parameters (physiologically-based pharmacokinetic models) and these will be discussed in more detail in the section on '*in silico* models'. The strategy for measuring pK_a and $\log P$ is determined by the solubility of a compound since it must be soluble and stable during any procedure to ensure equilibrium is maintained (Earll 1999). Earll provides a good review of the methods available for measuring $\log P$ and pK_a . One of the more popular and straightforward of the methods is the Sirius potentiometric one based on aqueous titration and utilizing the PCA101TM and GLpKaTM $pK_a/\log P$ analysers. Though still generally low-throughput, the newer GLpKaTM has an autosampler and can also do multiple titrations on each sample. A recent development by Sirius for use with the GLpKaTM is the D-PASTM probe that enables higher throughput spectrophotometric determinations of pK_a 's to be made (up to 30 in 24 h). However, perhaps the fastest method they have developed to date is a fully automated microplate format spectrophotometric technique for measuring pK_a called the Sirius Profiler SGATM, which relies on the change in UV spectra at different pHs and is capable of measuring pK_a at a rate of 2 min per sample (Box 2000).

Solubility can be measured using a number of generally low-throughput methods. The 'traditional' shake flask method involves shaking the compound in the solvent for 24 h, filtering and determining the concentration and repeating this until a constant value is achieved. A modified version of this method (pSOL by Sirius) does not require one to wait for equilibrium to be achieved and is faster but still throughput is low, generally just a few compounds per person per day. Nephelometry, which involves the measurement of the intensity of scattered light in a suspension and enables you to detect the exact point at which a solute dissolves, offers the best opportunity for a high-throughput method. Bevan and Lloyd (2000) presented a high-throughput method using laser nephelometry in 96-well microtitre plates that allow the rapid determination of solubility for compounds supplied as DMSO solutions. The latter authors compared this method with HPLC-based methods and concluded that drug solubility could be determined as accurately but a hundred times quicker using laser nephelometry. However, false-positives are possible with such procedures because imperfections on the base of plates or precipitation on the sides of wells can cause scattering of light.

Drug dissolution in particular, although one of the most important characteristics of an orally administered solid is often overlooked during early screening in favour of solubility assessments but dissolution profiles over time or as a function of pH in association with intestinal permeability data are generally a better

indicator of drug absorption than just inherent solubility. Conventional methods for determining drug dissolution are not generally amenable to high-throughput although some miniaturization of these methods has led to increases in throughput (Bertrand 2000).

Metabolic stability models

The presence or absence of first-pass intestinal or liver metabolism is of great importance in determining *in vivo* oral bioavailability. The greatest attention has been paid to the functionalization or Phase I reactions, in particular the oxidative metabolic reactions carried out by cytochrome P450s. CYP-catalysed reactions are often the main reason for drugs failing to make it to the market (Bajpai and Adkison 2000).

A number of models have been developed to examine the metabolic stability of compounds including liver slices, hepatocyte cultures and subcellular fractions, e.g. S9 and microsomes, but it is the hepatic microsomal model that is the simplest and most suited to high-throughput screening. In particular, the 96-well plate technique with automation has played an important part in providing this high-throughput (Korfmacher *et al.* 1999, Nordyke *et al.* 2000). Also usable in a high-throughput manner are recombinant human P450s (Crespi 1999a) and Ghosal *et al.* (2000) highlighted the advantages of microtitre plate fluorescent assays for the determination of activities of 13 recombinant human P450s. An interesting development for utilizing hepatic microsomes in a 96-well format has been the use of the oxygen biosensor fluorescence system (BD Biosciences, San Jose, CA, USA) to measure the rate of oxygen consumption during cytochrome P450 enzymatic reactions (Dike *et al.* 2000). An ultrafiltration technique has also been detailed (van Breemen 1998) where liver microsomal incubations are conducted in an apparatus continuously infused with buffer and electrospray mass spectrometry is used for the analysis of metabolites. Up to 60 incubations an hour can be performed with this system.

Bertrand *et al.* (2000) described a more detailed method in hepatic microsomes suitable for providing apparent K_m and V_m determinations for the overall metabolism of a drug based on computer fitting of *in vitro* disappearance curves using the Michaelis–Menten integrated equation. With automation, the use of cassette LC-MS/MS analyses and utilizing one very low substrate concentration to minimize the risk of metabolic saturation, the high-throughput determination of intrinsic clearance (Cl_{int}) is possible and a rank order of *in vitro* rates of metabolism can be obtained. However, it is important to check predictions of *in vivo* clearances from these *in vitro* data with actual *in vivo* data to see if the correlation is good within a series and whether the screen is valid. Importantly, predictions of *in vivo* bioavailability can be done using Cl_{int} (Carlile *et al.* 1997, Iwatsubo *et al.* 1997) and the results used by medicinal chemists to help in drug design; however, due to inaccuracies in absolute values it is often better simply to classify compounds as low, medium or high extraction. For a fuller discussion of the methods used for predicting human pharmacokinetics, see Obach *et al.* (1997), which examines several methods by which human pharmacokinetic parameters can be predicted from *in vitro* metabolism and/or preclinical pharmacokinetic data. Drawbacks of using liver microsomes include the lack of some enzymes, particularly those catalysing the Phase II reactions and the variations in enzyme content dependent

on preparation techniques, source and enzyme decay rates. Freshly isolated hepatocytes can offer a solution but their preparation and the sourcing of human liver can reduce their potential for use in high-throughput testing systems. Cryopreservation is one means of providing a regular supply of hepatocytes for screening purposes and Li (1999) reported on a 96-well hepatocyte method where activities of both Phase I and II enzymes have been well preserved.

According to Bertrand *et al.* (2000), it is doubtful that the determination of the extent of metabolism at the earliest phases has any other interest than to fill databases, even if this approach may be valuable for a few compounds to help the chemist apply metabolic stabilizing modifications. However, the latter authors go on to say that screening for potentially toxic metabolite intermediates could be very useful and highlight one useful high-throughput approach that utilizes glutamyl-cysteinylglycine (GSH) to trap reactive metabolite intermediates and LC-MS/MS to detect any resulting GSH-adducts.

Early on it was recognized that high-throughput could not be achieved when methods of analysis had to be generated for each individual compound so 'generic' methods were developed, typically LC-MS/MS and fluorescence assays (Crespi 1999a). The analytical methodologies applied can thus have a major influence on the throughput of *in vitro* screening systems and these will be discussed in more detail in the section on 'enabling technologies'.

Enzyme inhibition models

Inhibitory drug-drug interactions have led to issues with many marketed drugs and the withdrawal of some (Mullins *et al.* 1998). The screening for inhibition should therefore be an essential part of the discovery process. Enzyme inhibition lends itself to high-throughput formats because the same response is measured for each test compound in the same way that a single change is monitored in high-throughput biology screens. Many enzymic reactions can be inhibited but it is the CYP-mediated interactions that are considered most important (Lin and Lu 1998). CYP inhibition studies have typically been conducted using liver microsomes from humans and various other animal species with analysis of samples using HPLC with UV or fluorescence detection. The utilization of 96-cell plate formats with solid-phase extraction and pooling of samples for LC-MS/MS quantification (Ayrton *et al.* 1998, Yin *et al.* 2000), microtitre plate-based fluorometric assays (Crespi *et al.* 1997) and radiometric assays (Moody *et al.* 1999) have all been used to improve throughput. The fluorometric assays of Crespi *et al.* (1997, 1998) and Crespi (1999a) utilize single cytochrome P450 enzymes and represent a very rapid method for investigating the activities of the five most important drug metabolizing enzymes, namely CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A4. Favreau *et al.* (1999) presented a good comparison of a conventional dextromethorphan *O*-demethylase assay for CYP2D6 in human liver microsomes with a 96-well microtitre plate assay utilizing the recombinant baculovirus-infected insect cells expressing human CYP2D6 and concluded that this high-throughput method can produce data comparable with that of the conventional assay. However, they did point out that in the plate assay there was a risk of overestimating the IC₅₀s (false-positives) when longer incubations were used (45 instead of 10 min) and ~5% of the tested compounds proved to be false-negatives. Apart from hepatic

microsomes and recombinant cytochrome P450s, freshly prepared or cryopreserved hepatocytes can also be used in a higher throughput manner (Li *et al.* 1999).

An important distinction for cytochrome P450 screening and discussed in more detail in White (2000) is whether the observed inhibition is direct (reversible, non-covalent binding to enzyme), metabolism-based (reversible non-covalent binding of a metabolite product to enzyme tighter than parent) or mechanism-based (covalent binding to enzyme by a chemically reactive intermediate which inactivates the enzyme). Metabolism/mechanism-based inhibition increases with time while direct inhibition is constant or decreases with time so they can be distinguished experimentally in a high-throughput mode by determining the apparent IC_{50} of the test compound toward a standard substrate with or without a pre-incubation period of the enzyme with the test compound. A large decrease in the apparent IC_{50} after pre-incubation is evidence of metabolism/mechanism based inhibition. Typically, a compound is classified as potent if the IC_{50} is $> 1\mu M$. Single-point screening to predict IC_{50} s represent a means of further improving throughput. Johnson *et al.* (2000) described a single-point method for screening large numbers of structurally diverse compounds for their ability to interact with CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A4. The analytical methodologies available to measure CYP inhibition will be discussed further in the section on 'enabling technologies'.

Although the rapid estimation of the inhibitory potential of compounds is useful for the rank ordering of compounds, further characterization of compounds of interest should be done with CYP isoform-specific probe substrates to obtain definitive data to aid drug design and predict potential clinical implications for development candidates.

Enzyme induction models

The early identification of induction-based drug-drug interactions is also important during the selection of compounds. Although there are a number of inducible drug-metabolizing enzymes the cytochrome P450s are particularly important both because of their involvement in drug interactions and also because some can have carcinogenic effects. Traditionally, compounds were repeat-dosed in animals early in the development process to check for induction but this was not always predictive of human induction. A number of *in vitro* methods are available to investigate cytochrome P450 induction including liver slices, hepatocytes and cell-based gene reporter constructs. Perhaps the most commonly used *in vitro* induction method utilizes hepatocyte cultures and a number of papers exist on the technique (Langouët *et al.* 1997, Li *et al.* 1997, Silva *et al.* 1998, Madan *et al.* 1999). Primary cultures of fresh or cryopreserved hepatocytes are generally used but because of their more ready availability, cryopreserved human hepatocytes would appear to be most convenient for using in regular screening regimens. A detailed review of the utility of cryopreserved hepatocytes by Hengstler *et al.* (2000) described the use of these enzyme induction studies but noted that the technique requires further optimization. In spite of the distinct advantage of being able to look at human induction, these methods are still generally low-throughput but they are never the less proving very useful in the screening of key compounds arising from lead optimization. It should be borne in mind though that if fresh human liver is required it is normally of limited availability and in addition there is

often a larger intersubject variability in response to inducers than would be seen with inbred animal strains. Cell-based gene reporter constructs expressing all or part of the CYP regulation machinery and incorporating a product that can be measured spectrophotometrically lend themselves to high-throughput induction screening (Ogg *et al.* 1999). Also of note is the role of the novel orphan receptor, the pregnane responsive nuclear receptor (PXR), in regulating CYP3A expression that has provided a means for the rapid assessment of human CYP3A induction in a cell-based assay (Lehmann *et al.* 1998, Moore and Kliever 2000). The analytical methodologies available to measure induction events are an intrinsic part of the process and will be discussed further in the section on 'enabling technologies'. For a more detailed review of P450 induction, see Worboys and Carlile (2001).

Plasma protein binding models

Plasma protein binding is an important factor affecting the drug disposition and potency of drugs. Qualitative and quantitative differences in binding proteins, primarily albumin and α 1-acid glycoprotein can have important effects as can the extent and affinity of binding. *In vitro* plasma protein binding assays are therefore important during the optimization phase of drug discovery. A number of techniques have been used for protein-binding measurement including equilibrium dialysis and ultrafiltration (Bowers *et al.* 1984), spectrofluorometric methods (Epps *et al.* 1995) and microdialysis (Herrera *et al.* 1990). Pacifici and Viani (1992) provided an extensive review that in addition to the main methods of ultrafiltration and equilibrium dialysis also detailed other lesser used methods such as gel filtration and circular dichroism. The spectrofluorometric method of Parikh *et al.* (2000) shows some promise as a high-throughput method since measurements can be carried out with small samples in multiwell plates and no separation of bound and unbound species is required since the method relies on the quenching of the intrinsic tryptophan fluorescence of serum albumin and α 1-acid glycoprotein on binding of the drug. Another high-throughput method of note is that described by Wring *et al.* (2000) that utilizes 96-well ultrafiltration plates (Microcon[®] -96 YM-10, Millipore Corporation, Danvers, MA, USA) and dual LC-MS/MS bioanalysis with fast-gradient chromatography. The method is automated with a Tecan Genesis robot (Model RSP 150, Reading, UK) and it is claimed that 40 compounds can be processed in each run (6 h ultrafiltration and 23 h bioanalysis). Gu *et al.* (1999) described a pulsed ultrafiltration method with LC-MS detection suitable for investigating human serum albumin binding. By using smaller pulsed ultrafiltration chambers and investigating the binding affinities of three compounds simultaneously, the authors claim that this method offers a higher throughput alternative to conventional methods. Also available is a method utilizing multiple equilibrium dialysis cells (Spectrum[®] 20-cell equilibrium dialyser, Spectrum Laboratories, Rancho Dominguez, CA, USA) that allows the simultaneous assessment of protein binding of at least 20 compounds (or more if compounds are assessed in cassettes).

Though not widely reported, serum albumin (or plasma) is sometimes added to *in vitro* efficacy screens to allow for plasma protein binding and to determine if efficacy is maintained in its presence. Potentially, such methods offer the opportunity to screen in a high-throughput manner directly in conventional high-throughput activity screens whilst taking account of the effects of binding.

However, an accurate simulation of *in vivo* plasma protein composition is difficult and data can be misleading.

***In silico* screening methodologies**

An important development in recent years has been the adoption of *in silico* (computer) methods to help design libraries of compounds with appropriate characteristics and screen out compounds at each stage in discovery that are unlikely to 'make the grade'. Virtual libraries can now be generated and tested in computer models using information on receptors, enzymes and pharmacophores (Walters *et al.* 1998). Computer-aided drug design (CADD) departments are taking an ever more active role in drug discovery in helping to design libraries with appropriate chemical, physical, pharmacokinetic and biological properties (Tropsha 2000). CADD and structure-based design techniques were previously seen as ways of achieving 'super-potency' but now have a more realistic role in obtaining acceptable primary activity while achieving suitable ADME properties. Although required characteristics may differ for each target, relationships and criteria for ADME properties can be built up and carried over from one series to another (Hyde and Hersey 1996).

Absorption models

The rapid and accurate prediction of physicochemical parameters has become intrinsic to the success of high-throughput *in silico* models. Many parameters can be generated automatically from chemical structure (H-bonding, molecular weight, $\log P$, polar surface area (PSA), solubility, etc.); however, the accuracy of some of these, particularly $\log P$, PSA and solubility is often debated. Lipinski's rules, as discussed above, can be calculated solely from molecular structure and are commonly incorporated into company compound databases so those molecules with potentially poor absorption are flagged up. Some companies have written their own modified version of these rules. Organon Pharmaceuticals has its 'Monika' rules where compounds are judged as likely to have good intestinal absorption if they have a molecular weight < 500 , $C \log P - 2 < x < 5$, rotatable bonds < 12 , H-bond donors < 6 , H-bond acceptors < 11 and PSA < 110 . For CNS penetration, they recommend $C \log P 0 < x < 5$ and PSA < 60 (Kelder *et al.* 1999, Delbressine 2001). PSA, which in itself is based on the van der Waals surface area of all nitrogen and oxygen atoms plus their attached hydrogen atoms (Palm *et al.* 1997), has proven a very useful physicochemical parameter for predicting absorption. Companies have their own 'cut-off' values for PSA below which they will accept compounds but generally compounds possessing a value of between 40 and 80 are likely to be well absorbed. Clark (1999a) presented comparisons of methods for calculating PSA and showed that values can be calculated accurately and more rapidly from a single minimized structure. Solubility is also an important parameter and Ruelle and Kesselring (1998) described a universal solubility equation based on mobile order thermodynamics and applied it to the prediction of the solubility of diverse organic compounds but it does require some experimentally determined crystallinity parameters. Abraham and Le (1999) described a solubility prediction model based on solvatochromic parameters, descriptors thought to be important for molecular interactions in solution, calculated using

an amended linear solvation energy relationship (LSER) equation. Platts (2000) developed a rapid automated empirical fragment-based method for establishing LSER descriptors suitable for solubility predictions. Models of solubility also exist based on electrotopological state indices that are a measure of the electron accessibility of atoms in a molecule (Huuskonen *et al.* 2000). A number of other methods exist that utilize experimentally determined melting points and these are reviewed in Taskinen (2000). Linear regression and neural network methods can also be applied but the accuracy of these and other models of solubility are very dependent on the design of the training sets. Despite the advances in solubility prediction, Taskinen (2000) said that he could not yet recommend any of the published models as reliable tools in drug design.

A combination of physicochemical parameters has been used in models for predicting intestinal absorption. Van de Waterbeemd *et al.* (1996) constructed a series of linear models containing H-bonding and molecular weight terms, and Wessel *et al.* (1998) used a neural network approach with a genetic algorithm for descriptor selection to predict quantitatively human intestinal absorption. Complex multivariate models also exist (Camenisch *et al.* 1998). Computational modelling techniques to determine structural features necessary for the desired activity have much potential and some of the more common ones used have been compared by Tominaga (1999) and include artificial neural nets (ANN), partial least-squares (PLS) and soft independent modelling by class analogy (SIMCA).

Blood-brain barrier penetration models have also been developed (Abraham *et al.* 1995, Norinder *et al.* 1998, George 1999) but the prediction can be problematic (Pardridge 1998). Clark (1999b) proposed a predictive equation for blood-brain barrier penetration that is more rapid for calculating values than the semi-empirical method of Norinder *et al.* (1998) and better suited to automation. Abraham (2000) described high-throughput methods for predicting blood-brain barrier distribution and other parameters such as water solubility and intestinal absorption for up to 500 molecules per min using his Abraham solvation descriptors calculated with the Daylight software (Mission Viejo, CA, USA). Crivori *et al.* (2000) also described a fully automated virtual model of blood-brain barrier penetration based on 3D molecular structures which they demonstrated could correctly predict blood-brain barrier permeation in 90% of the cases in their training set. There are also a number of models designed to predict plasma protein binding from physicochemical parameters such as lipophilicity, pK_a and molecular weight (Herman and Veng-Pederson 1994).

Physiologically-based pharmacokinetic (PBPK) models utilize physicochemical parameters as well as physiological parameters such as blood flow, gastrointestinal tract content flow rates and tissue volumes along with experimentally obtained data like tissue partition coefficients and Caco-2 permeabilities to try and predict the DMPK characteristics of test compounds. Although these models are not generally high-throughput and require some experimentally determined data, they can prove to be very useful (Leahy *et al.* 1996, Sinko *et al.* 1996, Nesterov *et al.* 1997). In fact, although the Lipinski rules provide a reasonable qualitative measure, they do not provide a quantitative measure of the PK properties of drug absorption. Grass (1997), for instance, describes a PBPK intestinal model of drug absorption that extrapolates from *in vitro* measured parameters and accounts for drug and fluid movement within the human gastrointestinal tract. More robust models have been subsequently developed by Grass *et al.* at NaviCyte, Inc. (a

subsidiary of Trega Biosciences, Inc., San Diego, CA, USA) through a consortium of pharmaceutical Companies (Sinko 1999), and the result of this, the iDEA predictive ADME simulation system, is now available commercially. The absorption module has been trained with a proprietary database of *in vitro* and human *in vivo* data for a diverse set of drugs and drug candidates including ones that failed in the development process. By inputting a series of measurements such as solubility, dissolution and Caco-2 permeability, the program can make *in vivo* absorption predictions. A module for metabolism (drug elimination) is now being produced and it is planned to develop additional modules for the complete prediction of bioavailability. Another commercially available program is GastroPlus™ (Simulations Plus, Inc., Lancaster, CA, USA), which can also be used to help predict compartmental absorption from physicochemical parameters and physiological parameters and in its latest form (v.2.0) also includes a pH-dependent chemical degradation model to handle compounds that chemically degrade in the gastrointestinal tract. There have also been attempts to integrate PBPK models with quantitative structure-activity relationships (QSARs) modelling for toxicity (Leahy *et al.* 1996, Yang *et al.* 1998).

Predictions of drug absorption often do not take account of the effect of transporters and understanding the substrate specificity of these and building this into models will undoubtedly be a major growth area in the next few years. P-gp is probably one of the most well known and best understood transporters of relevance to DMPK and computational models exist that can flag up particular structural features of molecules, i.e. the distance between hydrogen bond acceptors, that make them more likely to be substrates for P-gp (Seelig 1998). Structural features that help predict what compounds are most likely to bind to bile acid carriers (Stein and Hoshen 1996) or intestinal peptide carriers (Swann and Tukker 1997) are also known.

Metabolism models

Drug metabolism data accumulated over many years have been implemented into rule-based expert systems that can be used in the virtual metabolism screening of combinatorial libraries. A number of expert systems have been developed both for prediction of toxicity and metabolism (DEREK, TOPKAT, CASE, HAZARDEXPERT, COMPACT, META, METABOLEXPert) and these are detailed more fully in Marchant and Combes (1996). Hawkins (1999) also reviews metabolism databases and predictive software. Of particular note is the COMPACT system, developed by Lewis *et al.* (1990) at the University of Surrey that uses measurements of molecular shape and orbital energies to determine the likely cytochrome P450 specificity and the METABOLEXPert system, which has a list of rules describing the bonds that are broken and created in the metabolic step under consideration and the structural features that need to be present or not in the target structure for the transformation to occur. Another useful system, META, contains > 750 Phase I and II biotransformations based on substructures and quantum mechanical calculations, but although it has been shown to give excellent predictions on test data, in a recent study where it was used to predict the metabolism of 42 polycyclic hydrocarbons, it over-predicted 29 and missed eight important epoxidations and 27 of the observed hydroxylations (Klopman *et al.* 1999). In another approach, CEREP (Rueil-Malmaison, France) has been devel-

oping a predictive program called BioPrintTM, a structure-driven system that takes *in vitro* information and 'clusters' compounds in terms of a variety of properties (potency, selectivity, solubility, cytotoxicity, absorption, metabolism, etc.). A considerable number of structures and associated pharmacological, DMPK and toxicity data derived from known pharmaceuticals (either on the market or in the clinic) and privileged libraries exist within the system. Such an approach could be very successful as long as the data inputted about your own compounds (and those already in the system) have been generated in a consistent manner and in fact some companies already use 'clustering' in-house to help them select compounds suitable for *in vivo* DMPK and pharmacology testing.

Molecular (homology) models now exist for many key CYPs and have been applied to the design of better drugs (Halliday *et al.* 1997, de Groot *et al.* 1999). Models exist for CYPs 2C9, 2C19 and 2D6 (Lewis *et al.* 1997, 1998) and 3A4 (Szlarz and Halpert 1997). Groot *et al.* (1999) detailed a combined protein and pharmacophore model for CYP2D6 derived from various computational chemistry techniques that has been used successfully to explain/predict the involvement of CYP2D6 in hydroxylation, *O*-demethylation and *N*-dealkylation reactions. In homology modelling, the 3D structure of a mammalian P450 can be predicted by comparing its primary sequence with that of crystallized bacterial P450s. The assumption is that the tertiary fold of P450s are well conserved during evolution because haem-incorporation and interaction with redox-partners are common to all P450s (Commandeur 1999). Also, novel *ab initio* quantum mechanical modelling techniques, requiring only knowledge of the positions and atomic numbers of the atoms, can be applied to determine CYP interactions. Specifically, the spin state of an Fe³⁺ ion present in the haem moiety on the binding of a particular substrate is calculated to decide if the catalytic reaction will proceed (Segall *et al.* 1997).

Improving in silico predictions

Limitations to the design of computer-based predictive systems include deficiencies in the understanding of the relationships of structure to DMPK and the quality and size of the datasets used to build the models. One unfortunate result of trying to increase throughput has been that this has sometimes led to a concomitant reduction in the quality of data. With this in mind, it would appear eminently sensible that data generated using high-throughput screens are better used to generate a rank order than establishing definitive DMPK. Ideally, the models themselves should be based on definitive data and it is hoped that with improvements in the knowledge of DMPK and growing databases the predictivity of *in silico* models will improve.

Some workers have tried using statistical methods to improve the predictivity of *in silico* models. Egan *et al.* (2000), for instance, used human absorption and Caco-2 data to build a statistical pattern recognition model of passive intestinal absorption that they claimed gave good predictions of between 74 and 92% depending on the dataset and exact criterion used. Other models using statistical methods that have given good predictions of intestinal absorption include Norinder *et al.* (1999), who used partial least-squares (PLS) regression models utilizing MolSurf calculated descriptors of H-bonding, acidity/basicity, surface area and polarizability, and Winiwarter *et al.* (1998), who used a PLS model that

utilized *C* log *P*, H-bond donor number and PSA. More recently, the program VolSurf (Multivariate Infometric Analysis, Perugia, Italy) and multivariate statistics have been used to correlate 3D molecular structures with physicochemical and pharmacokinetic properties (Cruciani and Guba 2000). Active transport and efflux mechanisms are often accounted for in the model-building process but some are better than others at identifying actively transported compounds. Egan *et al.* (2000) claimed that robust outlier detection allows actively transported compounds to be successfully identified in his model.

The use of physicochemical data along with *in vitro* data can help in the development of predictive models, but because such models are usually only as good as the data used to generate them, they may only be reasonably predictive for a specific chemical class. Pagliari *et al.* (1999) believed that no reliable, universal predictive model for drug absorption existed since all too often correlations break down when structural diversity is introduced. Having extensively reviewed the literature on models for predicting drug permeation, Pagliari *et al.* claimed that many seemingly general models published in the literature are in fact statistically unsound and misleading because of overfitting or hidden intercorrelation between 'independent' variables, or both.

Darvas *et al.* (2000) proposed a means for enhancing the ADME admissibility of combinatorial libraries by maximizing the diversity of a virtual library, the premise being that if the compounds in a library are equally distributed in the chemical space, the probability of finding a hit should be much higher. With the similar aim of maximizing diversity, Koehler *et al.* (1999) proposed an algorithm (LASSOO) that exploits differences in descriptor distributions to identify novel compounds that are most dissimilar in structure to the members of an existing screening library but possess the attributes associated with successful drug candidates.

Many companies have appreciated the need for handling larger and larger amounts of data and organizing this information into formats that are readily searchable and retrievable. A number of systems have been designed 'in-house' but others are commercially available (Erhardt 1998). Sadowski and Kubinyi (1998) and Ajay *et al.* (1998) described methods for scoring molecules in an automated neural network process where numerous molecular descriptors are compared with values for molecules in existing databases defined as 'drug-like' and 'non-drug-like'. A company's own compounds can be ranked alongside those in these databases and, in theory, assuming the quality of data in the databases is good, reasonable predictions of compounds with 'drug-like' characteristics can be made. However, as Johnson and Wolfgang (2000) noted, a gap in the current *in vivo* knowledge base could affect the utility of future computational approaches. The reason for this 'gap' is that the most extensive datasets in the public domain today are weighted towards 'safer' pharmaceutical compounds since most of the failed or toxic structures remain within company archives.

False-positives and -negatives

One of the problems with screening methodologies is that with increasing throughput there is usually a greater danger that individual compounds with good (or bad) properties will be wrongly characterized. False-positives refer to compounds that wrongly pass a particular minimum requirement, while false-

negatives are compounds that should have passed. False-negatives are most problematic because de-selection of these during screening can lead to the exclusion of promising candidates early on in a discovery programme. Adjusting the selection criteria can help reduce the number of false-positives and -negatives but where there are still too many of one or both then it is worth considering whether the screen is sufficiently relevant. Zhang *et al.* (2000) presented a statistical approach for dealing with the hit rate and false-positives and -negatives arising from screens. Such a tool is useful for the validation of high-throughput screens as well as for post-screening data analysis. Sometimes a screen may be sufficiently predictive for one set of compounds but not another; this is common where Caco-2 cultures are used to screen for intestinal absorption. Well-absorbed compounds with high aqueous solubility or high lipophilicity often display poor permeability in Caco-2 cultures, as do some compounds that utilize specific transporters absent in the model. Ideally screens should be validated using compounds with known DMPK characteristics so that the strengths and weaknesses of each is known and the screen can then be applied when and where it will be most useful and predictive. The unbound plasma levels in the target species are particularly important where false-negatives are concerned, i.e. plasma protein binding might be high in an *in vitro* screen, and suggest that insufficient 'free' drug is available for activity, but this may not be the case in the target species (human). When checking the predictivity of *in vitro* screens, false-negatives can also occur because of poor modelling of cytochrome P450 inhibition and influx transporters. Conversely, false-positives can occur because of the poor representation of non-microsomal clearance mechanisms and efflux transporters in conventional screens.

In the case of cassette-dosing studies there is always the possibility of drug-drug interactions affecting the results, but as such interactions are more likely to enhance oral bioavailability or reduce clearance (through CYP inhibition), then there are likely to be more false-positives than -negatives. Therefore, such a method runs less of a risk of deselecting otherwise promising candidates during screening (Watt *et al.* 2000).

Enabling technologies

Many of the *in vivo*, *in vitro* and *in silico* screening methodologies would not be possible without the enabling technologies of computing, automation, new sample preparation technologies, and highly sensitive and selective detection systems. Perhaps most important in the last 10 years has been the increased utilization of liquid chromatography with mass spectrometry to allow for fast, sensitive and selective drug analysis. Before the widespread adoption of mass spectrometry, bioanalysis was often carried out with less selective methods such as HPLC-UV, but with the advent of atmospheric pressure ionization (API)-MS the molecular ion could be detected. LC-MS/MS offered even greater specificity and it was this and the ability to analyse a number of components simultaneously without chromatographic resolution that has made it a powerful technique for supporting high-throughput approaches (Gilbert *et al.* 1996).

With the advent of generic mass spectrometry-based methods and the use of microtitre plates for *in vitro* incubations and sample preparation, there has been a large increase in the use of robotics (Simpson *et al.* 1998). Sample handling robots include the Beckman Biomek (Beckman Coulter Ltd, High Wycombe, UK),

Tecan Genesis (Reading, UK) and Packard Multiprobe (Canberra Packard Ltd, Pangbourne, UK). Watt *et al.* (2000) provided a good review of the current technologies available to increase the throughput of pharmacokinetic assays. The latter authors and Henion *et al.* (1998) described solid-phase extraction (SPE) and liquid-liquid extraction methods that have been used for sample preparation. In particular, 96-well plates are used both in SPE and liquid-liquid formats, while cartridges and columns that allow direct injection of plasma samples and fast gradient methods are also available.

The experimental design is also important. For instance, in the case of cassette-dosing studies, single samples will contain multiple compounds and mass spectrometry methods represent a means of quantifying these simultaneously. In particular, the multiple reaction monitoring (MRM) technique available with tandem mass spectrometry (LC-MS/MS) gives the selectivity and sensitivity required for mixtures of compounds (Korfmacher *et al.* 1997). Deciding which compounds to 'cassette' can in itself be very time-consuming since care must be taken to avoid molecular weight clashes and allowances made for possible metabolites and isotopes of carbon, chlorine and bromine. Bayliss and Frick (1999) described how they automated cassette design, mass spectrometer optimization and the calculation of concentrations to the point that tasks that once took hours such as working with a large list of compounds and deciding which to cassette can now be done in 1–2 min. In fact, with such potential to increase throughput, it has been tempting to make 'cassettes' as large as possible and Frick *et al.* (1998) describe one containing 90 compounds used in a study in the dog. As the number of samples being analysed increases, it is imperative that the data-handling aspects are sufficiently rapid so as not to act as a bottleneck. Typically, software is employed to export peak areas and sample-specific information from mass spectrometer data files to a spreadsheet where calibration curves are constructed and following predetermined rules the concentrations of the unknowns calculated. Concentration data are then processed further on preprepared spreadsheets to give pharmacokinetic data or exported into commercially available pharmacokinetics packages, e.g. WinNonLinTM (Scientific Consulting, Inc., Cary, NC, USA).

Various laboratory information management systems (LIMS) are used that help transfer data to in-house databases. Many companies are now investing heavily in such databases as their potential as a predictive tool is recognized. As well as enabling companies to handle large amounts of their own data, systems are also being developed as searchable repositories for all the information known on particular topics, e.g. cytochrome P450 interactions, drug transporters, etc., with links to original data in-house and outside of companies. Some specialized consortium databases exist, e.g. METEOR (LHASA Ltd, Leeds, UK), which has concentrated on the metabolic fate of compounds, and DMC-QSAR (Drug Metabolism Consortium-P450 QSAR Project, Dundee/Leicester, UK), which has a WEB-based site containing the latest available P450 structures and literature.

Another advantage of using LC-MS/MS for sample assay is that it can be used to examine plasma for simple metabolites by changes in mass. Hydroxylated products, for instance, are evident as having 16 mass units more than the parent while demethylation results in a loss of 14 mass units. Software has evolved to the stage where particular molecular ions and fragments can be flagged up automatically, thus allowing rapid metabolite screening. A number of metabolite identification methods exist that can be considered high-throughput such as the one where

liver microsomal incubations are carried out in ultrafiltration apparatus and protein-free effusate is analysed by direct injection into an LC-MS/MS (van Breemen *et al.* 1999). New developments in mass spectrometry, in particular quadrupole ion traps (Cooks *et al.* 1997), quadrupole time of flight (TOF) (Baillie and Pearson 2000) and Fourier transform ion cyclotron resonance (FT-ICR) (Easterling and Amster 1997), have great utility for rapid metabolite identification. The use of NMR may also have an increasing impact in metabolite identification, particularly for molecules containing fluorine, phosphorus or deuterium atoms. For instance, ^{19}F NMR spectroscopy has been applied successfully to the detection of fluorinated drug metabolites in whole urine from a cassette dose study (Corcoran *et al.* 1999). LC-NMR/MS techniques are also now being applied, albeit not in a high-throughput manner, and offer a means of obtaining definitive sets of metabolism data (Baillie and Pearson 2000).

According to Crespi (1999b), accelerated throughputs have been principally achieved by novel experimental designs that reduce the number of samples to be analysed and/or reduce the time and resources needed to analyse each sample. HPLC-UV methods were often utilized for assessing *in vitro* cytochrome P450 inhibition but were generally low-throughput. A number of high-throughput approaches now exist including fluorometric assays in a multiwell plate format (Crespi *et al.* 1997), LC-MS/MS analysis of mixtures (Crespi 1999a) and methods that utilize radiolabelled substrates with HPLC analysis (Wynalda and Wienkers 1997) and scintillation counting (Rodrigues *et al.* 1997).

Cytochrome P450 induction can be assessed by a number of methods including the utilization of probe substrates specific for a single isoform (Ekins *et al.* 1995) and the measurement of mRNA levels using reverse transcriptase polymerase chain reaction (RT-PCR) and Northern blotting methods (Ryu *et al.* 1996) or ribonuclease protection assays (Gonzalez *et al.* 1992). Also, ELISA and Western blotting techniques can be used where increases in apoprotein levels are assessed using antibodies raised against single CYP isoforms (Wortelboer *et al.* 1991). Generally, these methods are low-throughput, but Surry *et al.* (1999) described a method for determining CYP3A induction in primary rat hepatocytes utilizing a radiolabelled CYP3A antisense riboprobe, *in-situ* hybridization techniques and a ribonuclease protection assay to detect changes in mRNA expression in hepatocytes cultured on 96-well plates. Also, because conventional RT-PCR and Northern blot methods require relatively large amounts of total RNA and are typically only semiquantitative, new real-time quantitative RT-PCR methods utilizing Taqman technology (ABI Prism 7700, PE Biosystems, Foster City, CA, USA) have been developed that are suitable for high-throughput 96-well-based induction assays (Bowen *et al.* 2000, Pan *et al.* 2000).

It is possible though that microplate-based high-throughput methods may be superseded in the future by microchip-based screening methods. DNA microarray chips are being developed for the investigation of the mechanism of toxicity where hundreds of thousands of oligonucleotides are attached to a solid silicon surface in an ordered array and fluorescent labels incorporated so that each acts as an allele-specific probe. DNA alterations can then be identified using fluorescent scanning and computer analysis (Chee *et al.* 1996). These micro-array technologies may prove to have great utility for screening compounds for potential cytochrome P450 interactions (Johnson and Wolfgang 2000). Affymetrix, Inc. (Santa Clara, CA, USA) is one of the main companies developing the gene chip technology and

it has a number of collaborations with major pharmaceutical companies to use the technology for drug discovery (Wallace 1997).

Conclusions and future perspectives

Although major increases in DMPK screening have been achieved in the last 10 years and there have been some major technological developments, throughputs still do not generally match those of high-throughput biology activity screens. The important question is whether DMPK screening truly needs to be high-throughput in nature to have the greatest impact. In the future, with the maturing of *in silico* methods for designing libraries and screening out the less ideal candidates, the libraries requiring actual physical screening may not be as large as anticipated. In addition, increases in throughput often result in a reduction in the quality of the data, so ideally a balance will need to be achieved between quantity and quality for each particular screening method. For DMPK screening to be most effective, it is important that screens are applied at the right stage in the drug discovery process, and applying such screening before any potency has been achieved can be counterproductive, but conversely trying to optimize the DMPK of compounds after other characteristics have been optimized gives less scope for improvement.

Bertrand *et al.* (2000) believed that though progress has been very rapid in drug metabolism during the last 5 years, in one sense things have moved too fast with most of the automated *in vitro* tests being established before we have fully mastered the handling of the enormous amounts of data produced. However, this field is unlikely to stop evolving particularly with the advent of genomics. Such knowledge is now rapidly increasing our understanding of the molecular pathways responsible for genetic disease and interindividual differences that lead to varying responses to drug treatment. Using 'GeneChip' technology, not only should it be possible to screen out drugs with unwanted effects in the drug discovery phase, but also it should allow the design of individual drug therapy regimens (Wallace 1999). Genomic methods involving cytochrome P450 measurements are being used by a number of pharmaceutical companies, but there are limits to this approach. Whereas cytochrome P450 mRNA profiling can predict reasonably accurately the likely metabolic fate of a drug, it cannot say whether the metabolites are likely to be toxic. Also, as there can be doubt over the correlation of mRNA levels and the corresponding protein levels, a proteomic approach should have some value since proteins released in screens can act as markers of metabolism and toxicity (Page *et al.* 1999).

Advances in automation, software and analytical hardware will no doubt continue and some new developments are already on the horizon. Triple quadrupole MS systems offer improvements in sensitivity and Watt *et al.* (2000) believe that a hybrid of quadrupole-TOF technology allowing simultaneous parent and metabolite information to be obtained from a single chromatographic run will enter the mainstream in the same manner as MS/MS has done. In addition, manufacturers are continuing to update existing software and write new programs to aid in the development of methods and processing of data from samples arising from high-throughput screens.

CADD has a continuing and important role to play in supporting automation-based approaches to drug discovery and what should prove most useful in the future is the building of databases containing more consistent *in vitro* properties

relevant to the prediction of *in vivo* profiles. Also, it has been recognized that validation of these models with *in vivo* data is essential to aid their future predictivity, so I would expect that the generation of such data will continue to be important for some time to come.

It has not been the aim of this review to include details on toxicology screens, but as the two disciplines of DMPK and toxicology are quite closely related and important in the drug-selection process, it is worth mentioning that the establishing of high-throughput toxicology screens has lagged a little behind that of DMPK ones. There is clearly a need for early *in vitro* and *in silico* screens for potential toxicity. Cronin (1998) reviewed the computer-aided prediction of drug toxicity, and other approaches have been reviewed by Richard (1998), Todd and Ulrich (1999) and Robertson and Bulera (2000). Apart from the *in silico* approaches, the most promising high-throughput methods for the future are those that involve the assessment of gene and protein expression changes using micro-arrays, electrophoresis and mass spectrometry.

In spite of their obvious advantages, high-throughput *in vitro* and *in silico* DMPK models suffer from a number of drawbacks that will need addressing in the future. Generally existing datasets were not derived from sufficiently large numbers of compounds in a consistent manner, so for models to be most predictive, comparable *in vivo*, *in vitro* and *in silico* data needs to be generated on far more compounds using standardized methods. Some of the simpler absorption models will probably require less data to validate, but metabolism, because of the generally more complex relationships, could prove more difficult to model effectively. Smaller and newer companies without access to large compound libraries may be at a disadvantage in building models but could buy in libraries and screen them or access non-company databases. Also, it is likely that more well-validated models will become available commercially in the future based on the example of the Navicte Consortium approach that led to the development of the iDEA predictive ADME simulation system (Trega Biosciences, Inc., San Diego, CA, USA).

Rational high-throughput screening will rely on greater integration of *in silico*, *in vitro* and *in vivo* techniques. Along these lines, Rodrigues (1997) presented what he called the AVID approach (automation, validation, integration and database management) for high-throughput screening, which could act as a framework for rational drug discovery. Looking to the future, Rodrigues noted that for such approaches to be successful, greater cooperation would be required between technology- and project-based groups. It is evident that there will also need to be greater synergies between departments, for instance, CADD and physical chemistry groups often function separately from DMPK groups in organizations but for *in silico* models to have their greatest impact these disciplines must work more closely together. Reassuringly, the whole process of discovery has been undergoing a refocusing in many companies with 'lead generation' and 'lead optimization' groups being established that bring together all the associated disciplines. Having closer cooperation between disciplines as well as newer and more predictive models is essential, but it is also important to have efficient data-handling systems in place and to be assured that the data held within them are of a high enough quality to enable rational screening approaches to be effective.

There are many genuinely useful and potentially useful experimental and computational high-throughput DMPK approaches available for enhancing drug

selection and preventing this stage in the process of drug discovery becoming a bottleneck. However, it should not be assumed that the throughput of DMPK screens is always the most important factor as other steps in the discovery process such as the number of targets selected and validated per year, the rates of medicinal chemistry synthesis and biology screening and the availability of validated pharmacology models can also become a bottleneck.

Apart from improving throughput, *in silico* and *in vitro* techniques also offer the opportunity of replacing animals in the drug-discovery process. However, 10 years ago it was hoped that *in vitro* models would replace *in vivo* techniques and although some reductions in animal use have occurred, *in vivo* studies are still proving important. With future advances in high-throughput *in vitro* and *in silico* DMPK screening, it may be hoped that robust candidates can be selected for development and their fate accurately predicted in humans without the need for *in vivo* data. Although this is an admirable aim, we are probably still some way off this becoming a reality. Perhaps I will stand corrected in the 40th DMDG anniversary issue of *Xenobiotica*.

References

- ABRAHAM, M. H., 2000, High-throughput prediction of physicochemical and biochemical processes from structure, using calculated Abraham solvation descriptors. *Abstracts of Papers — American Chemical Society*, **220**, COMP-032.
- ABRAHAM, M. H., CHADHA, H. S. and MITCHELL, R. C., 1995, Hydrogen-bonding. Part 36. Determination of blood-brain distribution using octanol-water partition coefficient. *Drug Design and Discovery*, **13**, 123–131.
- ABRAHAM, M. H. and LE, J., 1999, The correlation and prediction of the solubility of compounds in water using an amended solvation energy relationship. *Journal of Pharmaceutical Sciences*, **88**, 868–880.
- ANNAERT, P. P., LECLUYSE, E. L. and BROUWER, K. L. R., 2000, Modulation of *P*-glycoprotein (*P*-GP)-mediated biliary excretion in sandwich-cultured (SC) rat hepatocytes. *Drug Metabolism Reviews*, **32**, 198.
- ARTURSSON, P. and KARLSSON, J., 1991, Correlation between oral drug absorption in humans and apparent drug permeability coefficients in human intestinal epithelial (Caco-2) cells. *Biochemical and Biophysical Research Communications*, **175**, 880–885.
- AYRTON, A. and MORGAN, P., 2001, Role of transport proteins in drug absorption, distribution and excretion. *Xenobiotica*, **31**, 399–427.
- AYRTON, J., PLUMB, R., LEAVENS, W. J., MALLETT, D., DICKINS, M. and DEAR, G. J., 1998, Application of a generic fast gradient liquid chromatography tandem mass spectrometry method for the analysis of cytochrome P450 probe substrates. *Rapid Communications in Mass Spectrometry*, **12**, 217–224.
- BAILLIE, T. A. and PEARSON, P. G., 2000, The impact of drug metabolism in contemporary drug discovery: new opportunities and challenges for mass spectrometry. In A. J. Burlingame, S. A. Carr and M. A. Baldwin (eds), *Mass Spectrometry in Biological Medicine* (Totowa: Humana), pp. 481–496.
- BAJPAI, M. and ADKISON, K. K., 2000, High-throughput screening for lead optimization: a rational approach. *Current Opinions in Drug Discovery and Development*, **3**, 63–71.
- BAYLISS, M. K. and FRICK, L. W., 1999, High-throughput pharmacokinetics: cassette dosing. *Current Opinions in Drug Discovery and Development*, **2**, 20–25.
- BERMAN, J., HALM, K., ADKISON, K. K. and SHAER, J. E., 1997, Simultaneous pharmacokinetic screening of a mixture of compounds in the dog using API LC/MS/MS analysis for increased throughput. *Journal of Medicinal Chemistry*, **40**, 827–829.
- BERTRAND, M., JACKSON, P. and WALTHER, B., 2000, Rapid assessment of drug metabolism in the drug discovery process. *European Journal of Pharmaceutical Sciences*, **11**, 61–72.
- BEVAN, C. D. and LLOYD, R. S., 2000, A high-throughput screening method for the determination of aqueous drug solubility using laser nephelometry in microtiter plates. *Analytical Chemistry*, **72**, 1781–1787.

- BOWEN, W. P., CAREY, J. E., MIAH, A., McMURRAY, H. F., MUNDAY, P. W., JAMES, R. S., COLEMAN, R. A. and BROWN, A. M., 2000, Measurement of cytochrome P450 gene induction in human hepatocytes using quantitative real-time reverse transcriptase-polymerase chain reaction. *Drug Metabolism and Disposition*, **28**, 781–788.
- BOWERS, W. F., FULTON, S. and THOMPSON, J., 1984, Ultrafiltration vs equilibrium dialysis for determination of free fraction. *Clinical Pharmacokinetics*, **9**, 49–60.
- BOX, K. J., 2000, High-throughput physicochemical profiling: a tool for lead optimization. Paper presented at the Cambridge Healthtech Institutes 2nd Annual Conference Smarter Lead Optimization, Baltimore, MD, 16–17 March.
- CALDWELL, G. W., 2000, Compound optimization in early- and late-phase drug discovery: acceptable pharmacokinetic properties utilizing combined physicochemical, *in vitro* and *in vivo* screens. *Current Opinions in Drug Discovery and Development*, **3**, 30–41.
- CAMENISCH, G., ALSENZ, J., VAN DE WATERBEEMD, H. and FOLKERS, G., 1998, Estimation of permeability by passive diffusion through Caco-2 cell monolayers using drugs' lipophilicity and molecular weight. *European Journal of Pharmaceutical Sciences*, **6**, 313–319.
- CAMENISCH, G., FOLKERS, G. and VAN DE WATERBEEMD, H., 1997, Comparison of passive drug transport through Caco-2 cells and artificial membranes. *International Journal of Pharmaceutics*, **147**, 61–70.
- CARLILE, D. J., ZOMORODI, K. and HOUSTON, J. B., 1997, Scaling factors to relate drug metabolic clearance in hepatic microsomes, isolated hepatocytes, and the intact liver — studies with induced livers involving diazepam. *Drug Metabolism and Disposition*, **25**, 903–911.
- CHEE, M., YANG, R., HUBBELL, E., BERNO, A., HUANG, X. C., STERN, D., WINKLER, J., LOCKHART, D. J., MORRIS, M. S. and FODOR, S. P. A., 1996, Assessing genetic information with high-density DNA arrays. *Science*, **274**, 610–614.
- CHIKHALE, E. G., NG, K. Y., BURTON, P. S. and BORCHARDT, R. T., 1994, Hydrogen bonding potential as a determinant of the *in vitro* and *in situ* blood–brain barrier permeability of peptides. *Pharmaceutical Research*, **11**, 412–419.
- CHONG, S., DANDO, S. A. and MORRISON, R. A., 1997, Evaluation of Biocoat® intestinal epithelium differentiation environment (3-day cultured Caco-2 cells) as an absorption screening model with improved productivity. *Pharmaceutical Research*, **14**, 1835–1837.
- CLARK, D. E., 1999a, Rapid calculation of polar surface area and its application to the prediction of transport phenomena. 1. Prediction of intestinal absorption. *Journal of Pharmaceutical Sciences*, **88**, 807–814.
- CLARK, D. E., 1999b, Rapid calculation of polar molecular surface area and its application to the prediction of transport phenomena. 2. Prediction of blood–brain barrier penetration. *Journal of Pharmaceutical Sciences*, **88**, 815–821.
- COMMANDEUR, J. N. M., 1999, Cytochrome P450 in drug metabolism. *Chimie Nouvelle*, **17**, 1994–1995.
- COOKS, R. G., CHEN, G. and WEIL, C., 1997, Quadrupole mass filter and quadrupole ion traps. *NATO ASI Series*, **504**, 213–238.
- CORCORAN, O., WILSON, I. D. and NICHOLSON, J. K., 1999, Rapid multi-component detection of fluorinated drug metabolites in whole urine from a 'cassette' dose study using high resolution ¹⁹F NMR spectroscopy. *Analytical Communications*, **36**, 259–261.
- COX, K. A., DUNN-MEYNELL, K., KORFMACHER, W. A., BROSKIE, L., NOMEIR, A. A., LIN, C. C., CAYEN, M. N. and BARR, W. H., 1999, Novel *in vivo* procedure for rapid pharmacokinetic screening of discovery compounds in rats. *Drug Discovery Today*, **4**, 232–237.
- CRESPI, C. L., 1999a, Higher-throughput screening with human cytochromes P450. *Current Opinions in Drug Discovery and Development*, **2**, 15–19.
- CRESPI, C. L., 1999b, Assessing the potential for drug–drug interactions in an accelerated throughput mode. *Pharmaceutical Science Technology Today*, **2**, 119–120.
- CRESPI, C. L., MILLER, V. P. and PENMAN, B. W., 1997, Microtiter plate assays for inhibition of human drug metabolizing cytochromes P450. *Analytical Biochemistry*, **248**, 188–190.
- CRESPI, C. L., MILLER, V. P. and PENMAN, B. W., 1998, High throughput screening for inhibition of cytochrome P450 metabolism. *Medicinal Chemistry Research*, **8**, 457–471.
- CRESPI, C. L., PENMAN, B. W. and HU, M., 1996, Development of Caco-2 cells expressing high levels of cDNA-derived cytochrome CYP3A4. *Pharmaceutical Research*, **13**, 1635–1641.
- CRIVORI, P., CRUCIANI, G., CARRUPT, P.-A. and TESTA, B., 2000, Predicting blood–brain barrier permeation from three-dimensional molecular structure. *Journal of Medicinal Chemistry*, **43**, 2204–2216.
- CRONIN, M., 1998, Computer-aided prediction of drug toxicity in high-throughput screening. *Pharmacy and Pharmacology Communications*, **4**, 157–163.
- CRUCIANI, G. and GUBA, W., 2000, VolSurf: a new tool for the pharmacokinetic optimization of lead compounds. *European Journal of Pharmaceutical Sciences*, **11**, S29–S39.
- DARVAS, F., DORMAN, G. and PAPP, A., 2000, Diversity measures for enhancing ADME admissibility of combinatorial libraries. *Journal of Chemical Information and Computer Sciences*, **40**, 314–322.

- DE GROOT, M. J., ACKLAND, M. J., HORNE, V. A., ALEX, A. A. and JONES, B. C., 1999, Novel approach to predicting P450-mediated drug metabolism: development of a combined protein and pharmacophore model for CYP2D6. *Journal of Medicinal Chemistry*, **42**, 1515–1524.
- DELBRESSINE, L., 2001, Organon's experience with knowledge management in preclinical development. Paper presented at the DMDG 1 Day Meeting, Informatics and *In Silico* Modelling in DM/PK, Ware, 15 February.
- DELIE, F. and RUBAS, W., 1997, A human colonic cell line sharing similarities with enterocytes as a model to examine oral absorption: advantages and limitations of the Caco-2 model. *Critical Review of Therapeutic Drug Carrier Systems*, **14**, 221–286.
- DIKE, L. E., GUARINO, R. and TIMMINS, M., 2000, Development of a high throughput, fluorescent assay system to determine relative rates of metabolism. *Drug Metabolism Reviews*, **32**, 183.
- DOPPENSCHMITT, S., LANGGUTH, P., REGARDH, C. G., ANDERSSON, T. B., HILGENDORF, C. and SPAHN-LANGGUTH, H., 1999, Characterisation of binding properties to human P-glycoprotein: development of a [³H]verapamil radioligand-binding assay. *Journal of Pharmacology and Experimental Therapeutics*, **288**, 348–357.
- EARLL, M., 1999, A guide to log P and pKa measurements and their use [<http://www.raell.demon.co.uk/chem/logp/logpka.html>].
- EASTERLING, M. L. and AMSTER, I. J., 1997, Fourier transform mass spectrometry. *NATO ASI Series*, **504**, 287–314.
- EDDERSHAW, P. J., BERESFORD, A. P. and BAYLISS, M. K., 2000, ADME/PK as part of a rational approach to drug discovery. *Drug Discovery Today*, **5**, 409–414.
- EGAN, W. J., MERZ, K. M. and BALDWIN, J. J., 2000, Prediction of drug absorption using multivariate statistics. *Journal of Medicinal Chemistry*, **43**, 3867–3877.
- EKINS, S., MURRAY, G. I., BURKE, M. D., WILLIAMS, J. A., MARCHANT, N. C. and HAWKSWORTH, G. M., 1995, Quantitative differences in phase I and II metabolism between rat precision-cut liver slices and isolated hepatocytes. *Drug Metabolism and Disposition*, **23**, 1274–1279.
- EPDS, D. E., RAUB, T. J. and KEZDY, F. J., 1995, A general, wide-range spectrofluorometric method for measuring the site-specific affinities of drugs toward human serum albumin. *Analytical Biochemistry*, **227**, 342–350.
- ERHARDT, P. W., 1998, Drug metabolism data: past and present status. *Medicinal Chemistry Research*, **8**, 400–421.
- FRICK, L. W., ADKISON, K. K., WELLS-KNECHT, K. J., WOOLAND, P. and HIGTON, D. M., 1998, Cassette dosing: rapid *in vivo* assessment of pharmacokinetics. *Pharmaceutical Science and Technology Today*, **1**, 12–18.
- GEORGE, A., 1999, The design and molecular modeling of CNS drugs. *Current Opinions in Drug Discovery and Development*, **2**, 286–292.
- GHOSAL, A., HAPANGAMA, N., YUAN, Y., FARREAU, L. V., PALAMANDER, J. R., PATRICK, J. E., CAYEN, M. N. and ZBAIDA, S., 2000, Microtiter plate-based assay for rapid determination of enzyme activities of 13 P450 supersomes and human liver microsomes. *Drug Metabolism Reviews*, **32**, 188.
- GIDDINGS, S. J., CLARKE, S. E. and GIBSON, G. G., 1997, CYP4A1 gene transfection studies and the peroxisome proliferator-activated receptor: development of a high-throughput assay to detect peroxisome proliferators. *European Journal of Drug Metabolism and Pharmacokinetics*, **22**, 315–319.
- GILBERT, J. D., OLAH, T. V. and McLoughlin, D. A., 1996, High-performance liquid chromatography with atmospheric pressure ionization tandem mass spectrometry as a tool in quantitative bioanalytical chemistry. *American Chemical Society Symposium Series*, **619**, 330–350.
- GONZALEZ, F. J., CRESPI, C. L., CZERWINSKI, M. and GELBOIN, H. V., 1992, Analysis of human cytochrome P450 catalytic activities and expression. *Tohoku Journal of Experimental Medicine*, **168**, 67–72.
- GRASS, G. M., 1997, Simulation models to predict oral drug absorption from *in vitro* data. *Advanced Drug Delivery Reviews*, **23**, 199–219.
- GU, C., NIKOLIC, D., LAI, J., XU, X. and VAN BREEMEN, R. B., 1999, Assays of ligand-human serum albumin binding using pulsed ultrafiltration and liquid chromatography mass spectrometry. *Combinatorial Chemistry and High Throughput Screening*, **2**, 353–359.
- HALLIDAY, R. C., JONES, B. C., PARK, B. K. and SMITH, D. A., 1997, Synthetic strategies to lower affinity for CYP2D6. *European Journal of Drug Metabolism and Pharmacokinetics*, **22**, 291–294.
- HAWKINS, D. R., 1999, Use and value of metabolism databases. *Drug Discovery Today*, **4**, 466–471.
- HENION, J., BREWER, E. and RULE, G., 1998, Sample preparation for LC/MS/MS: analyzing biological and environmental samples. *Analytical Chemistry*, **70**, 650–656.
- HERMAN, R. A. and VENG-PEDERSON, P., 1994, Quantitative structure–pharmacokinetic relationships for systemic drug distribution kinetics not confined to a congeneric series. *Journal of Pharmaceutical Sciences*, **83**, 423–428.
- HERRERA, A. M., SCOTT, D. O. and LUNTE, C. E., 1990, Microdialysis sampling for determination of plasma protein binding of drugs. *Pharmaceutical Research*, **7**, 1077–1081.

- HOLLENBERG, P. F. and SCARPELLI, D. G., 1988, Uptake of *N*-nitrosobis(2-oxopropyl)amine by isolated rat and hamster hepatocytes: species differences and evidence for an active carrier-mediated transport process. *Carcinogenesis*, **9**, 1763–1772.
- HOP, C. E., WANG, Z., CHEN, Q. and KWEI, G., 1998, Plasma-pooling methods to increase throughput for *in vivo* pharmacokinetic screening. *Journal of Pharmaceutical Sciences*, **87**, 901–903.
- HUUSKONEN, J., RANTANEN, J. and LIVINGSTONE, D., 2000, Prediction of aqueous solubility for a diverse set of organic compounds based on atom-type electrotopological state indices. *European Journal of Medicinal Chemistry*, **35**, 1081–1088.
- HYDE, R. M. and HERSEY, A., 1996, Screening and rational drug design — competitive or complementary? In M. G. Ford, R. Greenwood, G. T. Brooks and R. Frankel (eds), *Bioactive Compound Design: Possibilities for Industrial Use* (Oxford: Bios), pp. 37–45.
- IRVINE, J. D., TAKAHASHI, L., LOCKHART, K., CHEONG, J., TOLAN, J. W., SELICK, H. E. and GROVE, J. R., 1999, MDCK (Madin–Darby canine kidney) cells: a tool for membrane permeability screening. *Journal of Pharmaceutical Sciences*, **88**, 28–33.
- IWATSUBO, T., HIROTA, N., OOE, T., SUZUKI, H., SHIMADA, N., CHIBA, K., ISHIZAKI, T., GREEN, C. E., TYSON, C. A. and SUGIYAMA, Y., 1997, Prediction of *in vivo* drug metabolism in the human liver from *in vitro* metabolism data. *Pharmacology and Therapeutics*, **73**, 147–172.
- JOHNSON, D. E. and WOLFGANG, G. H. I., 2000, Predicting human safety: screening and computational approaches. *Drug Discovery Today*, **5**, 445–454.
- JOHNSON, D. L., KELLY, K. G., GAO, F., MEYER, R. D., MORRIS, J. C., WEST, M. and EKINS, S. E., 2000, Single point screening for high throughput methods to predict drug–drug interaction potential. *Drug Metabolism Reviews*, **32**, 185.
- KANSY, M., SENNER, F. and GUBERNATOR, K., 1998, Physicochemical high throughput screening: parallel artificial membrane permeation assay in the description of passive absorption processes. *Journal of Medicinal Chemistry*, **41**, 1007–1010.
- KASUHARA, H. and SUGIYAMA, Y., 2001, Efflux transport systems for drugs at the blood–brain barrier and blood–cerebrospinal fluid barrier (Part 1). *Drug Discovery Today*, **6**, 150–156.
- KELDER, J., GROOTENHUIS, D. J., BAYADA, D. M., DELBRESSINE, P. C. and PLOEMEN, J.-P., 1999, Polar molecular surface as a dominating determinant for oral absorption and brain penetration of drugs. *Pharmaceutical Research*, **16**, 1514–1519.
- KENNEDY, T., 1997, Managing the drug discovery/development interface. *Drug Discovery Today*, **2**, 436–444.
- KIM, R. B., 2000, Transporters and drug disposition. *Current Opinions in Drug Discovery and Development*, **3**, 94–101.
- KIM, R. B., FROMM, M. F., WANDEL, C., LEAKE, B., WOOD, A. J. J., RODEN, D. M. and WILKINSON, G. R., 1998, The drug transporter *P*-glycoprotein limits oral absorption and brain entry of HIV-1 protease inhibitors. *Journal of Clinical Investigation*, **101**, 289–294.
- KLOPMAN, G., TU, M. and FAN, B. T., 1999, META. Part 4. Prediction of the metabolism of polycyclic aromatic hydrocarbons. *Theoretical Chemistry Accounts*, **102**, 33–38.
- KOBAYASHI, K., SOGAME, Y., HARA, H. and HAYASHI, K., 1990, Mechanism of glutathione *S*-conjugate transport in canalicular and basolateral rat liver plasma membranes. *Journal of Biological Chemistry*, **265**, 7737–7741.
- KOEHLER, R. A., DIXON, S. L. and VILLAR, H. O., 1999, LASSOO: a generalised directed diversity approach to the design and enrichment of chemical libraries. *Journal of Medicinal Chemistry*, **42**, 4695–4704.
- KORFMACHER, W. A., COX, K. A., BRYANT, M. S., VEALS, J., NG, K., WATKINS, R. and LIN, C.-C., 1997, HPLC-API/MS/MS: a powerful tool for integrating drug metabolism into the drug discovery process. *Drug Discovery Today*, **2**, 532–537.
- KORFMACHER, W. A., PALMER, C. A., NARDO, C., DUNN-MEYNELL, K., GROTZ, D., COX, K., LIN, C. C., ELICONE, C. and DUCHOSLAV, E., 1999, Development of an automated mass spectrometry system for the quantitative analysis of liver microsomal incubation samples: a tool for rapid screening of new compounds for metabolic stability. *Rapid Communications in Mass Spectrometry*, **13**, 901–907.
- LEAHY, D. E., ARUNDEL, P. A., BLAKEY, G. E. and ROWLAND, M., 1996, Physiologic based pharmacokinetic modelling and QSAR. In M. G. Ford, R. Greenwood, G. T. Brooks and R. Frankel (eds), *Bioactive Compound Design: Possibilities for Industrial Use* (Oxford: Bios), pp. 147–151.
- LEE, C. P., DE VRUEH, R. L. A. and SMITH, P. L., 1997, Selection of development candidates based on *in vitro* permeability measurements. *Advances in Drug Delivery Reviews*, **23**, 47–62.
- LEHMANN, J. M., MCKEE, D. D., WATSON, M. A., WILLSON, T. M., MOORE, J. T. and KLIEWER, S. A., 1998, The human orphan nuclear receptor PXR is activated by compounds that regulate CYP3A4 gene expression and cause drug interactions. *Journal of Clinical Investigation*, **102**, 1016–1023.

- LEWIS, D. F., DICKINS, M., WEAVER, R. J., EDDERSHAW, P. G., GOLDFARB, P. S. and TARBIT, M. H., 1998, Molecular modelling of human CYP2C subfamily enzymes CYP2C9 and CYP2C19: rationalization of substrate specificity and site-directed mutagenesis experiments in the CYP2C subfamily. *Xenobiotica*, **28**, 235–268.
- LEWIS, D. F., EDDERSHAW, P. J., GOLDFARB, P. S. and TARBIT, M. H., 1997, Molecular modelling of cytochrome P4502D6 (CYP2D6) based on an alignment with CYP102: structural studies on specific CYP2D6 substrate metabolism. *Xenobiotica*, **27**, 319–339.
- LEWIS, D. F. V., IOANNIDES, C. and PARKE, D. V., 1990, A prospective toxicity evaluation (COMPACT) on 40 chemicals currently being tested by the National Toxicology Program. *Mutagenesis*, **5**, 433–435.
- LI, A., 1999, Higher throughput metabolism assays using human hepatocytes. Paper presented at the AAPS Annual Meeting, New Orleans, LA, Symposium Proceedings.
- LI, A. P., LU, C., BRENT, J. A., PHAM, C., FACKETT, A., RUEGG, C. E. and SILBER, P. M., 1999, Cryopreserved human hepatocytes: characterisation of drug-metabolizing enzyme activities and applications in higher throughput screening assays for hepatotoxicity, metabolic stability, and drug–drug interaction potential. *Chemico-Biological Interactions*, **121**, 17–35.
- LI, A. P., MAUREL, P., GOMEZ-LECHON, M. J., CHENG, L. C. and JURIMA-ROMET, M., 1997, Preclinical evaluation of drug–drug interaction potential: present status of the application of primary human hepatocytes in the evaluation of cytochrome P450 induction. *Chemico-Biological Interactions*, **107**, 5–16.
- LIN, J. H. and LU, A. Y., 1998, Inhibition and induction of cytochrome P450 and the clinical implications. *Clinical Pharmacokinetics*, **35**, 361–390.
- LIPINSKI, C. A., LOMBARDO, F., DOMINY, B. W. and FEENEY, P. J., 1997, Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. *Advanced Drug Delivery Reviews*, **23**, 3–25.
- MADAN, A., DEHAAN, R., MUDRA, D., CARROLL, K., LECLUYSE, E. and PARKINSON, A., 1999, Effect of cryopreservation on cytochrome P-450 enzyme induction in cultured rat hepatocytes. *Drug Metabolism and Disposition*, **27**, 327–335.
- MARCHANT, C. A. and COMBES, R. D., 1996, Artificial intelligence: the use of computer models in the prediction of metabolism and toxicity. In M. G. Ford, R. Greenwood, G. T. Brooks and R. Frankel (eds), *Bioactive Compound Design: Possibilities for Industrial Use* (Oxford: Bios), pp. 153–162.
- MCCARTHY, S., OSBOURNE, S. and KUHFELD, M., 1996, Automated permeability analysis of mixtures across Caco-2 cell monolayers. *Pharmacology Research*, **13**, S242.
- MICHNE, W. F., 1996, Hit-to-lead chemistry: a key element in new lead generation. *Pharmaceutical News*, **3**, 19–21.
- MOODY, G. C., GRIN, S. J., MATHER, A. N., MCGINNITY, D. F. and RILEY, R. J., 1999, Fully automated analysis of activities catalysed by the major human liver cytochrome P450 (CYP) enzymes: assessment of human CYP inhibition potential. *Xenobiotica*, **9**, 53–75.
- MOORE, J. T. and KIEWER, S. A., 2000, Use of the nuclear receptor PXR to predict drug interactions. *Toxicology*, **153**, 1–10.
- MULLINS, M. E., HOROWITZ, B. Z., LINDEN, D. H., SMITH, G. W., NORTON, R. L. and STUMP, J., 1998, Life-threatening interaction of mifepradil and beta blockers with dihydropyridine calcium channel blockers. *Journal of American Medical Association*, **280**, 157–158.
- NAVIA, M. A. and CHATURVEDI, P. R., 1996, Design principles for orally bioavailable drugs. *Drug Discovery Today*, **1**, 179–189.
- NESTEROV, I. A., AARONS, L. and ROWLAND, M., 1997, Physiologically based pharmacokinetic modelling of a homologous series of barbiturates in the rat: a sensitivity analysis. *Journal of Pharmacokinetics and Biopharmacy*, **25**, 413–447.
- NIINUMA, K., KATO, Y., SUZUKI, H., TYSON, C. A., WEIZER, V., DABBS, J. E., FROELICH, R., GREEN, C. E. and SUGIYAMA, Y., 1999, Primary active transport of organic anions on bile canalicular membrane in humans. *American Journal of Physiology*, **276**, G1153–G1164.
- NORDYKE, A., BESTOR, J. K., YERINO, P. P., HANSEN, D. K., BEHLOW, H. W., MILLER, T. D. and THOMPSON, T. N., 2000, Validation of an improved automated incubation procedure used to screen metabolic stability of multiple compounds in parallel. *Drug Metabolism Reviews*, **32**, 183.
- NORINDER, U., OSTERBERG, T. and ARTURSSON, P., 1999, Theoretical calculation and prediction of intestinal absorption of drugs in humans using MolSurf parametrization and PLS statistics. *European Journal of Pharmaceutical Sciences*, **8**, 49–56.
- NORINDER, U., SJOBERG, P. and OSTERBERG, T., 1998, Theoretical calculation and prediction of brain–blood partitioning of organic solutes using MolSurf parametrization and PLS statistics. *Journal of Pharmaceutical Sciences*, **87**, 952–959.
- OBACH, R. S., BAXTER, J. G., LISTON, T. E., SILBER, B. M., JONES, B. C., MACINTYRE, F., RANCE, D. J. and WASTALL, P., 1997, The prediction of human pharmacokinetic parameters from preclinical and *in vitro* metabolism data. *Journal of Pharmacology and Experimental Therapeutics*, **283**, 46–58.

- OGG, M. S., WILLIAMS, J. M., TARBIT, M., GOLDFARB, P. S., GRAY, T. J. B. and GIBSON, G. G., 1999, A reporter gene assay to assess the molecular mechanisms of xenobiotic-dependent induction of the human CYP3A4 gene *in vitro*. *Xenobiotica*, **29**, 269–279.
- OLAH, T. V., MCLOUGHLIN, D. A. and GILBERT, J. D., 1997, The simultaneous determination of mixtures of drug candidates by liquid chromatography/atmospheric pressure chemical ionization mass spectrometry as an *in vivo* drug screening procedure. *Rapid Communications in Mass Spectrometry*, **11**, 17–23.
- PACICI, G. M. and VIANI, A., 1992, Methods for determining plasma and tissue binding of drugs. *Clinical Pharmacokinetics*, **23**, 449–468.
- PAGE, M. J., AMESS, B., ROHL, C., STUBBERELD, C. and PAREKH, R., 1999, Proteomics: a major new technology for the drug discovery process. *Drug Discovery Today*, **4**, 55–62.
- PAGLIARI, A., REIST, M., GEINOZ, S., CARRUPT, P.-A. and TESTA, B., 1999, Evaluation and prediction of drug permeation. *Journal of Pharmacy and Pharmacology*, **51**, 1339–1357.
- PALM, K., STENBERG, P., LUTTMAN, K. and ARTURSSON, P., 1997, Polar surface properties predict the intestinal absorption of drugs in humans. *Pharmaceutical Research*, **14**, 568–571.
- PAN, J., XIANG, Q. and BALL, S., 2000, Use of a novel real-time quantitative reverse transcription-polymerase chain reaction method to study the effects of cytokines on cytochrome P450 mRNA expression in mouse liver. *Drug Metabolism and Disposition*, **28**, 709–713.
- PARDRIDGE, W. M., 1998, CNS drug design based in principles of blood–brain barrier transport. *Journal of Neurochemistry*, **70**, 1781–1792.
- PARIKH, H. H., MCELWAIN, K., BALUASUBRAMANIAN, V., LEUNG, W., WONG, D., MORRIS, M. E. and RAMANATHAN, M., 2000, A rapid spectrofluorometric technique for determining drug–serum protein binding suitable for high-throughput screening. *Pharmaceutical Research*, **17**, 632–637.
- PLATTS, J. A., 2000, Rapid automated prediction of Abraham LSER descriptors. *Abstracts of Papers — American Chemistry Society*, **220**, COMP-135.
- PIDGEON, C., ONG, S., LIU, H., QUI, X., PIDGEON, M., DANTZIG, A. H., MUNROE, J., HORNBACK, W. J., KASHER, J. S., GLUNZ, L. and SZCZERBA, T., 1995, IAM chromatography: an *in vitro* screen for predicting drug membrane permeability. *Journal of Medicinal Chemistry*, **38**, 590–594.
- PRENTIS, R. A., LIS, Y. and WALKER, S. R., 1988, Pharmaceutical innovation by the seven UK-owned pharmaceutical companies (1964–1985). *British Journal of Clinical Pharmacology*, **25**, 387–396.
- RICHARD, A. M., 1998, Commercial toxicology prediction systems: a regulatory perspective. *Toxicology Letters*, **102/103**, 607–612.
- ROBERTSON, D. G. and BULERA, S. J., 2000, High-throughput toxicology: practical considerations. *Current Opinions in Drug Discovery and Development*, **3**, 42–47.
- RODRIGUES, A. D., SURBER, B. W., YAO, Y., WONG, S. L. and ROBERTS, E. M., 1997, [O -ethyl ^{14}C]phenacetin O -deethylase activity in human liver microsomes. *Drug Metabolism and Disposition*, **25**, 1097–1100.
- ROSENBERG, S. H., SPINA, K. P., WOODS, K. W., POLAKOWSKI, J., MARTIN, D. L., YAO, Z., STEIN, H. H., COHEN, J. and BARLOW, J. L., 1993, Studies directed toward the design of orally active renin inhibitors. 1. Some factors influencing the absorption of small peptides. *Journal of Medicinal Chemistry*, **36**, 449–459.
- RUELLE, P. and KESSELING, V. W., 1998, The hydrophobic effect. 2. Relative importance of the hydrophobic effect on the solubility of hydrophobes and pharmaceuticals in H-bonded solvents. *Journal of Pharmaceutical Sciences*, **87**, 998–1014.
- RYU, D.-Y., LEVI, P. E., FERNANDEZ-SALGUERO, P., GONZALEZ, F. J. and HODGSON, E., 1996, Piperonyl butoxide and acenaphthylene induce cytochrome P450 1A2 and 1B1 mRNA in aromatic hydrocarbon response receptor knock-out mouse liver. *Molecular Pharmacology*, **50**, 443–446.
- SADOWSKI, J. and KUBINYI, H., 1998, A scoring scheme for discriminating between drugs and non-drugs. *Journal of Medicinal Chemistry*, **41**, 3325–3329.
- SAWAI, Y., YAMAOKA, K. and NAKAGAWA, T., 1998, Effect of coadministered uridine on intestinal first-pass metabolism of 5'-deoxy-5-fluorouridine in conscious rats — an evaluation by method of portal-systemic concentration difference. *Pharmaceutical Research*, **15**, 1007–1011.
- SEELIG, A., 1998, A general pattern for substrate recognition by P -glycoprotein. *European Journal of Biochemistry*, **251**, 252–261.
- SEGALL, M. D., PAYNE, M. C., ELLIS, S. W., TUCKER, G. T. and BOYES, R. N., 1997, *Ab initio* molecular modeling in the study of drug metabolism. *European Journal of Drug Metabolism and Pharmacokinetics*, **22**, 283–289.
- SHAH, A. V., WALTERS, W. P. and MURCKO, M. A., 1998, Can we learn to distinguish between 'drug-like' and 'non-drug-like' molecules? *Journal of Medicinal Chemistry*, **41**, 3314–3324.
- SHAROM, F. J., YU, X., LU, P., LIU, R., CHU, J. W. K., SZABO, K., MULLER, M., HOSE, C. D., MONKS, A., VARADI, A., SEPRODI, J. and SARKADI, B., 1999, Interaction of the P -glycoprotein multidrug transporter (MDR1) with high affinity peptide chemosensitizers in isolated membranes, reconstituted systems, and intact cells. *Biochemical Pharmacology*, **58**, 571–586.

- SHITARA, Y., SUZUKI, H., KATO, Y., SUGIYAMA, D., TOKUI, T., ABE, T., ITOH, T. and SUGIYAMA, Y., 2000, Comparative effects of inhibitors on rat OATP1- and OATP2-mediated transport: investigation of specific inhibitors. *Drug Metabolism Reviews*, **32**, 194.
- SILVA, J. M., MORIN, P. E., DAY, S. H., KENNEDY, B. P., PAYETTE, P., RUSHMORE, T., YERGEY, J. A. and NICOLL-GRITH, D. A., 1998, Refinement of an *in vitro* cell model for cytochrome P450 induction. *Drug Metabolism and Disposition*, **26**, 490–496.
- SIMPSON, H., BARTHEMY, A., BUHRMAN, D., BURTON, R., NEWTON, J., KEALY, M. and WU, D., 1998, High throughput liquid chromatography/mass spectrometry bioanalysis using 96-well disk solid phase extraction plates for the sample preparation. *Rapid Communications in Mass Spectrometry*, **12**, 75–82.
- SINKO, P. J., 1999, Drug selection in early drug development: screening for acceptable pharmacokinetic properties using combined *in vitro* and computational approaches. *Current Opinions in Drug Discovery and Development*, **2**, 42–48.
- SINKO, P. J., LEESMAN, G. D., WACLAWSKI, A. P., YU, H. and KOU, J. H., 1996, Analysis of intestinal perfusion data for highly permeable drugs using a numerical aqueous resistance-non-linear regression method. *Pharmaceutical Research*, **13**, 570–576.
- SMITH, D. A., 1998, High throughput drug metabolism. *Biomedical Health Research*, **25**, 137–143.
- STEIN, W. D. and HOSHEN, M. B., 1996, The role of ligand-binding proteins in increasing the transcellular flux of metabolites, in particular that of calcium, bilirubin, and fatty acids. *Comparative Biochemistry and Physiology, Part B: Biochemistry and Molecular Biology*, **115**, 307–312.
- STEWART, B. H., CHAN, O. H., JEZYK, N. and FLEISHER, D., 1997, Discrimination between drug candidates using models of intestinal absorption. *Advances in Drug Delivery Reviews*, **23**, 27–45.
- SUGIYAMA, Y. and SUZUKI, H., 2000, Role of MRP2 and MRP3 in the xenobiotics detoxification in the liver and small intestine. *Drug Metabolism Reviews*, **32**, 137.
- SURRY, D. D., MCALLISTER, G., MENESES-LORENTE, G. and EVANS, D. C., 1999, High throughput ribonuclease protection assay for the determination of CYP3A mRNA induction in cultured rat hepatocytes. *Xenobiotica*, **29**, 827–838.
- SWANN, P. W. and TUKKER, J. J., 1997, Molecular determinants of recognition for the intestinal peptide carrier. *Journal of Pharmaceutical Sciences*, **86**, 596–602.
- SZLARZ, G. D. and HALPERT, J. R., 1997, Molecular modeling of cytochrome P450 3A4. *Journal of Computer Aided Molecular Design*, **11**, 265–272.
- TAKAHASHI, L., 2000, Rapid permeability screening using MDCK cells in a novel device. Paper presented at the Cambridge Healthtech Institutes 2nd Annual Conference: Smarter Lead Optimization, Baltimore, MD, 16–17 March.
- TARBIT, M. H. and BERMAN, J., 1998, High throughput approaches for evaluating absorption, distribution, metabolism and excretion properties of lead compounds. *Current Opinion in Chemical Biology*, **2**, 411–416.
- TASKINEN, J., 2000, Prediction of aqueous solubility in drug design. *Current Opinions in Drug Discovery and Development*, **3**, 102–107.
- TODD, M. D. and ULRICH, R. G., 1999, Emerging technologies for accelerated toxicity evaluation of potential drug candidates. *Current Opinions in Drug Discovery Development*, **2**, 58–68.
- TOMINAGA, Y., 1999, Comparative study of class data analysis with PCA-LDA, SIMCA, PLS, ANNs and k-NN. *Chemometrics and Intelligent Laboratory Systems*, **49**, 105–115.
- TROPSHA, A., 2000, Recent trends in computer-aided drug discovery. *Current Opinions in Drug Discovery and Development*, **3**, 310–313.
- VAN BREEMEN, R. B., NIKOLIC, D. and BOLTON, J. L., 1998, Metabolic screening using on-line ultrafiltration mass spectrometry. *Drug Metabolism and Disposition*, **26**, 85–90.
- VAN DE WATERBEEMD, H., CAMENISCH, G., FOLKERS, G. and RAEVSKY, O. A., 1996, Estimation of Caco-2 cell permeability using calculated molecular descriptors. *Quantitative Structure Activity Relationships*, **15**, 480–490.
- WALLACE, R. W., 1997, High-throughput screening. HTS and gene chip technology. *Drug Discovery Today*, **2**, 557–558.
- WALLACE, R. W., 1999, Pharmacogenomics: the next logical step. *Drug Discovery Today*, **4**, 105–107.
- WALTERS, W. P., STAHL, M. T. and MURCKO, M. A., 1998, Virtual screening — an overview. *Drug Discovery Today*, **3**, 160–178.
- WATT, A. P., MORRISON, D. and EVANS, D. C., 2000, Approaches to higher throughput pharmacokinetics (HTPK) in drug discovery. *Drug Discovery Today*, **5**, 17–24.
- WESSEL, M. D., JURS, P. C., TOLAN, J. W. and MUSKAL, S. M., 1998, Prediction of human intestinal absorption of drug compounds from molecular structure. *Journal of Chemical Information and Computer Sciences*, **38**, 726–735.
- WINIWARTER, S., BONHAM, N. M., AX, F., HALLBERG, A., LENNERNAS, H. and KARLEN, A., 1998, Correlation of human jejunal permeability (*in vivo*) of drugs with experimentally and theoretically derived parameters. A multivariate data analysis approach. *Journal of Medicinal Chemistry*, **41**, 4939–4949.

- WOLF, R. C. and HENDERSON, C. J., 1998, Use of transgenic animals in understanding molecular mechanisms of toxicity. *Journal of Pharmacy and Pharmacology*, **50**, 567–574.
- WORBOYS, P. D. and CARLILE, D. J., 2001, Implications and consequences of enzyme induction on preclinical and clinical drug development. **31**, 469–486.
- WORTELBOER, H. M., DEKRUIF, C. A., VAN IERSEL, A. A. J., FALKE, H. E. and NOORDHOEK, J., 1991, Comparison of cytochrome P450 isozyme profiles in rat liver and hepatocyte cultures. The effects on apoproteins and biotransformation activities. *Biochemical Pharmacology*, **42**, 381–390.
- WRING, S. A., HARMAN, K. A., BRUNER, J., POLLI, J. W. and SERABJIT-SINGH, C., 2000, Automated plasma protein binding screen: rapid, robust and timely for drug discovery. *Drug Metabolism Reviews*, **32**, 179.
- WYNALDA, M. A. and WIENKERS, L. C., 1997, Assessment of potential interactions between dopamine receptor agonists and various human cytochrome P-450 enzymes using a simple *in vitro* inhibition screen. *Drug Metabolism and Disposition*, **25**, 1211–1214.
- YAMAZAKI, M., SUZUKI, H. and SUGIYAMA, Y., 1996, Recent advances in carrier-mediated hepatic uptake and biliary excretion of xenobiotics. *Pharmaceutical Research*, **13**, 497–513.
- YANG, R. S. H., THOMAS, R. S., GUSTAFSON, D. L., CAMPAIN, J., BENJAMIN, S. A., VERHAAR, H. J. M. and MUMTAZ, M. M., 1998, Approaches to developing alternative and predictive toxicology based on PBPK/PD and QSAR modeling. *Environmental Health Perspectives*, **106**, 1385–1393.
- YIN, H., LI, S., RACHA, J., OLEJNIK, N., SATOH, H. and MOORE, D., 2000, Automated high throughput human CYP isoform activity assay using SPE-LC/MS method: application in CYP inhibition evaluation. *Xenobiotica*, **30**, 141–154.
- ZHANG, J. H., CHUNG, T. D. Y. and OLDENBERG, K. R., 2000, Confirmation of primary active substances from high throughput screening of chemical and biological populations: a statistical approach and practical considerations. *Journal of Combinatorial Chemistry*, **2**, 258–265.

Utility of metabolic stability screening: comparison of *in vitro* and *in vivo* clearance

S. E. CLARKE* and P. JEFFREY

Drug Metabolism and Pharmacokinetics, GlaxoSmithKline Pharmaceuticals,
The Frythe, Welwyn AL6 9AR, UK

Received 15 February 2001

1. The ability of hepatic microsomal metabolic stability assessments to predict *in vivo* clearance in rat has been retrospectively evaluated for 1163 compounds from 48 programmes of chemistry. Using a simple binary classification system, the *in vivo* clearances of ~64% of the compounds were correctly classified.

2. About 24% of compounds were metabolically stable yet had clearance greater than half of liver blood flow *in vivo*. This might be expected as microsomes only contain a limited number of fully functioning drug-metabolizing enzymes and cannot be expected to account for extrahepatic or non-metabolic clearance processes.

3. About 13% of compounds had *in vivo* clearances of less than half liver blood flow despite being classified as metabolically unstable. Despite overcoming metabolic instability, these compounds had other undesirable properties and were generally more highly bound to plasma proteins, had smaller volumes of distribution (and shorter half-lives despite their clearance) and were more inhibitory against the major human cytochrome P450s.

4. Taking plasma protein binding into consideration reduced the proportion of misclassified low-clearance compounds but did not improve the overall success appreciably. Somewhat surprisingly, human microsomes were nearly as effective as rat microsomes at classifying rat *in vivo* clearance.

Introduction

The processes of using *in vitro* approaches to predict various aspects of the metabolism and pharmacokinetics of drugs is widely known and practised in the pharmaceutical industry (Rodrigues 1997). The ability to study the most relevant species (human) at the earliest stages, high throughput and the potential reduction in the requirement of animals offers both commercial and ethical advantages. Although there are many possible applications of various *in vitro* tools, one that is widely applied involves the use of hepatic subcellular fractions to predict metabolic clearance. The liver is the main organ involved in the metabolism of xenobiotics and this action is the major determinant of the clearance of many drugs.

The relationship between clearance and oral bioavailability is well established (Wilkinson and Shand 1975, Benet and Zia-Amirhosseini 1995) and reflects the fact that compounds with high hepatic extraction ratios will have high clearances and therefore the maximum oral bioavailability will be low. Conversely, compounds with low hepatic extraction ratios will have lower clearances and therefore the maximum oral bioavailability will be high (assuming complete absorption from

* Author for correspondence; stephen_e_clarke@gsk.com

the gastrointestinal tract and no pre-absorptive metabolism). Since clearance is the measure of the ability of the body to eliminate a drug, the impact on oral bioavailability can be determined from both *in vivo* and *in vitro* experiments, it is therefore often used as a pharmacokinetic screen in compound selection.

There have been many publications demonstrating a good relationship between *in vitro* and *in vivo* clearance for a particular drug and several attempts systematically to evaluate this type of approach with somewhat larger numbers of compounds (Houston and Carlile 1997, Carlile *et al.* 1999, Obach 1999, Schneider *et al.* 1999). However, the scale of these activities and the inability to publish 'failed' correlations does not allow an extensive evaluation of these methods in the optimization of clearance for new chemical entities. The present paper summarizes the effectiveness of the use of *in vitro* clearance to predict *in vivo* clearance for 1163 compounds from 48 programmes of chemistry at SmithKline Beecham Pharmaceuticals over the last 5 years.

Materials and methods

Determination of in vivo clearance

In vivo clearance was determined in rat following either intravenous (i.v.) infusion or infusion to steady-state. Following i.v. infusion (at doses ranging from 1 to 3 mg kg⁻¹ infused over 1 or 2 h, depending on compound solubility, pharmacological activity, etc.) serial blood (or plasma) samples were obtained and analysed for parent compound concentrations using LC/MS/MS methodologies. Terminal elimination rate constants (λ_z) were determined by linear regression of the log blood (or plasma) concentrations. The area under the blood (or plasma) concentration-time curve (AUC) was calculated to the last datum point using the combined linear-logarithmic trapezoidal method. AUC ∞ was calculated from extrapolation from the last datum point to infinity using λ_z . Clearance (CL) was calculated as CL $_i$ = dose/AUC ∞ . Volume of distribution at steady-state (V_{ss}) was calculated as V_{ss} = CL · MRT, where MRT is the mean residence time calculated as MRT = AUMC ∞ /AUC ∞ - τ /2, where AUMC ∞ is the area under the first moment curve extrapolated to infinity and τ is total infusion time.

Alternatively, following i.v. infusion to steady-state (at doses ranging from 0.25 to 1 mg kg⁻¹ h⁻¹ infused over 6–18 h, depending on compound solubility, pharmacological activity and half-life), blood (or plasma) samples were obtained during the last 2 h of the infusion to verify steady-state concentrations and CL was calculated using CL = R/C_{ss} , where R is the infusion rate and C_{ss} is the steady state blood (or plasma) concentration.

In the current evaluation there was no attempt to differentiate between compounds with respect to blood or plasma clearances and the values quoted were limited to the range 1–300 ml min⁻¹ kg⁻¹.

Determination of in vitro clearance

The microsomal fraction from the pooled livers of 10 male Sprague-Dawley rats (CrI:CD [SD] BR) (225–250 g) obtained from Charles River Breeding Laboratories (Raleigh, NC, USA) or five humans obtained from Vitron, Inc. (Tuscon, AZ, USA) or the International Institute for the Advancement of Medicine (Exton, PA, USA) was prepared by differential centrifugation as described by Clarke *et al.* (1994). The data presented here are from several such preparations over years. The microsomes were suspended in 50 mM potassium phosphate buffer (pH 7.4) at less than -70°C until use. To determine the metabolic stability the compounds, at a concentration of between 0.5 and 1 μ M, were incubated in this buffer at 0.5 mg microsomal protein ml⁻¹ at 37°C with 50 μ l ml⁻¹ of an NADPH generating system (7.8 mg glucose 6-phosphate, 1.7 mg NADP and 6 U glucose 6-phosphate dehydrogenase ml⁻¹ 2% w/v NaHCO₃) for up to 30 min. At various time points (generally eight) an aliquot (50 μ l) was taken, quenched with solvent, containing an appropriate internal standard, and analysed by LC/MS. Co-factor controls at zero and 30 min were also prepared and analysed. The *in vitro*, or intrinsic, clearance (CL_i) was determined from the first-order elimination constant by non-linear regression using Grafit vv. 3.09–4.12 (Erithacus Software, UK), using the actual volume of the incubation and assuming 52.5 mg microsomal protein g⁻¹ liver; clearances were expressed in units of ml min⁻¹ g⁻¹ liver. The lower limit of quantitation of clearance was determined as where <15% of the compound was metabolized by 30 min and this corresponded to 0.5 ml min⁻¹ g⁻¹ liver. The upper limit was determined as where compound could be quantified for at least the first three time points and this corresponded to 50 ml min⁻¹ g⁻¹ liver.

Determination of plasma protein binding

Duplicate samples of rat and human plasma (1 ml) containing compound (at typical target concentrations of 250, 100 and 5000 ng ml⁻¹) were dialysed (Diachema membrane discs, 10 000 molecular weight cut-off) against isotonic 0.01 M phosphate-buffered saline (PBS), pH 7.4 (1 ml), for 5 h at 37°C in a rotating (~30 rpm) dialyser (Spectra/Por equilibrium dialyser). Following dialysis, compound concentrations were determined in samples of plasma and PBS by LC/MS/MS.

Determination of cytochrome P450 inhibition

The cytochrome P450 inhibitory potential was determined against CYP1A2, 2C9, 2C19, 2D6 and 3A4 using microsomes from human β -lymphoblastoid cells expressing the appropriate cytochrome P450 (Gentest Corporation). The inhibition of activity was determined by a fluorescent plate reader methodologies similar to those described by Crespi *et al.* (1997) from an eight-point IC₅₀ curve.

Results and discussion

The comparison of rat intrinsic and *in vivo* clearance for 1163 compounds is shown in figure 1. As might be expected, there is a trend towards higher *in vivo* clearance with increasing intrinsic clearance. Since no model of hepatic function (e.g. venous equilibrium or 'well-stirred' model) has been applied to the *in vitro* data, a linear relationship would not be anticipated. Equally in the context of high throughput metabolism screening, a prediction for clearance is not the objective, it being rather a rank ordering of potential candidates or a broad classification. For this analysis the latter approach was used and compounds were classified as high or low clearance. The cut-offs (figure 1) are somewhat arbitrary and represent approximately half of liver blood flow (i.e. 45 ml min⁻¹ kg⁻¹) for the *in vivo* data

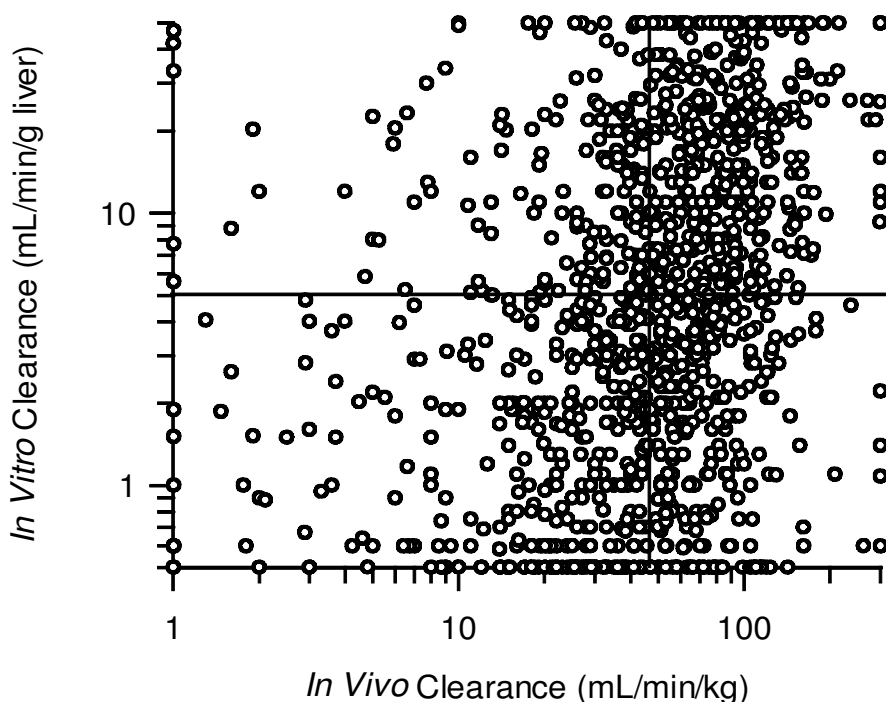


Figure 1. Comparison of rat *in vivo* and *in vitro* clearance for 1163 compounds. The horizontal and vertical lines represent the cut-offs used for the analysis in table 1.

and halfway between the upper and lower limit of quantitation for the intrinsic clearance ($5 \text{ ml min}^{-1} \text{ g}^{-1}$ liver). Clearly in practice one would not differentiate between 45 and $46 \text{ ml min}^{-1} \text{ kg}^{-1}$ or 5.0 and $5.1 \text{ ml min}^{-1} \text{ g}^{-1}$ liver, but, given the density of the data, providing an intermediate category does not appreciably alter the overall conclusions.

Least problematical are those compounds that are in the upper right and lower left quadrants, the true-negatives and true-positives, respectively. The true-negatives are those compounds that had poor metabolic stability properties (high intrinsic clearance) and also had high *in vivo* clearance. The true-positives being both metabolically stable and having low *in vivo* clearance. Together these can be considered to have been categorized correctly and this is the case for $\sim 64\%$ of the compounds (table 1). About 24% of compounds were false-positives, that is metabolically stable compounds that none the less had high clearance *in vivo*. Given the simplicity of subcellular fractions, it is not surprising that a significant number of compounds are false-positives. Microsomes may not contain certain drug-metabolizing enzymes (e.g. sulphotransferase, *N*-acetyl transferase, aldehyde oxidase) or the enzymes involved are relatively ineffective under the conditions used, even if the appropriate co-factors had been included (e.g. glucuronosyl-transferase). Equally such a system could not be expected to encompass extra-hepatic metabolism or non-metabolic clearance such as biliary or renal elimination through the action of transporters. Additionally the allosteric behaviour of certain cytochrome P450 enzymes, particularly CYP3A, would indicate that maximum intrinsic clearance would not be achieved at the low concentrations used (Kenworthy and Houston 2000). However, the dynamics of how such an *in vitro* tool is typically applied in the pharmaceutical industry are such that these misclassifications are not significant. Generally compounds would be tested *in vitro* first and those that appeared metabolically stable would then be evaluated *in vivo*. The false-positives would represent a disappointment but not a missed opportunity. Far more significant are the false-negatives, compounds that were metabolically unstable but had low clearance *in vivo*, since these compounds would have been rejected on the basis of the *in vitro* data alone.

Even if compounds are misclassified in this binary analysis, those compounds that are metabolically stable have a lower clearance than those that are metabolically labile. False-negatives have a median *in vivo* clearance of 30 compared with

Table 1. Binary analysis of the relationship between *in vitro* and *in vivo* clearance. Values are the percentage of compounds in each category. The cut-offs defining low and high clearance were $45 \text{ ml min}^{-1} \text{ kg}^{-1}$ (rat *in vivo*), $5 \text{ ml min}^{-1} \text{ g}^{-1}$ liver (rat and human *in vitro*) and $0.25 \text{ ml min}^{-1} \text{ g}^{-1}$ liver (rat using plasma protein binding, f_u).

	<i>n</i>		Rat <i>in vivo</i>	
			Low	High
Rat	1163	low	29.3	23.5
		high	12.9	34.3
<i>In vitro</i> rat with f_u	489	low	30.3	26.4
		high	8.1	35.2
	1127	low	27.7	23.7
Human		high	15.2	33.4

n, Number of compounds analysed.

$24\text{ ml min}^{-1}\text{ kg}^{-1}$ for true-positives, and false-positives are better than true-negatives, with median clearances of 68 and $83\text{ ml min}^{-1}\text{ kg}^{-1}$, respectively. So even where compounds are misclassified, the tendency is still towards selecting compounds with a better *in vivo* clearance. However, the existence of false-negatives represents the biggest barrier to the successful use of these *in vitro* approaches. Of course, if the *in vitro* criteria were applied rigidly, such shortcomings would not be uncovered. However, this data set includes compounds from many programmes of activity where *in vitro* criteria were applied spasmodically or not at all. Additionally, there was an attempt to obtain *in vitro* data whenever *in vivo* data were available. Whatever the limits of bias, it is important to investigate the nature of the false-negatives.

The most obvious parameter to consider is plasma protein binding since according to models of hepatic function (Houston and Carlile 1997) high protein binding would be expected to ameliorate the high intrinsic clearance. For 489 of the 1163 compounds, the fraction unbound had been determined in rat plasma. As might have been expected, the plasma protein binding of the false-negatives (data for 60 false-negatives) was significantly higher than the rest of the compounds (figure 2). For the false-negatives the incidence of compounds with $>99\%$ binding was twice that of the other compounds (figure 2). Repeating the analysis of figure 1 using $\text{CL}_{\text{i}}f_{\text{u}}$ did reduce the incidence of false-negatives to some extent (table 1), but otherwise had little significant on the overall classification accuracy. Hence plasma protein binding does allow the underlying mechanism for some false-negatives to be demonstrated, but it is questionable whether determining it is worthwhile as part of the primary screening process for clearance.

Compounds with very high protein binding may have a limited volume of distribution (V_{d}) and the V_{d} of the false-negatives (data for 117 compounds) was

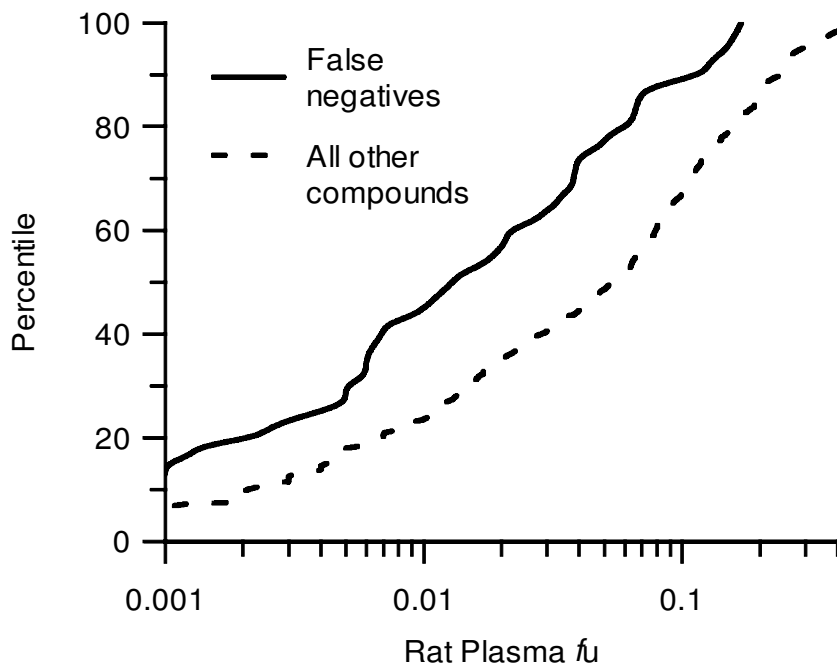


Figure 2. Probit plots for plasma protein binding. fu, Fraction of the compound unbound to plasma.

generally lower (figure 3) than the other compounds (data for 730); over twice the incidence of compounds with $V_d < 1 \text{ l kg}^{-1}$. As a consequence, despite their acceptable clearance, fewer of the false-negative compounds had an acceptable half-life (defined here as $> 1.5 \text{ h}$) than the true-positives (54 versus 66%). As well as a shorter half-life, compounds with a small V_d may have limited distribution to their site of action and be therapeutically ineffective.

Saturation of metabolism is another potential mechanism for low *in vivo* clearance despite metabolic instability. Many of the compounds (1058) in this data set have been evaluated for their ability to inhibit the major human cytochrome P450 enzymes (CYP1A2, 2C9, 1C19, 2D6 and 3A4) and the false-negatives (data for 140 compounds) are generally more inhibitory than the other compounds (figure 4). Since there are known to be significant differences between the inhibitory effects of xenobiotics on rat and human cytochrome P450s, this pattern can only be considered in general terms. However, saturation of metabolism *in vivo* has been shown, on several occasions, to be the likely mechanism for false-negative results for compounds in this data set.

Despite human intrinsic clearance explaining only 25% of the observed variability ($r^2 = 0.25$, $p < 0.001$) in rat intrinsic clearance (figure 5), the human data classified rat *in vivo* clearance nearly as well as the rat data (table 1). Despite several notable examples of species differences there would appear to be little advantage in determining the intrinsic clearance of both species in the context of high throughput metabolism screening. Conversely rat data might be expected to be similarly effective at classifying human *in vivo* clearance data. These results would suggest that human microsomes alone would be the most efficient approach. They are nearly as effective at classifying rat *in vivo* clearance as the rat microsomes and

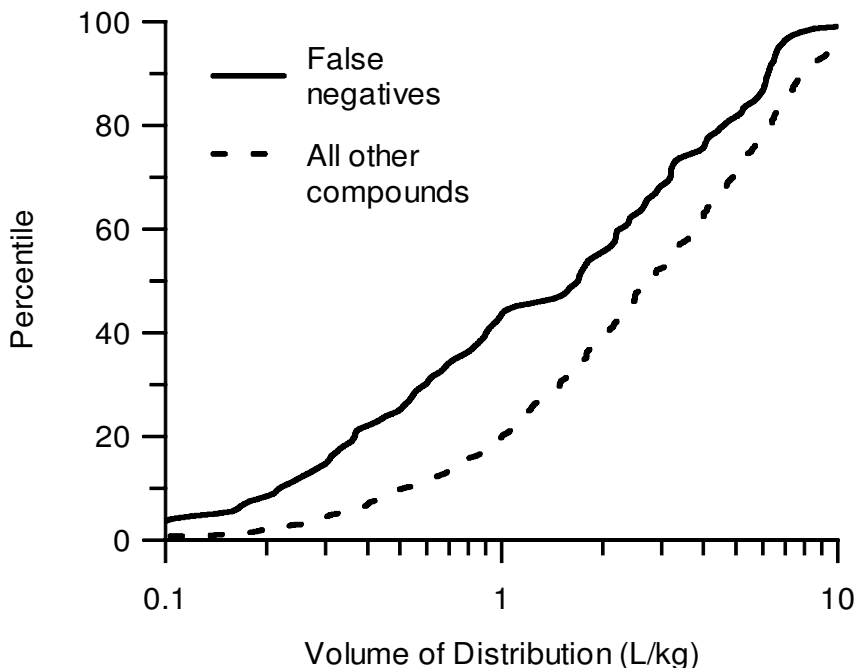


Figure 3. Probit plots for volume of distribution.

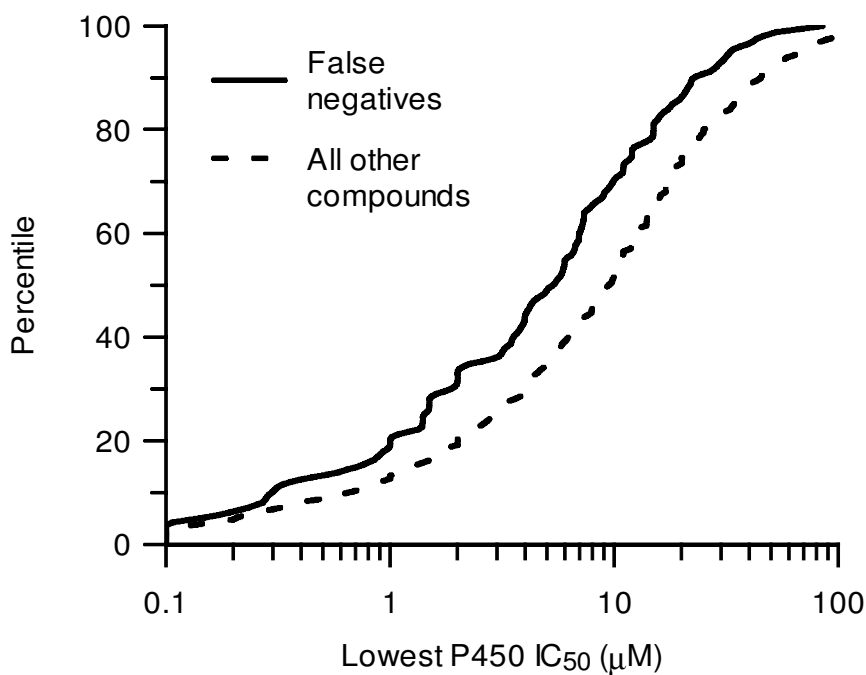


Figure 4. Probit plots for cytochrome P450 inhibition.

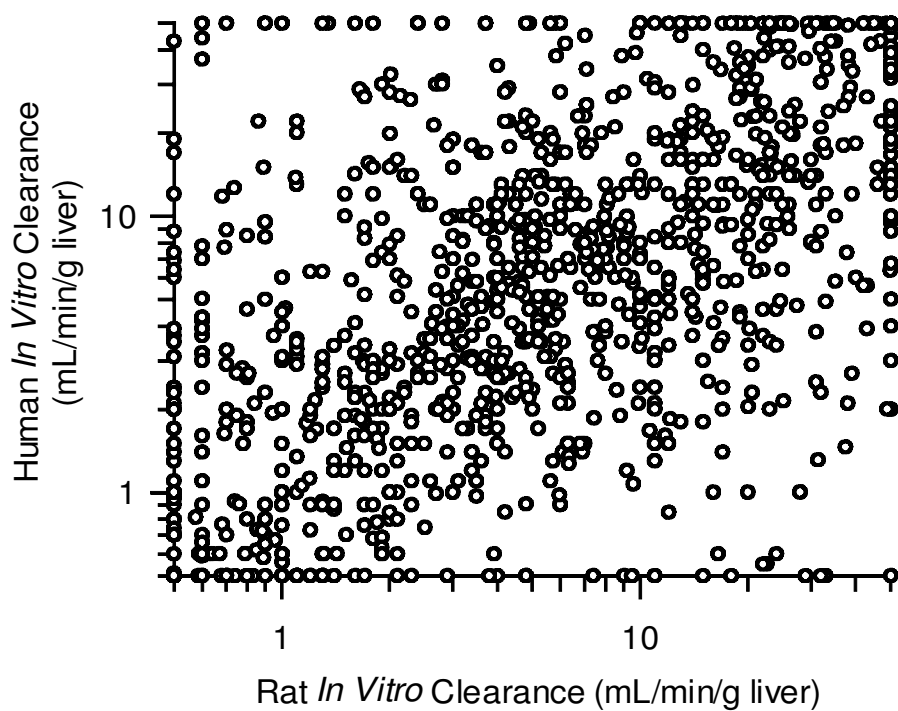


Figure 5. Comparison of rat and human *in vitro* clearance for 1127 compounds.

would be expected to be somewhat more effective at identifying compounds for the ultimate target species for pharmaceuticals – man. However, human tissue is a limited resource and another effective approach would be to use rat microsomes as the primary test and reserve human tissue for the secondary evaluation of those compounds which showed encouraging results, or for those programmes of chemistry where a consistent species difference has been identified.

Overall this data set clearly demonstrates some of the potential advantages and limitations of using *in vitro* methods as a high-throughput screening tool to predict clearance. The biggest limitation is the elimination of compounds from consideration that may have favourable clearance kinetics – the false-negatives. The compounds that achieve this do so in such a way that adversely effects other aspects of their pharmacokinetic profile, as described above. Eliminating those compounds that have these undesirable traits, despite their low clearance and applying a simple set of computational rules (e.g. Lipinski *et al.* 1997), leaves only seven compounds and none of these are being pursued further. Whatever their merits, it is probably not an effective use of resources to design a screening strategy that can account for atypical compounds that represent <1% of the total. Thus, overall, this *in vitro* approach is an effective means of selecting compounds that are likely to have desirable pharmacokinetic properties.

Acknowledgements

The authors acknowledge the staff, past and present, from the Drug Metabolism and Pharmacokinetics Department at SmithKline Beecham who were responsible for all the experimental results described.

References

- BENET, L. Z. and ZIA-AMIRHOSSEINI, P., 1995, Basic principles of pharmacokinetics. *Toxicologic Pathology*, **23**, 115–123.
- CARLILE, D. J., HAKOOZ, N., BAYLISS, M. K. and HOUSTON, J. B., 1999, Microsomal prediction of *in vivo* clearance of CYP2C9 substrates in humans. *British Journal of Clinical Pharmacology*, **47**, 625–635.
- CLARKE, S. E., BALDWIN, S. J., BLOOMER, J. C., AYRTON, A. D., SOZIO, R. S. and CHENERY, R. J., 1994, Lauric acid as a model substrate for the simultaneous determination of cytochrome P450 2E1 and 4A in hepatic microsomes. *Chemical Research in Toxicology*, **7**, 836–842.
- CRESPI, C. L., MILLER, V. P. and PENMAN, B. W., 1997, Microtiter plate assays for inhibition of human, drug-metabolizing cytochromes P450. *Analytical Biochemistry*, **248**, 188–190.
- HOUSTON, J. B. and CARLILE, D. J., 1997, Prediction of hepatic clearance from microsomes, hepatocytes, and liver slices. *Drug Metabolism Reviews*, **29**, 891–922.
- HOUSTON, J. B. and KENWORTHY, K. E., 2000, *In vitro-in vivo* scaling of CYP kinetic data not consistent with the classical Michaelis–Menten model. *Drug Metabolism and Disposition*, **28**, 246–254.
- LIPINSKI, C. A., LOMBARDO, F., DOMINY, B. W. and FEENEY, P. J., 1997, Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. *Advanced Drug Delivery Reviews*, **23**, 3–25.
- OBACH, R. S., 1999, Prediction of human clearance of twenty-nine drugs from hepatic microsomal intrinsic clearance data: an examination of *in vitro* half-life approach and nonspecific binding to microsomes. *Drug Metabolism and Disposition*, **27**, 1350–1359.
- RODRIGUES, A. D., 1997, Preclinical drug metabolism in the age of high-throughput screening: an industrial perspective. *Pharmaceutical Research*, **14**, 1504–1510.
- SCHNEIDER, G., COASSOLO, P. and LAVE, T., 1999, Combining *in vitro* and *in vivo* pharmacokinetic data for prediction of hepatic drug clearance in humans by artificial neural networks and multivariate statistical techniques. *Journal of Medicinal Chemistry*, **42**, 5072–5076.
- WILKINSON, G. R. and SHAND, G. G., 1975, A physiological approach to hepatic drug clearance. *Clinical Pharmacology and Therapeutics*, **18**, 377–390.

Quantitative analysis of pharmaceuticals in biological fluids using high-performance liquid chromatography coupled to mass spectrometry: a review

R. S. PLUMB, G. J. DEAR, D. N. MALLETT*, D. M. HIGTON, S. PLEASANCE and R. A. BIDDLECOMBE

Department of Bioanalysis and Drug Metabolism, GlaxoWellcome R&D, Park Road, Ware SG12 0DP, UK

Received 9 February 2001

1. The development of bio-analysis of drug molecules over the last 10 years is reviewed, focusing on advances in sample preparation, liquid chromatography and detection.
2. Developments have led to improvements in detection sensitivity, enhancements in specificity and increased capacity.
3. Emerging technologies such as monolithic column chromatography and miniaturized chip-based systems are discussed.

Introduction

The need for quantitative bio-analysis comes primarily from the requirement to demonstrate the exposure of preclinical species and human volunteers to candidate drug compounds during drug development studies. During the development of a drug molecule, it is essential to have a specific, sensitive bio-analytical method capable of resolving the dosed compound from its metabolites and from any endogenous compounds in the matrix. Such resolution can be achieved at the isolation stage by chromatographic resolution or by specificity at the detector. Quantitative drug analysis is also important at the early drug discovery phase, so that candidate molecules' pharmacokinetic parameters can be determined. This allows the comparison of candidate development compounds as well as a compound's elimination and distribution in different sexes and species.

Up until the early 1990s bio-analysis was a very labour-intensive process, both in terms of method development and sample processing. Analytical methods were based on either GC or HPLC employing UV, fluorescence or electrochemical detection. Sample preparation was normally carried out manually employing liquid/liquid extraction or solid-phase extraction (Kawai *et al.* 1995). Both the chromatographic and extraction methods were normally painstakingly developed to ensure sufficient resolution from interfering compounds and sufficient analyte recovery to achieve the required sensitivity. The HPLC methods were usually isocratic, employing columns of 15 or 25 cm in length and 4.6 mm i.d. These conditions typically resulted in analytical run times in the order of 15–30 min (Hempenius *et al.* 1994).

* Author for correspondence; e-mail: DNM7582@GlaxoWellcome.co.uk

The advent of parallel array synthesis has resulted in an increase in the number of compounds generated for pharmacokinetic evaluation. This has resulted in the need for more assays in shorter periods and in a greater number of samples requiring analysis. Along with the need for increased throughput has come the requirement for greater analytical sensitivity to cope with more potent drugs that are dosed at much lower levels, resulting in lower circulating concentrations. These new constraints have required a new generation of analytical technologies to be developed that allow faster method development, higher throughput and increased sensitivity.

The choices of analytical techniques available to the bio-analyst today are wide and varied. This paper has concentrated on an approach that has become the technique of choice for the vast majority of pharmaceutical bio-analysis, namely solid-phase extraction followed by high-performance liquid chromatography coupled to mass spectrometry.

Mass spectrometry

In today's drug-metabolism laboratories, LC/MS is the predominant means of analysis for routine drug quantification. This preference derives from the increased sensitivity, specificity and throughput afforded by LC/MS relative to conventional methodologies such as LC/UV. In the majority of cases LC/MS is now performed using atmospheric pressure ionization (API), where ions are formed from a liquid flow introduced into a source region maintained at atmospheric pressure, from which ions are then sampled into the mass analyser (Henion *et al.* 1993, Bruins 1994, Gelpi 1995).

Electrospray, in various guises, is the most widely used ionization process for drug quantification. It results in the formation of charged droplets from a liquid stream sprayed through a high-voltage capillary (Bruins *et al.* 1987, Fenn *et al.* 1990, Kebarle and Lang 1993). Analysis by electrospray requires the prior formation of analyte-related ions in solution. For non-ionic compounds, analyte-related ions are usually prepared by solution chemistry methods. Simple acid or base modifiers can be added to the HPLC solvents during LC/MS to promote the electrospray process. Ions are emitted into the gas phase as the solvent evaporates and the droplets shrink in size. Electrospray is a 'soft' ionization process and generates ions through simple proton addition (MH^+) or proton abstraction ($[M-H]^-$) in solution.

Pure electrospray is only suitable for capillary HPLC and CE, or conventional HPLC (typically 1 ml min^{-1}) where the post-column eluent is split down to a lower flow rate using a zero dead-volume T-piece. In an effort to extend the range of solvent flow rates amenable to electrospray, several modifications to the process have become common place. Principal among these modifications is pneumatic assistance (Bruins *et al.* 1987). Additional modifications include the application of heat during the droplet formation process to produce thermally assisted electrospray (Chowdhury *et al.* 1990, Lee and Henion 1992). The combination of heat and pneumatic assistance has increased the ease in which conventional HPLC can be coupled to a mass spectrometer fitted with an electrospray source, and this is currently the method of choice for drug quantification from biological extracts.

Another 'soft' ionization technique that has simplified the direct coupling of conventional HPLC and MS is the atmospheric pressure chemical ionization

(APCI) source. In this instance, a heated stream of nitrogen vaporizes the HPLC effluent, and the vapour is ionized by an electrical corona discharge. The discharge produces reagent ions from the HPLC solvent which then ionize the sample. Many references are available in the literature that demonstrate the use of APCI LC/MS for routine drug quantification (Fraser *et al.* 1995, Dear *et al.* 1998, Xu *et al.* 1999). In general, the detection of drugs derived from biological samples is less likely to be affected by co-eluting endogenous components (matrix suppression), when using APCI compared with electrospray. However, labile drug metabolites (e.g. *N*-oxides) are more prone to decomposition during the APCI process compared with electrospray. The choice of APCI versus electrospray for routine drug analysis is therefore compound- and matrix-dependent. The adverse effects of the matrix on analyte ionization can limit the overall accuracy and precision of an LC/MS-based assay; however, these effects can be reduced with a stable-isotope-labelled internal standard.

API interfaces are most commonly coupled with low-resolution quadrupole instruments, but in recent years have also been used for drug assays in conjunction with ion-traps (Wieboldt *et al.* 1998, Bartolucci *et al.* 2000, Darius and Banditt, 2000, Poch *et al.* 2000), magnetic sectors (Blanchflower *et al.* 1993, Poon *et al.* 1995) and time-of-flight mass spectrometers (Zhang *et al.* 2000a, b). Since both electrospray and APCI are soft ionization techniques, analytes of interest can be quantified using the relevant $[MH]^+$ or $[M-H]^{-1}$ ion. When coupled to single-quadrupole instruments, selected target analysis (selected ion monitoring) of the relevant ion can significantly enhance sensitivity, and many examples have been published (Kikuchi *et al.* 1998, Schanzle *et al.* 1999, Bogusz *et al.* 2000, Lachatre *et al.* 2000). However, to increase the specificity of a particular drug assay, it is common practice to use a triple-quadrupole instrument operating in tandem MS mode (MS/MS), whereby an analyte can be selectively quantified based on a parent ion to product ion transition (selected reaction monitoring). The selectivity of MS/MS reduces the need for complete chromatographic resolution of individual components allowing a reduction in analytical run time thus providing higher sample throughput (Chowdhury *et al.* 1999, Steinborner and Henion 1999, Xu *et al.* 2000, Zweigenbaum and Henion 2000). LC/MS/MS can significantly reduce the chromatographic method development time, and has led to the use of so-called 'generic methods' (Ayrton *et al.* 1998a, Scott *et al.* 1999). Figure 1 shows the ion-chromatograms for six cytochrome P450 probe substrates, in this case analysed using LC/MS/MS and selected reaction monitoring (Ayrton *et al.* 1998b). The combination of fast-gradient chromatography (see below) and MS/MS specificity result in an increased sample throughput of ~5–6-fold per sample, compared with the original LC/UV or LC/fluorescence-based assays.

Typically the combined increases in selectivity and sensitivity of LC/MS/MS-based methods provides a >10-fold improvement in the limit of quantification (LOQ) relative to conventional methods using UV or fluorescence detection. For example, Allanson *et al.* (1996) demonstrated an increase in LOQ by a factor of 20 for a 5-HT_{1D} receptor agonist upon redevelopment of the analytical method from HPLC with fluorescence detection to an electrospray-based LC/MS/MS method.

The advent of cheaper, more sensitive, bench-top mass analysers will ensure that LC/MS and LC/MS/MS remain at the cornerstone of the modern analytical laboratory. In addition, the coupling of MS/MS with faster high-performance

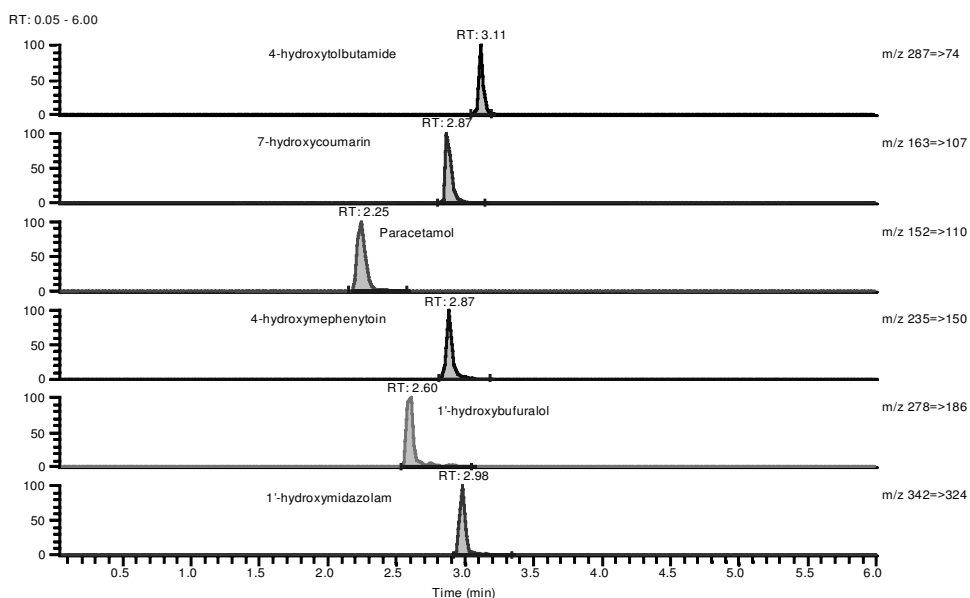


Figure 1. High-throughput analysis of six cytochrome P450 probe substrates using LC/MS/MS. Reprinted from Ayrton *et al.* (1998b) with the permission of John Wiley & Sons Ltd.

chromatographic methods (see below) will ultimately increase sample throughput even further.

Sample preparation

The aim of sample preparation is to isolate selectively the analyte of interest from the bulk of the matrix. The reason for the isolation may be to increase analyte concentration or to remove endogenous material in the sample that may be deleterious to the performance of the chromatography system, such as proteins. The three main sample preparation techniques used within bio-analysis are: solid-phase extraction (SPE), liquid-liquid extraction and protein precipitation (Wright *et al.* 1994, Majors 1998, Hubert *et al.* 1999, Sandahl *et al.* 2000).

SPE is traditionally performed using individual syringe barrel type cartridges, into which is packed bonded or bare silica with particle sizes in the range 40–100 μm . The typical bed size is 50–250 mg and bonding chemistries involved the incorporation of either aliphatic chains or ion-exchange functions. The SPE cartridges are placed on a vacuum box and samples are manually extracted in sequential mode using a method developed specifically for the analyte in question. This method usually consists of a solvent and aqueous prime, sample addition, sample washing and sample elution. The typical elution solvents are high eluotropic-strength HPLC solvents and the usual elution volumes employed are ~ 1 –2 ml. The eluting solvent is then evaporated to dryness and reconstituted in a reduced volume (typically 100–200 μl) of a solvent compatible with the chromatographic system. The extraction and evaporation process for a batch of 50 samples could take in the region of half a day.

Table 1. Comparison of elution volume required to achieve 80% analyte recovery.

	Solid-phase extraction format (all C18)			
	10 mg	25 mg	50 mg	Empore disk
Volume of methanol (μ l)	150	200	500	50

Format and chemistry

The introduction of disk extraction technology such as the Empore material by 3M (Wells *et al.* 1995) and the glass fibre material by Ansis offer the possibility of significantly lower elution volumes (Plumb *et al.* 1997). The elution volumes required to achieve 80% recovery for standard SPE cartridges and disk type materials are compared in table 1. As can be seen, disk-type materials only require elution volumes of $\sim 50 \mu$ l to achieve 80% analyte recovery. Such small elution volumes not only significantly reduce evaporation times, but also offer the possibility of directly injecting the eluent following appropriate dilution of the sample with water or buffer. The development of disk technology resulted in the re-evaluation of the traditional SPE cartridges (Plumb *et al.* 1997). This has led to a significant reduction in bed volume, typically 15 or 20 mg being sufficient for the processing of 100–200 μ l plasma. The use of lower bed volumes has allowed the volumes of wash solvent to be reduced, thus speeding up the process. The use of such low-bed volumes is now widespread and is rapidly becoming the method of choice. The combination of low-bed volume or disk SPE coupled to LC/MS has resulted in the development of rapid, robust, high-sensitivity assays (Blevins *et al.* 1998).

The move to a more rapid, simple method development and the wish to design one method that suits most analytes has resulted in the search for an SPE material that is suitable for a wide range of analyte properties from semi-polar to highly lipophilic. Although alkyl-bonded C18 silica supports have proved very successful as a generic-type phase, their applicability is limited by the pH range in which it can be operated (pH 2–9). The development of polymeric phases such as Waters Oasis has allowed the use of the entire pH range (Cheng *et al.* 1998) and has also offered a different type of chemistry that allowed the analysis of more polar analytes. The ability to use the entire pH range and mixed mode phases allows more selective wash protocols to be used, resulting in cleaner eluents and more sensitive assays.

Automation

The aim of automation is to relieve the scientist of the more mundane manual processes, freeing time for more challenging work. One of the first and most successful attempts to automate the processing of conventional SPE cartridges was the Gilson ASPEC. This system combined the autosampler with the sample processor, performing SPE before injecting the sample onto the chromatography system. Whilst being successful, with many applications being developed (Molina *et al.* 1996), the instrument did have several limitations. For example, it had no

facility for evaporation, therefore the sample had to be eluted in the chromatographic mobile phase, or if the sample was eluted in pure organic solvent only a small fraction of the eluent could be used. The instrument had just one needle, and it operated in a serial-processing mode and was quite slow with one sample taking ~10 min to process before injection.

A successful, if more expensive, alternative to the ASPEC is the Zymate robot (Stubbs and Harker 1995). Zymate systems are multifunctional with a robot arm moving samples from one module to another where a specific process is carried out. On such a machine, several (two to four) samples can be worked on at once. The system can perform SPE, liquid-liquid extraction, pipetting, gravimetric weighing, evaporation, reconstitution, derivatization and injection onto a HPLC system. The disadvantages of such a system are its size, the need for skilled operators, the high cost and the need to dedicate the system to a specific method. However, the system has proved very successful and has been employed for several different methods. It is especially beneficial in the analysis of hazardous samples including the analysis of HIV-positive samples for the development of AIDS treatments (Harker *et al.* 1994).

The drawbacks of these systems were not important whilst isocratic HPLC with UV or fluorescence detection was employed with long run times. The advent of fast chromatography and mass spectrometric detection significantly reduced analytical run times resulting in the requirement for a new approach to sample preparation. The need for faster analysis was answered by the development of 96-well SPE (Allanson *et al.* 1996, Harrison and Walker 1998). This is in essence, a 96 deep-well plate filled with 100 or 200 mg SPE material with a frit and nozzle at the bottom of each well, allowing liquid to pass through the plate once connected to a vacuum manifold. The sample eluent is collected in another 96-well plate, which can then be evaporated on a 96-well dry block, reconstituted and placed directly in the autosampler. These 96-well plates are in the ideal format for processing by a robotic sample processor, such as a Tecan or Multiprobe. These instruments are equipped with either four or eight probes, allowing parallel sample processing, significantly increasing sample extraction throughput (Callejas *et al.* 1998).

It was at this stage that two emerging technologies were combined to give an extremely powerful tool. The reduced sorbent bed and disk materials were combined in a new 96-well format to give fast, parallel sample processing that could reduce the time taken to process a batch of 96 samples to just 30 min (Biddlecombe and Pleasance 1997). This type of system did, however, require a manual intervention step, namely replacing the waste container with a collection plate, which limits the automation. This problem was addressed in the same system by the use of a fully automated robotic system, similar to that employed by Harker *et al.* (1994), but in this case the robot was used to manipulate plates rather than tubes. In such a system, the 96-well plates are moved around to various locations in the working envelope where different tasks are performed on the sample, such as pipetting, extraction, evaporation, reconstitution and plate sealing. These systems allow multi-user and -method interfacing with samples stored in a queue to be prepared and finished sample blocks being stored in a cooled sample 'hotel'. The system has also been extended to place the prepared sample directly onto the HPLC autosampler for injection onto an LC/MS/MS system. An example of such a system is given in figure 2.

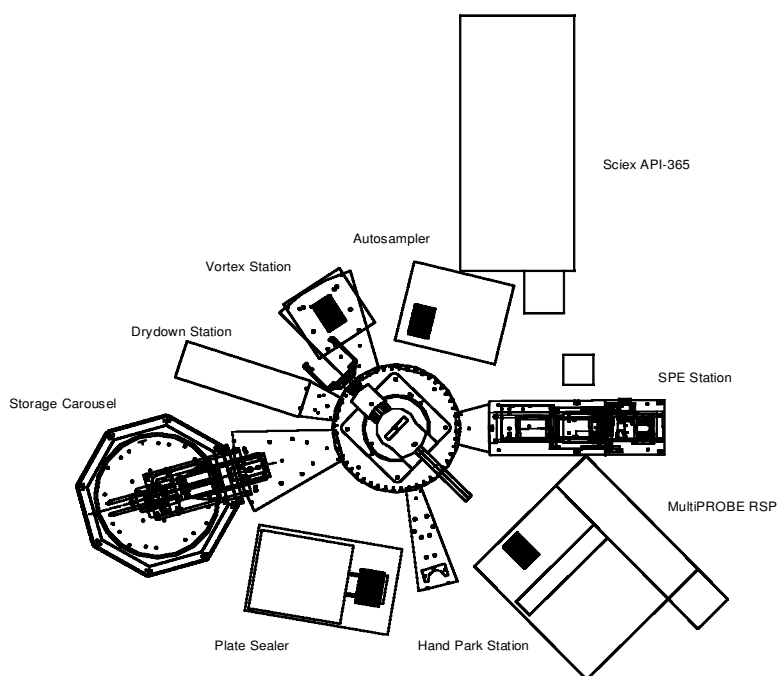


Figure 2. Schematic of a robotic sample processor integrated with an LC/MS system.

The use of protein precipitation is very widespread within the early drug discovery/development area. This process has traditionally been performed manually and indeed is still carried out in this manner for small batch sizes. The robotic sample processing systems have been adapted to allow the processing of samples via protein precipitation using 96-well plates with a simple filter in the bottom (Biddlecombe and Pleasance 1999).

An alternative approach to the use of SPE followed by LC/MS for speeding up sample throughput is the use of on-line sample extraction followed by MS/MS detection; such a system is the ProspektTM from Spark Holland (Hedenmo and Eriksson 1994). This approach employs a small extraction cartridge packed with bonded small particle silica (8–30 μm) and high volumetric flow rate washing, typically 4 ml min^{-1} . Following priming with solvent and aqueous buffer, raw sample is loaded onto the cartridge. The sample is then washed with buffer and then eluted with pure solvent at $100\text{--}200\text{ }\mu\text{l min}^{-1}$ into the mass spectrometer. Such an approach has resulted in high sensitivity methods, in the 1 ng ml^{-1} range, with cycle times of 3–5 min. An example of such a method is the analysis of a 5- α -reductase inhibitor in human plasma (Bowers *et al.* 1997). In this assay a sample throughput of 15 samples h^{-1} was achieved with sensitivity in the pg ml^{-1} range. This approach to sample preparation has been employed with great success in several laboratories.

The automated SPE and ProspektTM type systems are best used in an area where a large number of samples regularly require rapid analysis. To gain maximum advantage from either technology it is best if a decision is made to use just one exclusively. Such focusing of capital investment and training offers benefits from economies of scale.

Chromatography

The advent of combinatorial chemistry and the resulting exponential growth in the number of potential drug compounds entering the early phases of drug development has brought about major changes in the area of quantitative analysis. The emphasis in method development has shifted from a specific assay with conditions tailored to one or two analytes towards a generic approach in which a single method can be used for the bio-analysis of many putative drug candidates. In chromatographic terms, the only way to achieve this aim has been to adopt a gradient HPLC protocol.

In reversed-phase gradient HPLC, the mobile phase changes composition over a specified time. A steady increase in the percentage of organic solvent leads to an increase in the mobile phase elutropic strength and this makes it possible for highly retained non-polar analytes to be analysed within the same run as poorly retained polar analytes. This approach has been extremely successful (Bowers *et al.* 1997, Kerns *et al.* 1997) and has been widely adopted, particularly for drug metabolite profiling (Dear *et al.* 1995). A further development has been to use elevated flow rates and shorter columns, the so-called fast-gradient approach. Within our laboratory, it is fair to say that the great majority of bio-analyses are performed using fast-gradient HPLC on short (5 cm length) narrow-bore (~ 2 mm i.d.) columns with mass spectrometric detection (usually MS/MS). On such systems, where the chromatography is performed at about four times the optimal flow rate of the specified columns, satisfactory resolution and efficiency have been achieved to allow quantification of drugs and metabolites to low ng ml^{-1} sensitivity for biological samples (Ayrton *et al.* 1998). Figure 3 shows clearly that resolution is not compromised as a result of operating fast-gradient analysis on short columns compared with the more traditional gradient HPLC format.

Further to the need for ever-faster method development and, therefore, sample 'turnaround', there has been a requirement for improved sensitivity. There have been two drivers for this; (1) enhanced drug potencies are resulting in lower dosages and thus lower concentrations of drug in biological fluids, and (2) the desire to obtain pharmacokinetic data ever earlier in the drug-discovery process has led to the wish to perform these studies in smaller, and fewer animals, thereby using less drug compound. The practical outcome is that small volumes of sample are obtained from the serial bleeding of small rodents. The challenge posed to the bio-analyst by small volume samples is to achieve the same concentration sensitivity when analysing a 50 or 100 μl sample, as was possible from a 1 ml sample. The solution to this need to enhance sensitivity by an order of magnitude is provided by chromatographic miniaturization.

As most LC detectors are concentration sensitive, higher sensitivities can be realised by the elution of the analyte from the column in a reduced volume of mobile phase (Church and Justice 1989). For a conventional HPLC column of 4.6 mm i.d., a flow rate of 1 ml min^{-1} is typical. To maintain the same linear velocity for the mobile phase, a 300 μm i.d. column would need to be operated at $\sim 4 \mu\text{l min}^{-1}$. For equivalent efficiency systems, i.e. maintenance of the same peak width, this represents an ~ 250 -fold increase in concentration of an analyte as it elutes from the column and passes the point of detection. Although the theoretical gains in sensitivity are not generally achieved, sufficient gains have been achieved for capillary-scale LC to provide the required improved sensitivity (Plumb *et al.*

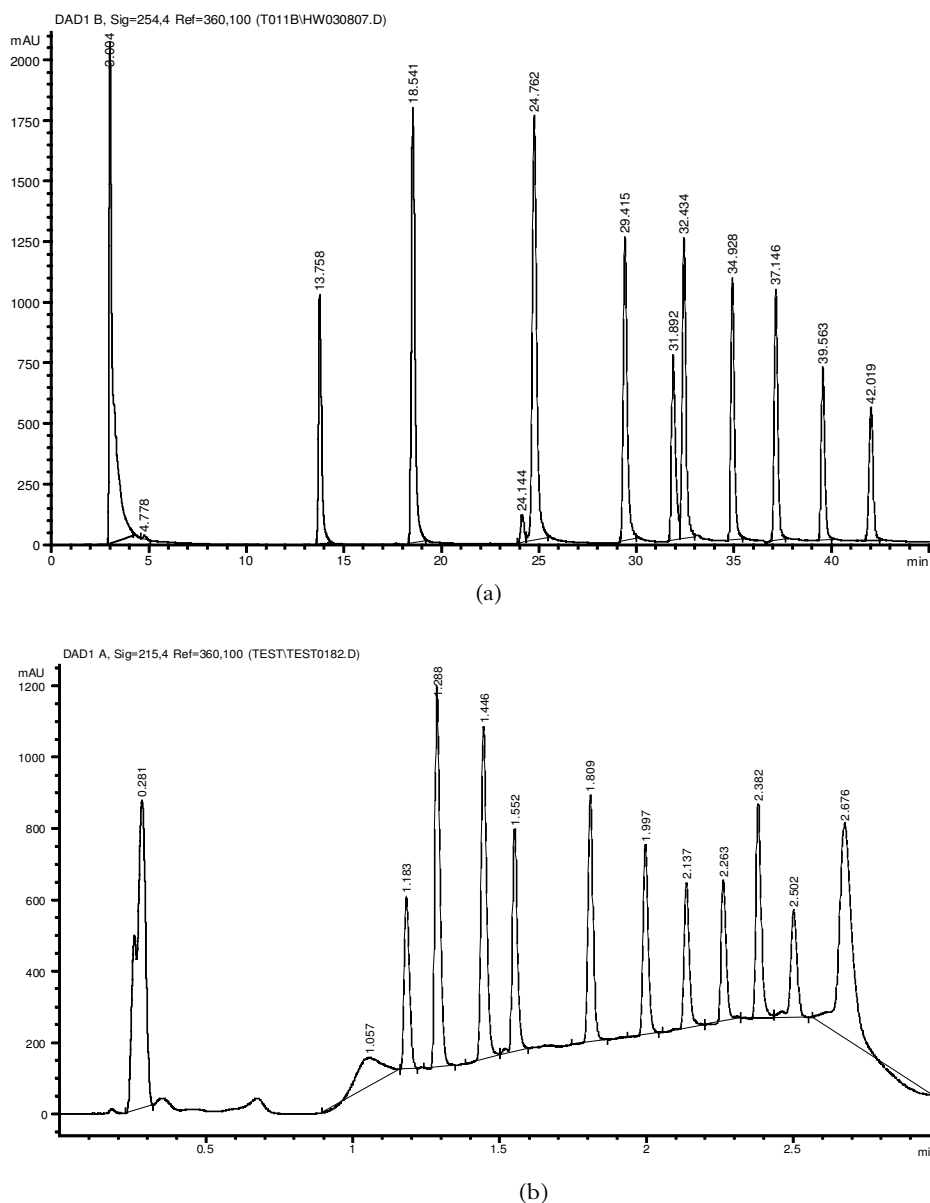


Figure 3. (a) Injection of an 11-component test mix onto a 150 × 2 mm C18 column; (b) injection of an 11-component test mix onto a 50 × 2 mm C18 column. Reprinted from Ayrtón *et al.* (1998a) with the permission of Elsevier Science.

1999) or an equivalent sensitivity for a greatly reduced sample volume (Dear *et al.* 1999, Fraser *et al.* 1999).

High-throughput bio-analysis

Meeting the demands of drug discovery and the seemingly ever increasing numbers of analytes and samples has led to the adoption of generic method

development strategies aiming for short analysis times per sample. The most widespread technique resulting from this philosophy is fast-gradient, reversed-phase HPLC on short, narrow columns, as described above. Typically, the vast majority of drug candidates can be quantified in biological matrices using one chromatographic methodology following sample pretreatment by protein precipitation or solid-phase extraction, with cycle times per sample of 3–4 min, i.e. a throughput of 15–20 samples h^{-1} .

Until recently, such a throughput would have been universally acceptable, and indeed this technique is the most widely applied in our laboratory. However, it is clear that when quantifying a single component, a run time of 3–4 min means that for large periods of each analytical run the mass spectrometer is effectively collecting baseline data. This has in part been addressed by the introduction of parallel two-column systems and staggering sample injection times such that as one column is re-equilibrating at the end of its gradient cycle, the mass spectrometer is collecting data from a second injection on a second column. Subsequently, while the second column re-equilibrates, the mass spectrometer switches back to collecting data from a further sample injection upon the first column. This approach allows an approximate doubling of the throughput (Higton, GlaxoWellcome unpublished data 1999, Wu 2001). Furthermore, a system has recently been demonstrated whereby four columns are operated in parallel utilizing differing lengths of mobile phase delay tubing before each column to stagger the times at which the analytes elute from the columns and enter the mass spectrometer (van Pelt *et al.* 2000)

Note that absolute sample throughput is determined by the entire analysis process, i.e. sample preparation must be considered as well as the chromatographic/detection process. In terms of the sample throughput, it is meaningless to reduce the chromatographic analysis time to, say, 2–3 min per sample if the sample preparation step is the rate-limiting 'bottleneck'. As discussed, work has been carried out to automate the most common sample preparation protocols in pharmaceutical bio-analysis, solid-phase extraction, protein precipitation and liquid–liquid extraction, particularly since the advent of 96-well microtitre plate technology (Allanson *et al.* 1996, Biddlecombe and Pleasance 1999, Zhang and Henion 1999, Zweigenbaum and Henion 2000). It should be noted though that it is a prerequisite of on-line SPE systems that the solvent required for elution from the cartridge is not of a nature or volume that is incompatible with direct injection to the reversed-phase HPLC system.

The approach that is gaining increasing usage, involves the use of large particle size stationary phase (30–50 μm) columns (50 $\times$ 1 mm) onto which plasma samples can be directly injected, with subsequent rapid development of a gradient elution profile at ultrahigh flow rates. Typically, the only sample preparation step required is the addition of an internal standard. This technique was introduced as turbulent flow chromatography (Quin and Takarewski 1997) and first applied to the quantitative analysis of pharmaceuticals in plasma in our laboratory (Ayrton *et al.* 1997). Following subsequent refinement of the gradient, a sample run time of 1.2 min was achieved allowing a throughput of 50 samples h^{-1} (Ayrton *et al.* 1998c). Such high chromatographic throughput coupled with the removal of the need for prior sample preparation means the overall productivity of the technique is very attractive. However, the lack of sample preparation means that there is no sample concentration step and the sensitivity of the technique is often limited to

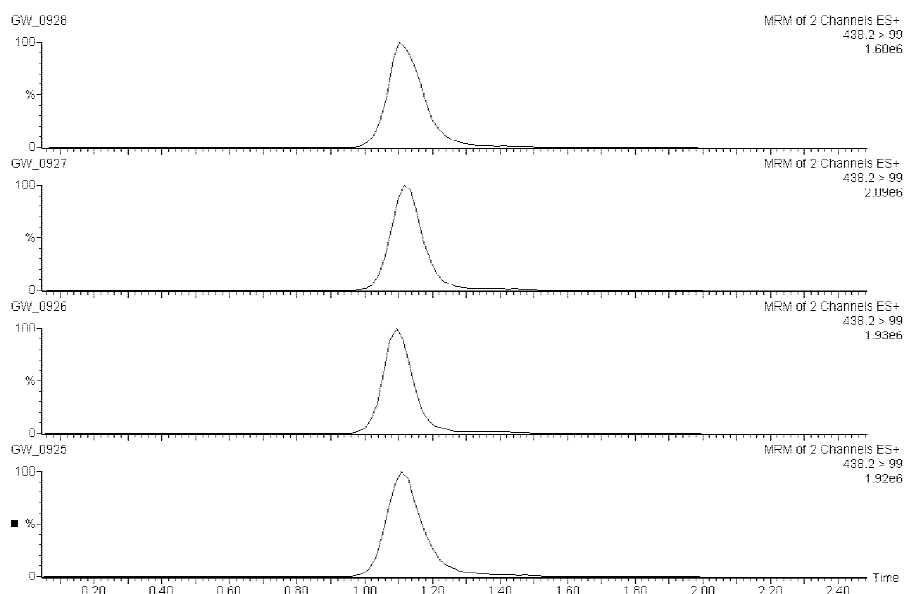


Figure 4. Chromatograms showing parallel 20 μ l injections of aqueous standards of an isoquinoline drug on four 50 $\times$ 1 mm 30- μ m columns with multiplex MS-MS detection. Reprinted from Bayliss *et al.* (2000) with the permission of John Wiley & Sons Ltd.

the low nanogram ml^{-1} range. It has been demonstrated, however, that this technique can be applied in the capillary scale (Ayrton *et al.* 1999) and the resulting increased concentration of the analyte in the chromatographic peak enabled the accurate and precise quantification of subnanogram ml^{-1} concentrations of a candidate drug from low μ l sample volumes. The development of the gradient at these reduced flow rates led to a slightly increased cycle time and a reduction in throughput to 30 samples h^{-1} . Recently, we have demonstrated the use of four such columns in parallel, in both the capillary and narrow-bore scales, that used in tandem with a multiplex interface to the mass spectrometer enable the quantification of four samples in parallel leading to a throughput of up to 120 samples h^{-1} (Bayliss *et al.* 2000). Typical chromatograms obtained for samples injected simultaneously onto the four-column system are shown in figure 4. These parallel systems were shown to exhibit comparable sensitivities to the single-column system and, importantly, no significant 'cross-talk' between the interface channels was observed. It should be noted that the use of large particle size stationary phases implies a fairly low efficiency separation. Consequently, some laboratories have preferred to use these columns as on-line extraction columns, with subsequent further chromatography on a conventional analytical column (Jemal *et al.* 1999, Jemal 2000), sometimes using two extraction columns to feed one analytical column for enhanced throughput (Xia *et al.* 2000).

Future developments

Emerging technologies that may offer great benefits to future high-throughput bio-analysis include monolithic columns (rods) and microfabricated chip-based systems; these are discussed in more detail below.

The dilemma that the analyst faces between high throughput and the need for chromatographic resolution may be answered, in the short-term, by the development of monolithic columns (Minakuchi *et al.* 1996). These columns offer, due to their novel construction, small particle performance with very low back pressures. The low back pressure and flatness of the van Deemter curve allow these phases to be operated at very high mobile phase linear velocities whilst maintaining good performance. The potential of such a material for pharmaceutical analysis was demonstrated by Cabrera *et al.* (2000). Recently it has been demonstrated how these monolithic columns can be applied to bio-analysis (Dear *et al.* 2001). In this latter study, the separation of six metabolites of debrisoquine was achieved on a monolithic column in 1 min, with no loss of resolution, compared with the 10 min required for a conventional particle packed column (figure 5). These columns are still in their initial stages of development but offer the potential to revolutionize high throughput chromatography.

Increased speed and resolving power could be achieved by coupling capillary HPLC with electrically driven LC (CEC). The potential of such an approach has been demonstrated (Stol *et al.* 1999) using high porosity phases, and very high linear flow velocities have been achieved. This approach along with the monolithic column technology offers the opportunity of significantly shortening analysis times without compromising performance. The advantages of combining capillary LC with monolithic column technology have been identified (Minakuchi *et al.* 1996, Tanaka *et al.* 2000). This work holds great potential for fast analysis, excellent specificity and high sensitivity.

A very interesting recent advancement is the application of LC/ICP/MS to drug metabolism studies (Nicholson *et al.* 2000). This technique offers for molecules containing some heteroatoms, e.g. bromine, the potential for assays with very high specificity and sensitivity.

More sensitive MS instruments and faster smaller scale HPLC will again put a strain on the sample preparation process with samples needing to be processed faster and in a smaller format. This may be achieved by the move to 384- (Biddlecombe *et al.* 2001) or 1536-well format SPE that will require the use of a new generation of robotic sample processors capable of addressing several hundred samples at a time. Other developments in sample preparation such as the use of molecular imprinted polymers (MIPS) and immuno-affinity phases may add extra specificity to the isolation process (Martin *et al.* 2000, Stevenson 2000).

To achieve the kind of quantum change in bio-analysis seen in the last 10 years, we have to look to new types of technology. Such concepts as 'lab on a chip' with multi-array detection operating in parallel mode may well offer this kind of quantum leap. These devices can offer all of the functionality of a standard analytical system but contained on a small piece of glass the size of a microscope slide. In such devices, separations are carried out in etched channels in either capillary electrophoresis (CE), capillary electrochromatography (CEC) or pressure-assisted electrochromatography mode and detection is made by either laser-induced fluorescence (LIF) or by MS. The size of these devices makes the parallel operation of many units relatively simple and as the separation can be electrically driven, there is no problem in flow splitting. Multichannel-type COSMOS chip technology has been demonstrated (He and Regnier 1998), and the use of non-MS detection, such as back-scattering interferometry, on chip-based detectors offers a glimpse of the future. Such technologies may allow on-chip isolation, separation

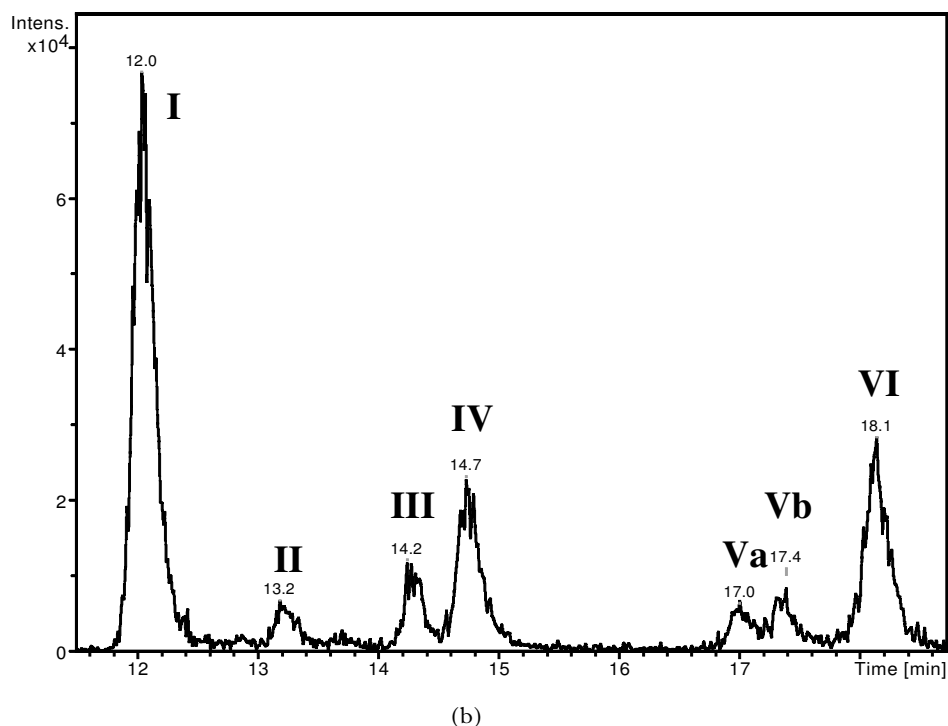
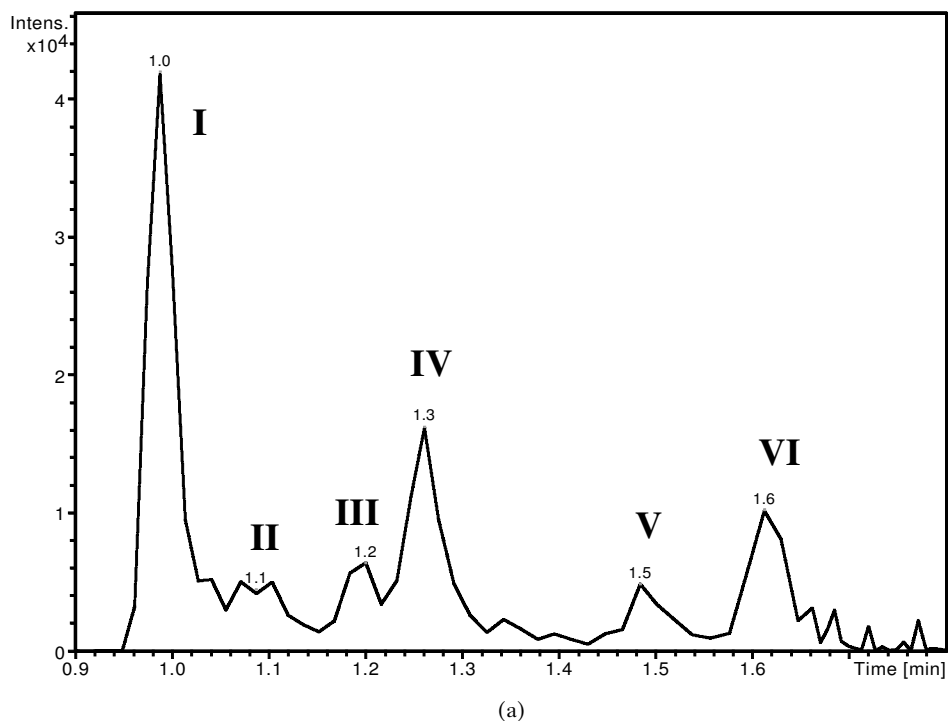


Figure 5. (a) Separation of the hydroxylated metabolites of debrisoquine on a 5 cm x 4.6 mm C18 bonded silica rod; (b) separation of the hydroxylated metabolites of debrisoquine on a 15 cm x 2.1 mm C18 bonded 5μm particulate column. Reprinted from Dear *et al.* (2001) with the permission of John Wiley & Sons Ltd.

and detection of the analyte. In the future such devices may find their way into the field allowing on-the-spot analysis and the continuous monitoring of patients or clinical trial volunteers. Indeed, a small chip-based analyser for RNA is already commercially available. This system has the capability to perform separations that traditionally took 1–2 h in just 2 min.

Conclusion

The process of bio-analysis is now unrecognizable from where it was 10 years ago. We have moved from the point of using complicated sample preparation, long columns of 4.6 mm i.d. diameter, 20–30-min run time and UV detection to simple sample preparation, short narrow columns with 1–2-min run times and mass spectrometric detection. The process of sample preparation has been streamlined and automated with open-access systems implemented to allow overnight, unattended operation. Other more direct methods have been developed that, when coupled to mass spectrometric detection, result in high-sensitivity assays and throughputs of up to 120 samples h⁻¹.

These developments have allowed us to produce a method development strategy that will facilitate the successful development of a moderate-to-high throughput assay for the vast majority of small molecules encountered in drug development. A schematic of such a method development strategy is shown in figure 6. It is based around using the simplest approach with the fastest throughput to gain the desired results. Thus, if a single component assay is required from

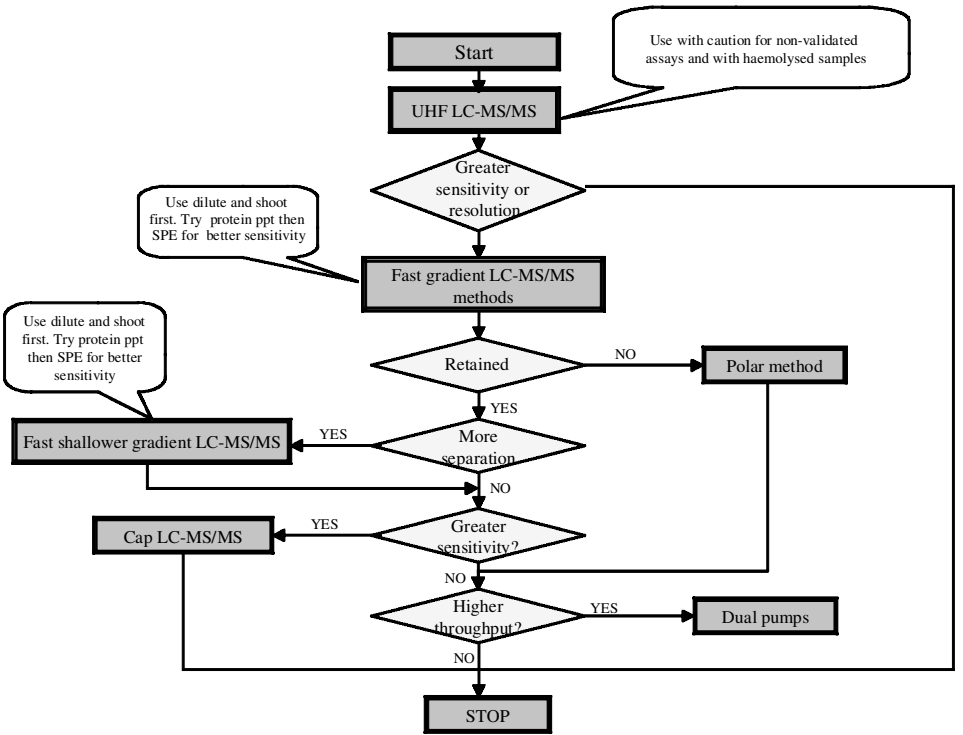


Figure 6. Flow diagram for a bio-analytical method development strategy.

plasma with an LOQ of $\sim 5 \text{ ng ml}^{-1}$, then the technique of ultrahigh flow direct analysis is recommended. If greater chromatographic resolution or more sensitivity is required, then a rapid gradient elution system with MS/MS detection is recommended, with sample preparation by protein precipitation as the technique of choice. An additional improvement in sensitivity can be achieved by the use of solid-phase extraction of a larger volume of sample or if sample is limited by employing capillary LC/MS/MS. By employing this simple flow chart, the most appropriate method can be selected, thereby fulfilling the requirements of the project in terms of sensitivity and speed of analysis. In the near future, the use of smaller-scale, narrow-bore and capillary-scale HPLC coupled to mass spectrometric detection will become widespread offering greater sensitivity, reduced solvent consumption and better performance.

In the last 10 years enormous progress has been made; the improvements in detection sensitivity, the enhancement in specificity and capacity achieved in bio-analysis have shaped our current strategy in the areas of drug metabolism and pharmacokinetics. We can only look forward with enthusiasm to what the next 10 years will bring.

References

- ALLANSON, J. P., BIDDLECOMBE, R. A., JONES, A. E. and PLEASANCE, S., 1996, The use of automated solid phase extraction in the '96-well' format for high throughput bioanalysis using liquid chromatography coupled to tandem mass spectrometry. *Rapid Communications in Mass Spectrometry*, **10**, 811–816.
- AYRTON, J., CLARE, R. A., DEAR, G. J., MALLETT, D. N. and PLUMB, R. S., 1999, Ultra-high flow rate capillary liquid chromatography with mass spectrometric detection for the direct analysis of pharmaceuticals in plasma at sub-nanogram per millilitre concentrations. *Rapid Communications in Mass Spectrometry*, **13**, 1657–1662.
- AYRTON, J., DEAR, G. J., LEAVENS, W. J., MALLETT, D. N. and PLUMB, R. S., 1997, The use of turbulent flow chromatography/mass spectrometry for the rapid, direct analysis of a novel pharmaceutical compound in plasma. *Rapid Communications in Mass Spectrometry*, **11**, 1953–1958.
- AYRTON, J., DEAR, G. J., LEAVENS, W. J., MALLETT, D. N. and PLUMB, R. S., 1998a, Use of generic fast gradient liquid chromatography-tandem mass spectroscopy in quantitative bioanalysis. *Journal of Chromatography B, Biomedical Sciences and Applications*, **709**, 243–254.
- AYRTON, J., DEAR, G. J., LEAVENS, W. J., MALLETT, D. N. and PLUMB, R. S., 1998c, Optimisation and routine use of generic ultra-high flow-rate liquid chromatography with mass spectrometric detection for the direct on-line analysis of pharmaceuticals in plasma. *Journal of Chromatography A*, **828**, 199–207.
- AYRTON, J., PLUMB, R. S., LEAVENS, W. J., MALLETT, D. N., DICKINS, M. and DEAR, G. J., 1998b, Application of a generic fast gradient liquid chromatography tandem mass spectrometry method for the analysis of cytochrome P450 probe substrates. *Rapid Communications in Mass Spectrometry*, **12**, 217–224.
- BARTOLUCCI, G., PIERACCINI, G., VILLANELLI, F., MONETTI, G. and TRIOLO, A., 2000, Liquid chromatography tandem mass spectrometric quantitation of sulfamethazine and its metabolites: direct analysis of swine urine by triple quadrupole and by ion trap mass spectrometry. *Rapid Communications in Mass Spectrometry*, **14**, 967–973.
- BAYLISS, M. K., LITTLE, D., MALLETT, D. N. and PLUMB, R. S., 2000, Parallel ultra-high flow rate liquid chromatography with mass spectrometric detection using a multiplex electrospray source for direct, sensitive determination of pharmaceuticals in plasma at extremely high throughput. *Rapid Communications in Mass Spectrometry*, **14**, 2039–2045.
- BIDDLECOMBE, R. A., BENEVIDES, C. and PLEASANCE, S., 2001, A clinical trial on a plate? The potential of 384-well format solid phase extraction for high-throughput bioanalysis using liquid chromatography/tandem mass spectrometry. *Rapid Communications in Mass Spectrometry*, **15**, 33–40.
- BIDDLECOMBE, R. A. and PLEASANCE, S., 1997, Fully automated SPE-LC-MS-MS. In *Proceedings of the International Symposium on Laboratory Automation and Robotics (ISLAR)*, Boston, MA, 19–22 October, pp. 389–403.

- BIDDLECOMBE, R. A. and PLEASANCE, S., 1999, Automated protein precipitation by filtration in the 96-well format. *Journal of Chromatography B, Biomedical Sciences and Applications*, **734**, 257–265.
- BLANCHOWER, W. J., HEWITT, S. A., CANNAVAN, A., ELLIOTT, C. T. and KENNEDY, D. G., 1993, Detection of clenbuterol residues in bovine liver, muscle, retina and urine using gas chromatography/mass spectrometry. *Biological Mass Spectrometry*, **22**, 326–330.
- BLEVINS, D. D. and HALL, D. O., 1998, Recent advances in disk-format solid-phase extraction. *LC GC — Magazine of Separation Science*, (suppl. S).
- BOGUSZ, M. J., KRUGER, K. D. and MAIER, R. D., 2000, Analysis of underivatized amphetamines and related phenethylamines with high-performance liquid chromatography-atmospheric pressure chemical ionization mass spectrometry. *Journal of Analytical Toxicology*, **24**, 77–84.
- BOWERS, G. D., CLEGG, C. P., HUGHES, S. C., HARKER, A. J. and LAMBERT, S., 1997, Automated spe and tandem ms without hplc columns for quantifying drugs at the picogram level. *LC GC — Magazine of Separation Science*, **15**.
- BRUINS, A. P., 1994, Atmospheric-pressure-ionisation mass spectrometry I and II. *Trends in Analytical Chemistry*, **13**, 37 and 81.
- BRUINS, A. P., COVEY, T. R. and HENION, J. D., 1987, Ion-spray interface for combined liquid chromatography/ atmospheric pressure ionisation mass spectrometry. *Annals of Chemistry*, **59**, 2642.
- CABRERA, K., LUBDA, D., EGGENWEILER, H. M., MINAKUCHI, H. and NAKANISHI, K., 2000, A new monolithic-type HPLC column for fast separations. *HRC — Journal of High Resolution Chromatography*, **23**, 93–99.
- CALLEJAS, S. L., BIDDLECOMBE, R. A., JONES, A. E., JOYCE, K. B., PEREIRA, A. I. and PLEASANCE, S., 1998, Determination of the glucocorticoid fluticasone propionate in plasma by automated solid-phase extraction and liquid chromatography tandem mass spectrometry. *Journal of Chromatography B, Biomedical Sciences and Applications*, **718**, 243–250.
- CHENG, Y. F., NEUE, U. D. and BEAN, L., 1998, Straightforward solid-phase extraction method for the determination of verapamil and its metabolite in plasma in a 96-well extraction plate. *Journal of Chromatography A*, **828**, 273–281.
- CHOWDHURY, S. K., KATTA, V. and CHAIT, B. T., 1990, An electrospray-ionisation mass spectrometer with new features. *Rapid Communications in Mass Spectrometry*, **4**, 81–87.
- CHOWDHURY, S. K., LAUDICINA, D., BLUMENKRANTZ, N., WIRTH, M. and ALTON, K. B., 1999, An LC/MS/MS method for the quantitation of MTIC (5-(3-N-methyltriazene-1-yl)-imidazole-4-carboxamide), a bioconversion product of temozolomide, in rat and dog plasma. *Journal of Pharmaceutical and Biomedical Analysis*, **19**, 659–668.
- CHURCH, W. H. and JUSTICE, J. B., 1989, On-line small-bore chromatography for neurochemical analysis in the brain. *Advances in Chromatography*, **28**, 165.
- DARIUS, J. and BANDITT, P., 2000, Validated method for the therapeutic drug monitoring of flunitrazepam in human serum using liquid chromatography-atmospheric pressure chemical ionization tandem mass spectrometry with an ion trap detector. *Journal of Chromatography B, Biomedical Sciences and Applications*, **738**, 437–441.
- DEAR, G. J., AYRTON, J., PLUMB, R. and FRASER, I. J., 1999, The rapid identification of drug metabolites using capillary LC coupled to an ion trap mass spectrometer. *Rapid Communications in Mass Spectrometry*, **13**, 456–463.
- DEAR, G. J., FRASER, I. J., PATEL, D. K., LONG, J. and PLEASANCE, S., 1998, Use of liquid chromatography-tandem mass spectrometry for the quantitative and qualitative analysis of an antipsychotic agent and its metabolites in human plasma and urine. *Journal of Chromatography A*, **794**, 27–36.
- DEAR, G. J., HARRELSON, J. C., JONES, A. E., JOHNSON, T. E. and PLEASANCE, S., 1995, Identification of the urinary and biliary conjugated metabolites of the neuromuscular blocker 51W89 by liquid chromatography/mass spectrometry. *Rapid Communications in Mass Spectrometry*, **9**, 1457–1464.
- DEAR, G. J., PLUMB, R. S. and MALLETT, D. N., 2001, The use of monolithic silica columns to increase analytical throughput for metabolite identification by LC/MS/MS. *Rapid Communications in Mass Spectrometry*, **15**, 152–158.
- FENN, J. B., MANN, M., MENG, C. K., WONG, S. F. and WHITEHOUSE, C. M., 1990, Electrospray ionisation — principles and practice. *Mass Spectrometry Review*, **9**, 37.
- FRASER, I. J., CLARE, R. A. and PLEASANCE, S., 1995, Determination of the acylcoenzyme A cholesterol acyltransferase inhibitor 447C88 in plasma using gas chromatography-mass spectrometry and liquid chromatography atmospheric pressure chemical ionisation tandem mass spectrometry. *Journal of Chromatography B, Biomedical Sciences and Applications*, **663**, 275–287.
- FRASER, I. J., DEAR, G. J., PLUMB, R. S., L'ANEUR, M., FRASER, D. and SKIPPEN, A. J., 1999, The use of capillary high-performance liquid chromatography with electrospray mass spectrometry for the analysis of small volume blood samples from serially bled mice to determine the pharmacokinetics of early discovery compounds. *Rapid Communications in Mass Spectrometry*, **13**, 2366–2375.

- GELPI, E., 1995, Biomedical and biochemical applications of liquid chromatography-mass spectrometry. *Journal of Chromatography A*, **703**, 59–80.
- HARKER, A. J., EVANS, G. L., HAWLEY, A. E. and MORRIS, D. M., 1994, High-performance liquid chromatographic assay for 2-deoxy-3-thiacytidine in human serum. *Journal of Chromatography B, Biomedical Sciences and Applications*, **657**, 227–232.
- HARRISON, A. C. and WALKER, D. K., 1998, Automated 96-well solid phase extraction for the determination of doramectin in cattle plasma. *Journal of Pharmaceutical and Biomedical Analysis*, **16**, 777–783.
- HE, B. and REGNIER, F., 1998, Microfabricated liquid chromatography columns based on collocated monolith support structures. *Journal of Pharmaceutical and Biomedical Analysis*, **17**, 925–932.
- HEDENMO, M. and ERIKSSON, B. M., 1994, On-line solid-phase extraction with automated cartridge exchange for liquid chromatographic determination of lipophilic antioxidants in plasma. *Journal of Chromatography*, **661**, 153–159.
- HEMPENIUS, J., HENDRIKS, G., HINGSTMAN, J., MENSINK, C. K., JONKMAN, J. H. G. and LIN, C. C., 1994, An automated analytical method for the determination of felbamate in human plasma by robotic sample preparation and reversed-phase high performance liquid chromatography. *Journal of Pharmaceutical and Biomedical Analysis*, **12**, 1443–1451.
- HENION, J., WACHS, T. and MORDEHAJ, A., 1993, Recent developments in electrospray mass spectrometry including implementation on an ion trap. *Journal of Pharmaceutical and Biomedical Analysis*, **11**, 1049–1061.
- HUBERT, P., CECCATO, A., CHIAP, P., TOUSSAINT, B. and CROMMEN, J., 1999, Sample preparation of biological matrix for chromatographic methods. *STP Pharmaceutical Sciences*, **9**, 160.
- JEMAL, M., 2000, High-throughput quantitative bioanalysis by LC/MS/MS. *Biomedical Chromatography*, **14**, 422–429.
- JEMAL, M., OUYANG, Z., XIA, Y.-Q. and POWELL, M. L., 1999, A versatile system of high-flow high performance liquid chromatography with tandem mass spectrometry for rapid direct-injection analysis of plasma samples for quantitation of a β -lactam drug candidate and its open-ring biotransformation product. *Rapid Communications in Mass Spectrometry*, **13**, 1462–1471.
- KAWAI, T., MIZUNUMA, K., YASUGI, T., HORIGUCHI, S., IGUCHI, H., MUTTI, A., GHITTORI, S. and IKEDA, M., 1995, Monitoring of exposure to methylpentanes by diffusive sampling and urine analysis for alcoholic metabolites. *Occupational and Environmental Medicine*, **52**, 757–763.
- KEBARLE, P. and LANG, L., 1993, From ions in solution to ions in the gas phase — the mechanism of electrospray mass spectrometry. *Annals of Chemistry*, **65**, 972A.
- KERNS, E. H., ROURICK, R. A., VOLK, K. J. and LEE, M. S., 1997, Buspirone metabolite structure profile using a standard liquid chromatographic-mass spectrometric protocol. *Journal of Chromatography B, Biomedical Sciences and Applications*, **698**, 133–145.
- KIKUCHI, Y., TERAMURA, T., SEKINO, J., NISHIMURA, T., MIURA, H., WATANABE, T. and HIGUCHI, S., 1998, Sensitive and specific method for the determination of josamycin in human plasma by liquid chromatography-mass spectrometry. *Journal of Chromatography B, Biomedical Sciences and Applications*, **720**, 81–87.
- LACHATRE, F., MARQUET, P., RAGOT, S., GAULIER, J. M., CARDOT, P. and DUPUY, J. L., 2000, Simultaneous determination of four anthracyclines and three metabolites in human serum by liquid chromatography-electrospray mass spectrometry. *Journal of Chromatography B, Biomedical Sciences and Applications*, **738**, 281–291.
- LEE, E. D. and HENION, J. D., 1992, Thermally assisted electrospray interface for liquid chromatography/mass spectrometry. *Rapid Communications in Mass Spectrometry*, **6**, 727–733.
- MAJORS, R. E., 1998, A review of modern solid-phase extraction. *LC GC — Magazine of Separation Science*, (suppl. S).
- MARTIN, P., WILSON, I. D. and JONES, G. R., 2000, Application and elution conditions for the selective elution of analytes from molecular imprinted polymers after extraction from aqueous samples: application to beta-blockers. *Chromatographia*, **52**, S19–S23.
- MINAKUCHI, H., NAKANISHI, K., SOGA, N., ISHIZUKA, N. and TANAKA, N., 1996, Octadecylsilylated porous silica rods as separation media for reversed-phase liquid chromatography. *Annals of Chemistry*, **68**, 3498–3501.
- MOLINA, C., GRASSO, P., BENFENATI, E. and BARCELO, D., 1996, Automated sample preparation with extraction columns followed by liquid chromatography ionspray mass spectrometry — interferences, determination and degradation of polar organophosphorus pesticides in water samples. *Journal of Chromatography A*, **737**, 47–58.
- NICHOLSON, J. K., LINDON, J. C., SCARFE, G., WILSON, I. D., ABOU-SHAKRA, F., CASTRO-PEREZ, J., EATON, A. and PREECE, S., 2000, High-performance liquid chromatography and inductively coupled plasma mass spectrometry (HPLC-ICP-MS) for the analysis of xenobiotic metabolites in rat urine: application to the metabolites of 4-bromoaniline. *Analyst*, **125**, 235–236.

- PLUMB, R. S., DEAR, G. J., MALLETT, D. N., FRASER, I. J., AYRTON, J. and IOANNOU, C., 1999, The application of fast gradient capillary liquid chromatography/mass spectrometry to the analysis of pharmaceuticals in biofluids. *Rapid Communications in Mass Spectrometry*, **13**, 865–872.
- Plumb, R. S., Gray, R. D. M. and JONES, C. M., 1997, Use of reduced sorbent bed and disk membrane solid-phase extraction for the analysis of pharmaceutical compounds in biological fluids, with applications in the 96-well format. *Journal of Chromatography B, Biomedical Sciences and Applications*, **694**, 123–133.
- POCH, G. K., KLETTE, K. L. and ANDERSON, C., 2000, The quantitation of 2-oxo-3-hydroxy lysergic acid diethylamide (O-H-LSD) in human urine specimens, a metabolite of LSD: comparative analysis using liquid chromatography-selected ion monitoring mass spectrometry and liquid chromatography-ion trap mass spectrometry. *Journal of Analytical Toxicology*, **24**, 170–179.
- POON, G. K., MISTRY, P., RAYNAUD, F. I., HARRAP, K. R., MURRER, B. A. and BARNARD, C. F., 1997, Determination of metabolites of a novel platinum anticancer drug JM216 in human plasma ultrafiltrates. *Journal of Pharmaceutical and Biomedical Analysis*, **13**, 1493–1498.
- QUIN, H. M. and TAKAREWSKI, J. J., 1997, High performance liquid chromatography method and apparatus. International Patent WO/97/16724 1997.
- SANDAHL, M., MATHIASSEN, L. and JONSSON, J. A., 2000, Determination of thiophanate-methyl and its metabolites at trace level in spiked natural water using the supported liquid membrane extraction and the microporous membrane liquid–liquid extraction techniques combined on-line with high-performance liquid chromatography. *Journal of Chromatography*, **893**, 123–131.
- SCHANZLE, G., LI, S., MIKUS, G. and HOFMANN, U., 1999, Rapid, highly sensitive method for the determination of morphine and its metabolites in body fluids by liquid chromatography-mass spectrometry. *Journal of Chromatography B, Biomedical Sciences and Applications*, **721**, 55–65.
- SCOTT, R. J., PALMER, J., LEWIS, I. A. and PLEASANCE, S., 1999, Determination of a 'GW cocktail' of cytochrome P450 probe substrates and their metabolites in plasma and urine using automated solid phase extraction and fast gradient liquid chromatography tandem mass spectrometry. *Rapid Communications in Mass Spectrometry*, **13**, 2305–2319.
- STEINBORNER, S. and HENION, J., 1999, Liquid–liquid extraction in the 96-well plate format with SRM LC/MS quantitative determination of methotrexate and its major metabolite in human plasma. *Analytical Chemistry*, **71**, 2340–2345.
- STEVENSON, D., 2000, Immuno-affinity solid-phase extraction. *Journal of Chromatography B, Biomedical Sciences and Applications*, **745**, 39–48.
- STOL, R., KOK, W. T. and POPPE, H., 1999, Capillary electrochromatography with macroporous particles. *Journal of Chromatography*, **853**, 45–54.
- STUBBS, R. J. and HARKER, A. J., 1995, Automated high-performance liquid chromatographic method for the determination of a neuraminidase inhibitor (gg167) in human serum by pre-column fluorescence derivatisation using benzoin. *Journal of Chromatography B, Biomedical Sciences and Applications*, **670**, 279–285.
- TANAKA, N., NAGAYAMA, H., KOBAYASHI, H., IKEGAMI, T., HOSOYA, K., ISHIZUKA, N., MINAKUCHI, H., NAKANISHI, K., CABRERA, K. and LUBDA, D., 2000, Monolithic silica columns for HPLC, micro-HPLC, and CEC. *HRC — Journal of High Resolution Chromatography*, **23**, 111–116.
- VAN PELT, C. K., CORSO, T. N., SCHULTZ, G. A., LOWES, S. and HENION, J., 2000, A novel four-column parallel chromatography system for gradient SRM LC/MS bioanalytical analyses. In *Proceedings of 48th ASMS Conference on Mass Spectrometry and Allied Topics*, Long Beach, CA.
- Wells, D. A., Lensmeyer, G. L. and WIEBE, D. A., 1995, Particle-loaded membranes as an alternative to traditional packed-column sorbents for drug extraction — in-depth comparative study. *Journal of Chromatographic Science*, **33**, 386–392.
- WIEBOLDT, R., CAMPBELL, D. A. and HENION, J., 1998, Quantitative liquid chromatographic-tandem mass spectrometric determination of orlistat in plasma with a quadrupole ion trap. *Journal of Chromatography B, Biomedical Sciences and Applications*, **708**, 121–129.
- WRIGHT, A. W. E., WATT, J. A., KENNEDY, M., CRAMOND, T. and SMITH, M. T., 1994, Quantitation of morphine, morphine-3-glucuronide, and morphine-6-glucuronide in plasma and cerebrospinal fluid using solid-phase extraction and high-performance liquid chromatography with electrochemical detection. *Therapeutic Drug Monitoring*, **16**, 200–208.
- WU JING-TAO, 2001, The development of a staggered parallel separation liquid chromatography/tandem mass spectrometry system with on-line extraction for high-throughout screening of drug candidates in biological fluids. *Rapid Communications in Mass Spectrometry*, **15**, 73–81.
- XIA, Y.-Q., WHIGAN, D. B., POWELL, M. L. and JEMAL, M., 2000, *Rapid Communications in Mass Spectrometry*, **14**, 105–111.
- XU, K., ARORA, V. K., CHAUDHARY, A. K., COTTON, R. B. and BLAIR, I. A., 1999, Quantitative analysis of cimetidine in human plasma using LC/APCI/SRM/MS. *Biomedical Chromatography*, **13**, 455–461.

- XU, K., LANUTI, M. LAMBRIGHT, E. S. FORCE, S. D. ALBELDA, S. M. and BLAIR, I. A., 2000, A rapid and sensitive method for the quantification of ganciclovir in plasma using liquid chromatography/selected reaction monitoring/mass spectrometry. *Biomedical Chromatography*, **14**, 93–98.
- ZHANG, H., HEINIG, K. and HENION, J., 2000a, Atmospheric pressure ionization time-of-flight mass spectrometry coupled with fast liquid chromatography for quantitation and accurate mass measurement of five pharmaceutical drugs in human plasma. *Journal of Mass Spectrometry*, **35**, 423–431.
- ZHANG, H. and HENION, J., 1999, Quantitative and qualitative determination of estrogen sulfates in human urine by liquid chromatography/tandem mass spectrometry using 96-well technology. *Annals of Chemistry*, **71**, 3955–3964.
- ZHANG, N., FOUNTAIN, S. T., BI, H. and ROSSI, D. T., 2000b, Quantification and rapid metabolite identification in drug discovery using API time-of-flight LC/MS. *Analytical Chemistry*, **72**, 800–806.
- ZWEIGENBAUM, J. and HENION, J., 2000, Bioanalytical high-throughput selected reaction monitoring-LC/MS determination of selected estrogen receptor modulators in human plasma: 2000 samples/day. *Analytical Chemistry*, **72**, 2446–2454.

Accelerator mass spectrometry (AMS): recent experience of its use in a clinical study and the potential future of the technique

G. YOUNG^{†*}, W. ELLIS[†], J. AYRTON[†], E. HUSSEY[‡] and B. ADAMKIEWICZ[‡]

[†] Division of Bioanalysis and Drug Metabolism, Glaxo Wellcome Research and Development Ltd, Park Road, Ware SG12 ODP, UK

[‡] Division of Clinical Pharmacology, Glaxo Wellcome, Inc., Research Triangle Park, NC 27709, USA

Received 22 January 2001

1. The technique of accelerator mass spectrometry (AMS) is outlined.
2. The use of AMS in an initial validation study in animals is outlined. As part of the validation of the technique, samples from the animal study were analysed by both liquid scintillation counting (LSC) and, following dilution, by AMS. The results were similar.
3. The use of AMS in support of a clinical study is described. Six healthy male human volunteers were administered 2.7 mg [¹⁴C]-GI1817771 (121 Bq; 3.3 nCi) to produce an exposure to ionizing radiation of 0.06 µSv. Mass balance in recovery of administered radioactivity was achieved and information about the presence of systemically circulating metabolites was gained.
4. The future potential of the technique of AMS is discussed.

Introduction

Accelerator mass spectrometry (AMS) is a very sensitive nuclear physics technique that was developed in America in the 1970s. AMS has been used in the analysis of a wide range of elements and its use in measurement of radiocarbon (¹⁴C) to date archaeological samples first appeared in the literature in the 1970s (Bennett *et al.* 1977, Nelson *et al.* 1977). AMS uses a tandem van de Graaff accelerator to provide the potential energy to strip off the valency electrons of negatively ionized atoms generating positively charged atoms which can then be separated using conventional mass spectrometry. In the measurement of carbon by AMS, the three isotopic species (i.e. ¹⁴C, ¹³C and ¹²C) are separated from each other by virtue of their different energies and mass:charge ratios. AMS can measure radiocarbon concentrations down to 1000th of the natural levels found in living tissue and so is significantly more sensitive than liquid scintillation counting (LSC). AMS as an analytical tool for the pharmaceutical industry has been recently comprehensively reviewed (Garner 2000).

The use of AMS for analysis of drugs and metabolites in biological matrices has been discussed extensively but little original data have been presented in the literature. A short communication demonstrating the feasibility of using AMS for drug measurement has been published by Kaye *et al.* (1997), as has a paper detailing validation of AMS for analysis of radiocarbon in biomedical samples

*Author for correspondence; e-mail: gcy1283@glaxowellcome.co.uk

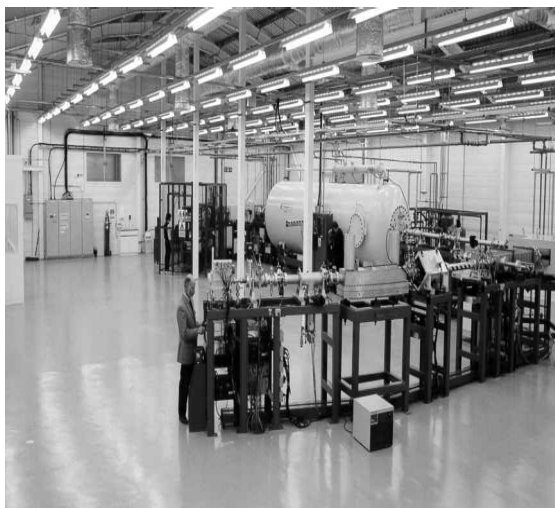


Figure 1. Accelerator mass spectrometry instrument at The Centre for Biomedical Accelerator Mass Spectrometry Ltd, Sand Hutton, York. Reproduced by kind permission of CBAMS Ltd.

(Garner *et al.* 2000). The AMS instrument upon which the validation work described here was conducted (figure 1) was established at The Centre for Biomedical Accelerator Mass Spectrometry (CBAMS) Ltd, Sand Hutton, York, UK. CBAMS Ltd became operational in 1998 and was cofunded by a group of companies from the pharmaceutical industry, along with the UK Ministry of Agriculture, Fisheries and Food (Central Science Laboratory) and the University of York.

This paper describes the approach taken by Glaxo Wellcome Research and Development Ltd to using the technology of AMS in the establishment of a facility to handle samples that contain very low quantities of radiocarbon-labelled material in the initial validation study. The paper also describes a clinical study where only 121 Bq (3.3 nCi) of a radioactively labelled new chemical entity ($[^{14}\text{C}]$ -GI181771) was administered by the oral route to six healthy male human volunteers to allow estimation of a mass balance of radioactivity of this compound in humans. The paper also discusses the potential future of AMS in biomedical applications and its possible impact on drug discovery and development in the pharmaceutical industry.

Validation of AMS

As part of the validation of the technique of AMS in biomedical use, we conducted an excretion balance and pharmacokinetics study in male Han Wistar rats using $[^{14}\text{C}]$ -fluticasone propionate ((6 α ,11 β ,16 α ,17 β)-6,9-difluoro-11-hydroxy-16-methyl-3-oxo-17-propionyloxy-androsta-1,4-diene-17-carbothioic acid *S*-fluoromethyl ester), a chemical in general medical use for the treatment of asthma and rhinitis. Samples of blood plasma and excreta from the animal study were analysed by both LSC and, following dilution, by AMS, and the results were similar. The design of the study and the results obtained are covered in detail in Garner *et al.* (2000). It should be noted that when handling the low levels of

radiocarbon in the samples destined for analysis by AMS, we took the approach of designating a laboratory area and isolating it, and the equipment in it, from all other routine use of radiocarbon. The laboratory was established in a temporary building with access limited to only those persons who were directly involved in work with very low level radiocarbon samples. 'Clean laboratory' techniques were employed to minimize further the risk of contamination from other laboratory areas. Following successful validation of the technique of AMS, from the perspective of Glaxo Wellcome Research and Development Ltd, using the animal study samples, the laboratory was equipped with everything that was required to support the preliminary preparation of samples generated from a small clinical study. This included sample storage, weighing, aliquotting, homogenizing, and separation of drugs and metabolites by high-performance liquid chromatography (HPLC) with fraction collection.

Clinical study

Materials and methods

Radiolabelled compound. GI181771 was radiolabelled by introduction of a ^{14}C -atom in a position considered unlikely to be vulnerable to metabolic transformation. The radiolabelled drug (0.04 mg) was diluted with unlabelled GI181771 (3.03 g) to produce a specific activity of 44.65 Bq mg^{-1} . Radiochemical purity of the labelled drug prior to dilution with unlabelled drug was 99.8%. The chemical purity after dilution was 95.2% with the main impurities being water and solvents. The radiochemical purity was not rechecked following dilution due to the limitations in assaying drug of such low specific activity.

Stable isotopic-labelled compound. $[\text{}^2\text{H}_5]$ -GI181771 was prepared for use as the internal standard for the assay of GI181771 by liquid chromatography with tandem mass spectrometric detection (LC-MS-MS). The chemical purity of this material was >98.6%.

Dosing and sample collection. Six healthy male human volunteers, aged 23–37 years, received a single oral administration of $[\text{}^{14}\text{C}]$ -GI181771 at a mean dosage of 2.7 mg in 5 ml polyethylene glycol 400 (Union Carbide Corporation, Danbury, CT, USA). The radiolabel dose was to be limited to that which would produce an exposure to ionizing radiation of $1 \mu\text{Sv}$, which is equivalent to a 4-h exposure to environmental (background) ionizing radiation in the UK. This exposure is the limit below which one is exempt from requiring a certificate from the UK Department of Health's Administration of Radiolabelled Substance Advisory Committee (ARSAC) to administer radioactive medicinal products to human subjects in the UK. The initial target exposure was $0.9 \mu\text{Sv}$, but the actual average dose of radioactivity administered to each volunteer was 121 Bq, producing exposure to ionizing radiation of $0.06 \mu\text{Sv}$. The exposure was as low as this for specific logistical reasons, which arose during the planning of the study. The total dose to be administered was reduced from the initially planned target dose and the radioactive dose was also reduced as a direct result. It was felt that reliable data could still be obtained at this dose level and the study therefore proceeded using this lower total dose and resultant lowered exposure to ionizing radiation.

Blood samples were collected predose and at 0.25, 0.5, 0.75, 1, 1.5, 2, 2.5, 3, 4, 6, 8, 12, 16 and 24 h post-dose. After clotting and centrifugation, serum was transferred to polypropylene tubes and stored frozen. Urine samples were collected predose and 0–12, 12–24, 24–48, 48–72, 72–96 and 96–120 h post-dose. The weights of the collections were recorded and an aliquot stored frozen prior to analysis.

Faeces samples were collected predose and 0–24, 24–48, 48–72, 72–96 and 96–120 h post-dose. The complete collections were stored frozen and weights recorded prior to processing.

Preliminary preparation of faeces and urine samples for analysis by AMS. Faeces samples (50–180 g) were weighed into 1.2 litre containers and smaller samples were weighed into 250 ml containers. Making the assumption that 1 g faeces was equivalent to 1 ml, 3 vols methanol (Rathburn Chemicals Ltd, Walkerburn, UK) were added. Samples were thawed and homogenized with an Ultra Turrax homogenizer (Brunner Scientific, Unit 4C, Hunmanby, Filey, UK). The final homogenate appeared as a fine suspension and was stored at nominally -20°C . Triplicate aliquots were taken from different positions from within each resuspended homogenate and were sent on solid CO_2 to CBAMS Ltd for

analysis by AMS. Aliquots of neat urine were sent frozen to CBAMS Ltd for analysis by AMS. Samples sent to CBAMS Ltd could be shipped as non-radioactive due to the extremely low quantities of radioactivity involved, with consequent reductions in costs and bureaucracy.

Serum sample preparation for analysis by AMS. Aliquots of neat serum were sent frozen to CBAMS Ltd for analysis by AMS. This method of analysis could not distinguish the very low concentrations of radioactivity present in serum samples against the endogenous background radiocarbon in neat serum, following administration of such a low dose of radioactivity. In an attempt to enhance the signal:noise ratio, the endogenous radioactivity was reduced through removal of carbon-containing components by precipitation of the serum proteins prior to analysis by AMS. An aliquot (400 µl) of each serum sample was vortex mixed with 800 µl acetonitrile (Rathburn Chemicals). After centrifugation, 800 µl of the supernatant was subjected to analysis by AMS.

Faecal extracts: preparation prior to fraction collection from HPLC. To allow metabolic profiling of the faecal samples, selected predose and 0–48 h post-dose collection faecal samples were extracted and fractionated by HPLC. Twenty millilitres of faecal homogenate from each sample was centrifuged. The supernatant was concentrated by evaporation under a stream of nitrogen to a final volume of up to 2 ml. Aliquots (100 µl) were fractionated by HPLC and sent to CBAMS Ltd for analysis by AMS. Radiochromatograms were constructed from the radioactivity in the HPLC fractions.

Serum samples: preparation prior to fraction collection from HPLC. Serum samples from all of the volunteers were combined into three pools according to times after dosing: 0.5–0.75, 2–6 and 12–16 h. This resulted in up to 2 ml serum from each of these times after dosing. Acetonitrile (2 vols) was added to each pooled sample, vortex mixed and centrifuged. The supernatant was concentrated by evaporation under a stream of nitrogen and reconstituted in ~150 µl 50% acetonitrile:50% water (v/v). An aliquot (100 µl) was fractionated by HPLC and the fractions were sent to CBAMS Ltd for analysis by AMS.

HPLC fractionation and metabolite profiling of serum and faeces. Aliquots (100 µl) of the sample of precipitated serum or faecal extract were injected on to an Inertsil ODS2 (250 × 4.6 mm i.d., 5 µm) analytical column, which was eluted under gradient conditions at a flow rate of 1 ml min⁻¹. Mobile phase A was 0.1% v/v trifluoroacetic acid in water and mobile phase B was 95% v/v acetonitrile in 0.1% v/v trifluoroacetic acid (in water). The mobile phase was a linear gradient which started at 75% A and 25% B, rose to 90% B over 30 min and returned to the starting conditions from 35 to 45 min. The column was maintained at ambient temperature. The column eluant was monitored at A_{240nm}. Column eluant fractions were collected every 0.5 min from 5 to 6 and from 14 to 30 min from the point of injection. A Gilson 233XL Fraction Collector (Anachem Ltd, Luton, UK) run from Unipoint v.1.02 was used. On this system, the retention times of GI181771 and its glucuronide were 24 and 20 min respectively. The column eluant fractions (0.5 ml) were sent frozen on solid carbon dioxide to CBAMS Ltd for analysis by AMS.

Graphitization of samples. In order to measure the ¹⁴C content of samples by AMS, the carbon in the samples was first converted to graphite by CBAMS Ltd using a previously described method (Vogel 1992). In instances where insufficient carbon was present in samples, a carrier of known total carbon content and carbon isotope inventory was added. The carrier used was usually tributyrin (1,2,3-tributyrylglycerol; Sigma-Aldrich Company Ltd, Poole, UK).

C,H,N analysis. C,H,N analysis was conducted by CBAMS Ltd on samples submitted for analysis by AMS. It was necessary to determine the percentage carbon content in samples so as to assess the volume or mass of sample required to produce good graphite. The determination also allowed conversion of the results output from the AMS which was expressed as ¹⁴C mg⁻¹ carbon, to total ¹⁴C sample⁻¹ and subsequent conversion to the commonly used units of radioactivity of dpm ml⁻¹ or dpm g⁻¹ of sample. Following sample addition and any pretreatment, tin capsules were closed and pressed prior to being loaded into the autosampler of a C,H,N analyser (Model NA2100 Brew Analyser, Elemental MicroAnalysis, Okehampton, UK). Pretreatment of samples included drying for faecal homogenates and serum extracts. A calibration curve of urea standards was prepared and used to determine the carbon content of each sample.

AMS analysis. The AMS sample wheel into which graphite-containing cathodes were placed was inserted into the ion source of the AMS instrument (5 MV 15SDH-2 Pelletron AMS system, National Electrostatics Corporation, Middleton, WI, USA) at CBAMS Ltd. The multicathode negative-ion source generated a caesium (Cs⁺) ion beam that was accelerated onto each cathode's graphite surface in sequence. The resulting negative carbon ion beam contained ¹²C⁻, ¹³C⁻ and ¹⁴C⁻ and other ions such as ¹⁶O⁻.

The carbon ion beam was pre-accelerated, passed through a spherical electrostatic analyser and then progressed towards the injection magnet. Output of $^{12}\text{C}^-$ was typically 1–100 μA . The magnet was set to inject $^{12}\text{C}^-$ (150 μs), $^{13}\text{C}^-$ (600 μs) and $^{14}\text{C}^-$ (0.1 s) ions sequentially normally at 68 keV; one combined measurement on each isotope in turn corresponded to one cycle. The carbon ion beam was accelerated towards the positive centre terminal of the tandem Pelletron accelerator through an Einzel lens. The terminal voltage used for this series of analyses was 4.5 MV with a particle energy of ~ 22.5 MeV. At the central terminal, electrons were stripped from the carbon atom in an argon gas collision cell to yield positively charged carbon ions ($^{12,13,14}\text{C}^{+1}$ to $+6$). C^{4+} ions were selected for measurement as these were the most abundant at this energy. These ions were accelerated away from the positive centre terminal and onwards towards the electrostatic quadrupole triplet and analysing magnet.

Immediately past the post-analysing magnet, $^{12}\text{C}^{4+}$ and $^{13}\text{C}^{4+}$ ions were measured as an ion current in offset Faraday cups. $^{14}\text{C}^{4+}$ ions were passed down the high-energy beam line, through an electrostatic quadrupole doublet and a cylindrical electrostatic analyser. From here, the ions entered a gas ionization detector where they were collected on anodes (four in total), which measured the energy loss and total energy of each ion. Other interfering non- $^{14}\text{C}^{4+}$ ions were generally prevented from entering the gas ionization detector by the combinations of electrostatic analysers, magnets, slits and charge state separation. Vacuum pressures of $\sim 10^{-9}$ Torr were maintained in the beam line and 10^{-6} Torr in the ion source. Ion transmission through the instrument was between 30 and 60%. A schematic of the AMS instrument is shown in figure 2.

Overall time for analysis of each sample ranged from 5 to 25 min consisting of a burn-in time of 600 cycles (60.4 s) and sample analysis of 500–5000 cycles (50.4–504 s) conducted a minimum of three times and a maximum of five. In some instances long analysis times were required to improve the precision of the data from samples, such as serum and serum extracts, containing very low levels of radiocarbon against relatively high background levels.

For each AMS run, in addition to cathodes containing graphitized samples, cathodes containing standards or blanks were included as quality controls.

Determination of limit of quantification (LOQ) for analysis by AMS. The determination of LOQ for analysis of samples by AMS was of critical importance since all samples analysed contained measurable background levels of radiocarbon. Analysis of controls and predose samples were used to estimate contributions of radiocarbon present as background and those values were subtracted from the post-dose sample results.

Limits of quantification for faeces, urine, neat serum and precipitated serum extracts were calculated from an analysis of variance of the predose controls and final sample equivalents measured in dpm ml^{-1} or dpm g^{-1} . Two background readings per subject, deemed to be ideally required in the calculation of the LOQ, were not available from samples generated from within the study. Samples from collections at the latest time points after dosing were thus considered to be equivalent to predose samples since by this time the levels of radioactivity appeared to have returned to predose levels. Limits of quantification, for

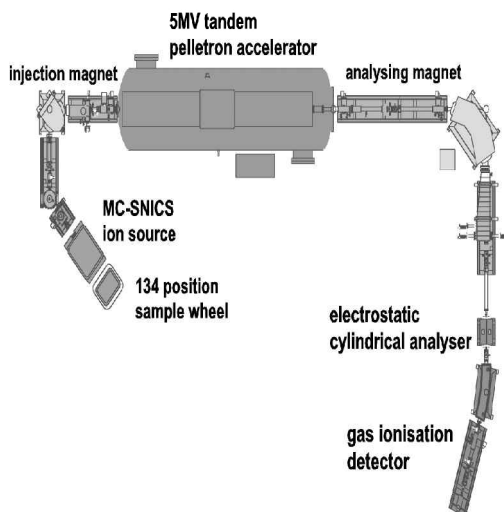


Figure 2. Schematic of the AMS instrument at The Centre for Biomedical Accelerator Mass Spectrometry Ltd. Reproduced by kind permission of CBAMS Ltd.

reconstructed radiochromatograms of HPLC fractions, were calculated from an analysis of variance of the fractions contained within defined background regions of interest, in dpm per fraction.

Two components of variance were calculated: between-subject variation and within-subject variation of predose or backgrounds. Both contributed to the overall variation associated with the LOQ.

A pooled estimated standard deviation was calculated as follows:

$$\sigma = \sqrt{\sigma_B^2 + \sigma_W^2}$$

where σ_B^2 is between-subject predose or background variance and σ_W^2 is within-subject predose or background variance.

Using criteria as defined in a Glaxo Wellcome Research and Development internal policy (based on the International Committee on Harmonization (ICH) Guideline 1996) for faeces, urine, neat serum and precipitated serum extracts, the limit of quantification was estimated using the increase above the background readings (5σ), whereas for HPLC fractions the LOQ determination included background ($5\sigma + \text{mean background}$). 95% confidence intervals of the limits of quantification were also calculated.

LC-MS-MS analysis of GI181771. Serum samples were prepared for analysis of GI181771 by liquid-chromatography with detection by tandem mass spectrometry. Calibration standards of GI181771 in human serum were prepared over the range 10–1500 pg ml⁻¹. All samples and standards were extracted by solid-phase extraction on a Packard MultiPROBE (Packard Instruments Ltd, Pangbourne, UK). Extraction was carried out on 50 mg Varian C18 Microlute II SPE 96-well blocks (Porvair Sciences Ltd, Shepperton, UK). The blocks were conditioned with 0.5 ml methanol (Fisher Scientific, Loughborough, UK) followed by 0.5 ml water. The blocks were loaded with 0.5 ml serum sample diluted with 0.5 ml internal standard in 20% v/v formic acid (BDH, Poole, UK) in water. The blocks were washed sequentially with 0.5 ml 20% v/v formic acid then 0.5 ml 40% v/v methanol and analytes of interest were then eluted with 0.75 ml ethyl acetate (Fisher Scientific). The resulting extracts were evaporated to dryness under a stream of nitrogen (nominally heated to 37°C) and reconstituted in 100 µl 50% mobile phase A, 50% mobile phase B prior to analysis by liquid chromatography with tandem mass spectrometric detection (LC-MS-MS). Mobile phase A was 0.01% formic acid in water:methanol (95:5 v/v) and mobile phase B was 0.01% formic acid in water:methanol (5:95 v/v). The serum extracts (50 µl) were analysed on a Perkin-Elmer Sciex API 3000 mass spectrometer (PE Sciex, Foster City, CA, USA) using Turbolonspray and selected ion monitoring. The liquid chromatography was performed on a 50 × 2 mm i.d. Phenomenex Luna C18 (5 µm) analytical column maintained at nominally 40°C. The mobile phase was a linear gradient which started at 50% A and 50% B, rose to 80% B over 2 min and returned to the starting conditions from 3.1 to 4 min, at a flow rate of 0.8 ml min⁻¹. The transitions of the protonated molecule, m/z 606.2 to the fragment ion m/z 443.2 were monitored for GI181771 and transitions of the protonated molecule m/z 611.2 to the fragment ion m/z 448.2 were monitored for the internal standard [²H₅]-GI181771. The method was validated and quality control acceptance criteria were applied to indicate inaccuracy of the method to be <15%.

Results

The recovery of the administered radioactive dose in faeces from six human volunteers over 5 days was determined (table 1). The majority of the dose, >59%, was excreted in the faeces within the first 48 h. After 5 days, a mean total of 92% of the dose could be accounted for in the faeces. Volunteer 64 was excluded from calculation of the means, as collection of faeces from this volunteer was incomplete. In four of the six volunteers an average of 99.2% of the dose was recovered in the faeces over the 5 days.

The recovery of the administered dose in urine from six human volunteers over the first 2 days after dose was determined (table 2). All of the values obtained from urine were below LOQ. The mean total recovery in urine was <6.5% of the dose with LOQ from 0.4 to 3.8% of the dose.

Animal studies with oral administration of [¹⁴C]-GI181771 showed low recovery of radiocarbon in the urine suggesting that <1% of the dose was absorbed (unpublished data). The results in this study in human volunteers indicate similarly low urinary excretion of drug-related material in humans.

Table 1. Percent recovery of radiocarbon in faeces following oral administration of 2.7 mg [^{14}C]-GI181771 to male human volunteers.

Collection time (h)	Volunteer number						Mean (%)	SD (%)
	61	62	63	64*	65	66		
0–24	11.8	<6.3	34.0	38.0	43.6	103	38.5	38.9
24–48	85.5	44.6	53.4	n.s.	15.8	<6.0	39.9	28.7
48–72	1.2	19.2	8.7	n.s.	40.3	<5.8	13.9	16.1
72–96	<6.2	<7.4	<2.2	n.s.	<3.3	<6.4	<5.1	n.c.
96–120	n.s.	<3.7	<3.2	<5.2	<6.4	<8.9	<5.5	n.c.
Total	98.5	63.8	96.1	38*	99.7	103	92.2	16.1

* Excluded from mean calculations.

n.c., Not calculated; n.s., no sample.

Table 2. Percent recovery of radiocarbon in urine following oral administration of 2.7 mg [^{14}C]-GI181771 to male human volunteers.

Collection time (h)	Volunteer number						Mean (%)
	61	62	63	64	65	66	
0–12	<3.7	<2.4	<1.4	<0.4	<1.3	<3.7	<2.2
12–24	<2.7	<1.5	<1.4	<1.8	<1.4	<0.9	<1.6
24–48	<3.8	<3.2	<2.5	<1.6	<2.0	<3.1	<2.7
Total	<10.2	<7.1	<5.3	<3.8	<4.7	<7.7	<6.5

Selected neat serum samples, those taken at 0.25–1.5 h after dose, were initially analysed directly by AMS to produce the concentration of radioactivity in ng GI181771 equivalents ml^{-1} serum. All concentrations were below the LOQ of 161 ng ml^{-1} . The parent drug concentrations at these times, analysed separately, rose to $\sim 3 \text{ ng ml}^{-1}$. The sensitivity of the AMS assay was such that the serum concentration of radiocarbon could not be compared with the concentrations of parent drug.

Subsequent sample preparation of all serum samples by protein precipitation resulted in an improvement in sensitivity. Removal of the majority of carbon in the sample, not derived from the dose, lead to reduction in the background signal. Results were corrected for recovery of radioactivity based on the known extraction efficiency of 105% for [^{14}C]-GI181771 by serum protein precipitation (table 3). This, >100% recovery, indicated exclusion of [^{14}C]-GI181771 from the pellet such that the apparent concentration measured in the extract supernatant was an overestimate of the true concentration. The assumption was made that the [^{14}C]-GI181771 related compounds found in the serum of dosed volunteers showed the same degree of recovery as [^{14}C]-GI181771. The radiocarbon present in the precipitated serum samples was expressed as ng equivalents of GI181771 ml^{-1} after subtraction of the predose levels. Over 25% of the values were quantifiable, above the LOQ of $\sim 2.0 \text{ ng ml}^{-1}$, with concentrations ranging up to $\sim 9.6 \text{ ng ml}^{-1}$.

An example of the data produced, for serum samples, by AMS and LC-MS-MS is shown in figure 3. The profiles obtained indicated that the concentration of radioactive drug-related material in serum was generally higher than concentra-

Table 3. Concentration of radioactivity in extracted serum (ng equivalents GI181771 ml⁻¹) following oral administration of 2.7 mg [¹⁴C]-GI181771 to male human volunteers.

Sampling time (h)	Volunteer number					
	61	62	63	64	65	66
Predose	0.00	0.00	0.00	0.00	0.00	0.00
0.25	<2.02	<2.02	2.66	<2.02	<2.02	<2.02
0.5	2.97	3.95	9.55	<2.02	2.46	<2.02
0.75	3.28	3.97	8.92	<2.02	2.49	3.97
1	<2.02	2.48	7.45	<2.02	<2.02	4.71
1.5	<2.02	<2.02	5.38	<2.02	<2.02	2.66
2	n.s.	<2.02	3.56	<2.02	<2.02	<2.02
2.5	<2.02	n.s.	4.31	<2.02	<2.02	<2.02
3	<2.02	<2.02	3.79	<2.02	<2.02	<2.02
4	2.63	<2.02	3.25	n.s.	<2.02	<2.02
6	3.85	<2.02	<2.02	<2.02	<2.02	<2.02
8	<2.02	<2.02	<2.02	<2.02	<2.02	<2.02
12	n.s.	<2.02	<2.02	<2.02	<2.02	<2.02
16	<2.02	<2.02	<2.02	<2.02	<2.02	<2.02
24	<2.02	<2.02	<2.02	<2.02	<2.02	<2.02

n.s., No sample.

tions of parent drug. Although there were insufficient data to allow for a calculation of areas under the serum radiocarbon:time curve (AUC), peak concentrations of radiocarbon may be up to eight times higher than concentrations of parent drug in serum of some volunteers.

A trend was evident indicating that the volunteers with the higher radiocarbon concentrations in serum also had the higher concentrations of GI181771 in serum, adding confidence that the data obtained were reliable.

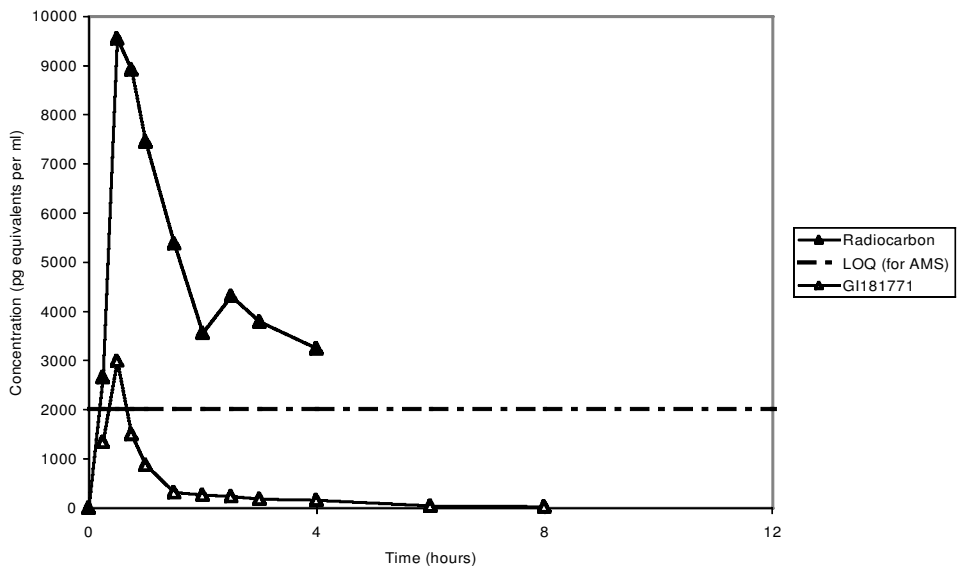


Figure 3. Example profiles of concentrations of radiocarbon due to drug-related material and concentrations of GI181771 in serum from a human male volunteer (no. 63) following oral administration of 2.7 mg [¹⁴C]-GI181771.

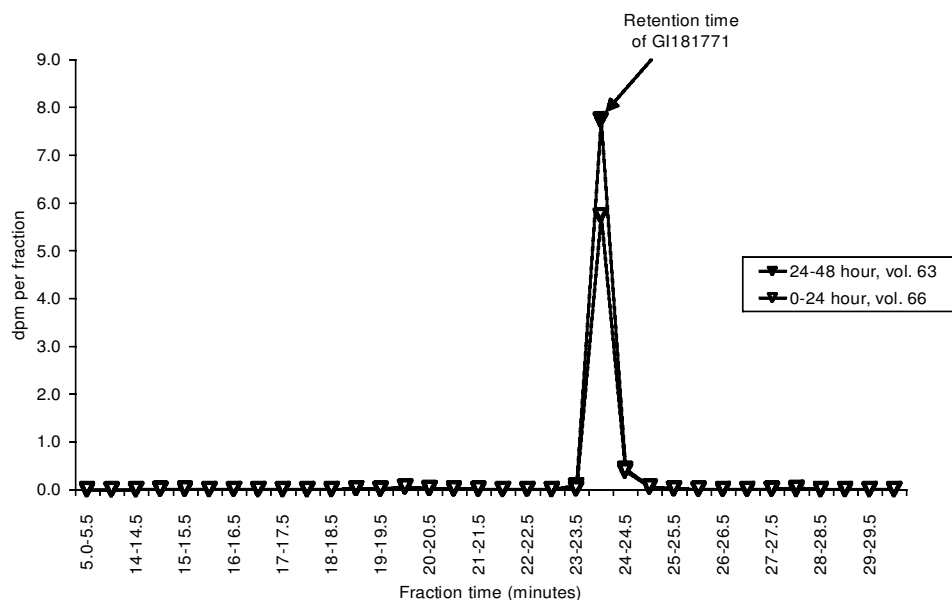


Figure 4. Reconstructed radiochromatograms (AMS results) of HPLC separation of faecal extracts from volunteers 63 and 66 following oral administration of 2.7 mg [^{14}C]-GI181771.

A reconstructed radiochromatogram of HPLC separations from 10 times concentrated faecal extracts taken from selected predose and post-dose samples is shown in figure 4. The data indicated that radioactive material was mostly excreted as unchanged GI181771. The same data are shown in figure 5 using a magnified scale and they indicate that $\sim 0.2\%$ of the radioactivity detected corresponds to the retention time of a metabolite previously observed in rat bile and in human, rat and cynomolgus monkey liver preparations (unpublished data).

Results from the HPLC fractions of the pooled serum extracts showed no obvious drug-related components. These samples had been converted to graphite using an alternative to tributyrin as the carbon carrier, which contained minimal radiocarbon, to reduce the background and improve sensitivity. Unfortunately due to the very low levels of radioactivity ($<0.01 \text{ dpm ml}^{-1}$) in all of the fractions, background regions of interest could not be defined, the limit of quantification could not be calculated and therefore drug-related radioactivity could not be determined.

In this study, LOQ for a range of biological matrices was determined (table 4). Serum samples obtained from a placebo group dosed orally with the same dosing vehicle (polyethylene glycol 400) as used in this study were processed to help further the determination of LOQ. To provide an estimate of LOQ for each of the matrices, a statistical analysis of background results was carried out. HPLC fractions coinciding with background regions of interest were treated as background measurements for this purpose. LOQ was taken as five times the pooled standard deviation of the background measurements derived from the between- and within-subject variations. LOQ was $\sim 0.9 \text{ dpm g}^{-1}$ for faeces homogenate, 0.4 dpm ml^{-1} for neat serum and 0.2 dpm ml^{-1} for neat urine. LOQ were lower for

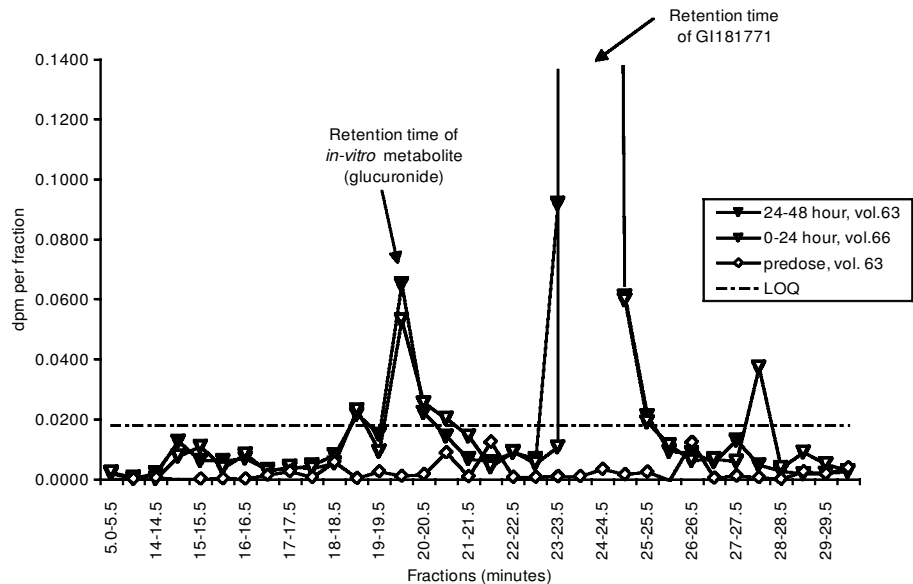


Figure 5. Reconstructed radiochromatograms (AMS results) of HPLC separation of faecal extracts from volunteers 63 and 66 following oral administration of 2.7 mg [¹⁴C]-GI181771 – expanded y-axis.

Table 4. Limits of quantification for analysis of biomedical matrices by AMS, as defined for this study within Glaxo Wellcome Research and Development Ltd.

		dpm g ⁻¹ , dpm ml ⁻¹ or dpm/fraction				LOQ after predose of background has been subtracted	
Sample description	Matrix	Volume or weight analysed	Mean predose or background	SD (pooled)	CV (%)	Best estimate	95% Confidence interval
Faeces	homogenate	50 mg	0.60	0.17	28	0.86	0.58–1.97
Urine	neat	200 µl	0.11	0.040	36	0.20	0.15–0.37
Serum	neat	60 µl	0.57	0.087	15	0.43	0.28–0.96
Serum extract ¹	supernatant	800 µl	0.021	0.0011	5	0.005	0.004–0.011
Serum extract ²	supernatant	800 µl	0.025	0.0042	17	0.021	0.017–0.026
							LOQ including background
HPLC fraction of faecal extract ³	mobile phase	100 µl	0.0027	0.0030	111	0.018	0.014–0.025
HPLC fraction of serum extract ⁴	mobile phase	150 µl	0.0011	0.0016	145	0.009	0.008–0.012

¹ Volunteer 63 was excluded, through application of a statistical test, as an outlier from the LOQ calculation.

² Data generated from serum samples from subjects administered only with dosing vehicle (PEG 400). Calculation assumes 100% recovery after precipitation.

³ Pre- and post-dose fractions, contained within the defined background regions of interest (5–6, 14–18 and 28–30 min), were used in calculation of LOQ.

⁴ Fractions were converted to graphite using an older (undisclosed) carbon source than Tributyrin. As background regions of interest could not be defined all fractions were used in calculation of LOQ.

samples that were processed further such that LOQ for serum extracts ranged from 0.02 to 0.005 dpm ml⁻¹. Fractions from HPLC of faecal extracts produced an LOQ = 0.02 dpm ml⁻¹ and for serum extracts LOQ = 0.009 dpm ml⁻¹. As expected, samples containing either the least amount of total carbon or naturally occurring radiocarbon have the least variation in background and have the lowest LOQ for radiocarbon determinations. The data shown here represent a crude estimation of the LOQ that may be found in these matrices. The values are based on limited predose samples and approximations to predose samples, which were analysed in triplicate for a set period: 100–500 s.

Discussion

AMS is now recognized as a useful addition to the tools available to those involved in drug discovery and development. Glaxo Wellcome Research and Development Ltd has been involved in the validation of AMS for biomedical use through the conduct of studies in animals and has established a dedicated laboratory for the preliminary sample handling involved. In the pilot clinical study described, it has been successfully demonstrated that AMS can be used as a tool for determining routes of excretion following administration of a very low dosage of radiocarbon-labelled drug. Throughout the conduct of the work on the clinical study and samples generated, contamination of samples with radiocarbon was not encountered and this may well endorse the contamination containment precautions taken by all involved. The approach taken by Glaxo Wellcome Research and Development Ltd with regard to contamination containment was that of minimizing risk and may have been overcautious, i.e. a specialized laboratory may not be required so long as appropriate precautions are taken.

A mean dose of 2.7 mg, 121 Bq (3.3 nCi) [¹⁴C]-GI181771 was administered to six healthy human male volunteers and mass balance was achieved between administered and recovered radiocarbon. The mean faecal recovery of radiocarbon was 92% of the dose, mostly recovered in the first 48 h in the form of unchanged drug. Radiocarbon was not detected in any of the samples of urine implying that urine collected in the first 48 h following dosing contained no more than 6.5% of the dose. Concentrations of radioactivity in serum were measured using a precipitation step to increase sensitivity and contained up to 10 ng GI181771 equivalents ml⁻¹. Comparison with parent drug levels indicated the presence of systemically circulating metabolites.

It is clear that the LOQ for analysis of biomedical samples by AMS is improved when 'cleaner' samples (those containing lowered levels of endogenous material) are analysed as the impact of background radiocarbon is reduced. Signal:noise ratios and hence limits of detection were improved significantly (ranging from 20- to 80-fold) by use of protein precipitation to 'clean-up' the serum prior to analysis. The assumption was made that a high recovery of radioactive drug-related components was achieved through this sample preparation procedure. The estimate for LOQ obtained for serum extracts from the dosed subjects, after excluding an outlier from the calculation, was 0.005 dpm ml⁻¹, with a coefficient of variation (CV) = 5%. This LOQ was used in calculating the results for precipitated serum samples from the subjects dosed with drug. The LOQ determined from the placebo-dosed subjects in a separate study was 0.021 dpm ml⁻¹, with CV = 17%. This value was similar to that obtained

from subjects dosed with drug, with the outlier included ($0.020 \text{ dpm ml}^{-1}$) and may be a more typical value. The background level of radiocarbon in extracts of serum may vary from one particular group of subjects to another, possibly due to dietary differences and thus result in a different LOQ being determined for each individual study. It is likely that the use of plasma rather than serum may reduce variability by removal of the variable protein recoveries associated with the clotting process and leading to lower LOQ. Indeed CBAMS Ltd has determined $\text{LOQ} = 0.01 \text{ dpm ml}^{-1}$ for neat plasma from studies conducted for other sponsors (unpublished data). There is an intention to assess the variability in background levels of radiocarbon in plasma from human volunteers in an AMS study, with a view to recommending that plasma is used rather than serum in all future AMS studies, conducted within Glaxo Wellcome Research and Development Ltd.

With regard to the LOQ determined in this study, precision would be improved by increasing the AMS analysis time, but the time constraints encountered when processing hundreds of biomedical samples would need to be borne in mind. A further study to examine specifically the limits of quantification for various matrices is, in our opinion, warranted.

Valuable lessons have been learned during the conduct of this study, which will aid in the design of future studies where AMS is used. Administration of a dose of radiolabel that produces the maximum exposure to ionizing radiation of $1 \mu\text{Sv}$, where no certification is required from ARSAC, would likely improve sensitivity ~ 17 -fold over that observed in this study, where the exposure was only $0.06 \mu\text{Sv}$. A robust determination of limits of quantification for each matrix requires at least two predose samples per subject, ideally taken several hours apart. These extra predose samples would need to be included in any future study design. If concentrations of drug-related radioactivity in serum equating to $<0.4 \text{ dpm ml}^{-1}$ are expected, then a precipitation step may be required to provide adequate sensitivity, involving both an estimation of recovery and carbon analysis of the supernatant.

Future potential of AMS

AMS has the potential to provide supplemental information in studies where conventional doses have been administered and sensitivity becomes an issue, for example at later time-point sample collections, or to enable conduct of studies that are currently not possible. The inherent sensitivity of this technique could be harnessed to enable monitoring of drug-related analytes following administration of very low doses. Such low dose studies, which have been termed 'microdosing' studies, may be of interest to the pharmaceutical industry. Through the use of sample separation techniques coupled with AMS for off-line detection, as shown here, pharmacokinetics of both parent drug molecule and total drug-related material (drug and metabolites), could be elucidated. With the use of high specific activity radiocarbon labelled drugs incorporated into a small, perhaps low μg or even sub- μg , total dose it is envisaged that sensitivities in the high fg ml^{-1} range may be achievable. It is possible that the administration of such a small dose may not require very much in the way of supporting safety information from toxicology and ADME (absorption, distribution, metabolism and elimination) studies, thereby speeding up the progression from drug discovery to first clinical investigation. There may well be many caveats placed upon information obtained from

microdosing studies, but should there be a desire to rank compounds according to their pharmacokinetics or susceptibility to metabolism then this could be achieved in human subjects earlier in a drug development programme than is currently the norm. The authors believe that this type of approach, and its pros and cons, is under consideration in a number of companies within the pharmaceutical industry.

AMS could also be of use in the reduction of numbers of animals used in drug discovery pharmacokinetic and excretion balance studies. The small sample size required for analysis by AMS, as low as 5 µl for plasma or serum, means that serial blood sampling from small animals such as rodents is viable and the need for composite study designs is reduced with consequent reduction in the number of animals required.

One of the biggest drawbacks to the use of AMS in early drug discovery is potentially the requirement for incorporation of the radioisotopic label within the molecule of interest. If the technique is ever to be used as part of a screening strategy for drug discovery candidates then incorporation of, for example, radio-carbon would need to be higher in throughput than is currently the case.

With the advent of the use of AMS in the pharmaceutical industry, the way is opened to reducing the use of relatively high levels of radioactivity for drug discovery and development. Reduction in levels of radioactivity handled brings with it not only ethical advantages associated with clinical use, but also a reduction in the environmental issues involved in the handling and disposing of radioactivity.

This paper has focussed on the use of AMS for the measurement of radio-carbon, but it should be noted that other elemental isotopes may well be of interest, for example ^{41}Ca and ^{129}I , both of which can be analysed using this technique. ^{41}Ca has been used to label human bones in studies to measure bone turnover (Freeman *et al.* 1997). ^{131}I and ^{125}I are frequently used in radio-immunoassays and AMS provides the alternative of using the more radiologically safe ^{129}I . Labelling of proteins or antibodies with ^{129}I and subsequent analysis by AMS could be of use in developing new therapies or diagnostic reagents.

AMS will no doubt be more widely used in biomedical investigations in the future. The rate-limiting step in the analysis of samples is the sample preparation which for radiocarbon involves the technically challenging combustion and graphitization steps. It is envisaged that advances will be sought in interfacing the AMS with some sort of on-line chromatography system for increased sample throughput and improved ease of use. At present there is only one commercial AMS facility in the UK for analysis of biomedical samples, but it may be that as the technique matures in the biomedical area and as AMS instrument size and cost potentially reduce, more will become available.

Acknowledgements

The authors are grateful for the analytical service and input to study design provided by Professor Colin Garner, Sally Gregory and staff at The Centre for Biomedical Accelerator Mass Spectrometry (CBAMS) Ltd, Sand Hutton, York. The authors acknowledge staff at the Glaxo Wellcome Clinical Pharmacology Unit, Northwick Park Hospital, London, for conducting the clinical study and they also thank the staff of the Lawrence Livermore National Laboratory, Livermore, CA, for assistance in the early days. The authors also acknowledge

the assistance of Michael Aylott, Statistical Services at Glaxo Wellcome Research and Development Ltd.

References

- ARSAC, Application for Certificate to Administer Radioactive Medicinal Products; obtained from ARSAC Support Unit, National Radiological Protection Board, Chilton, Didcot, UK.
- BENNETT, C. L., BEUKENS, R. P., CLOVER, M. R., GOVE, H. E., LIEBERT, R. B., LITHERLAND, A. E., PURSER, K. H. and SONDEHEIM, W. E., 1977, Radiocarbon dating using electrostatic accelerators: negative ions provide the key. *Science*, **198**, 508–510.
- FREEMAN, S. P. H. T., KING, J. C., VIEIRA, N. E., WOODHOUSE, L. R. and YERGEY, A. L., 1997, Human calcium metabolism including bone resorption measured with ^{41}Ca tracer. *Nuclear Instruments and Methods in Physics Research*, **B123**, 266–270.
- GARNER, R. C., 2000, Accelerator mass spectrometry in pharmaceutical research and development – a new ultrasensitive analytical method for isotope measurement. *Current Drug Metabolism*, **1**, 205–213.
- GARNER, R. C., BARKER, J., FLAVELL, C., GARNER, J. V., WHATTAM, M., YOUNG, G. C., CUSSANS, N., JEZEQUEL, S. and LEONG, D., 2000, A validation study comparing accelerator mass spectrometry and liquid scintillation counting for analysis of [^{14}C]-labelled drugs in plasma, urine and faecal extracts. *Journal of Pharmaceutical and Biomedical Analysis*, **24**, 197–209.
- ICH GUIDELINE 1996, *The International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) Guideline, 1996, Q2B Validation of Analytical Procedures: Methodology*.
- KAYE, B., GARNER, R. C., MAUTHE, R. J., FREEMAN, S. P. H. T. and TURTELTAUB, K. W., 1997, A preliminary evaluation of accelerator mass spectrometry in the biomedical field. *Journal of Pharmaceutical and Biomedical Analysis*, **16**, 541–543.
- NELSON, D. E., KORTEING, R. G. and SCOTT, W. R., 1977, Carbon-14: direct detection at natural concentrations. *Science*, **198**, 507–508.
- VOGEL, J. S., 1992, Rapid production of graphite without contamination for biomedical AMS. *Radiocarbon*, **34**, 344–350.

Pharmacokinetic/pharmacodynamic assessment of the effects of E4031, cisapride, terfenadine and terodiline on monophasic action potential duration in dog

R. WEBSTER†*, G. ALLAN†, K. ANTO-AWUAKYE†,
A. HARRISON†, T. KIDD†, D. LEISHMAN‡, J. PHIPPS‡ and
D. WALKER†

† Department of Drug Metabolism and ‡ Department of Discovery Biology, Pfizer Global Research and Development, Sandwich CT13 9NJ, UK

Received 22 January 2001

1. Torsades de pointes (TDP) is a potentially fatal ventricular tachycardia associated with increases in QT interval and monophasic action potential duration (MAPD). TDP is a side-effect that has led to withdrawal of several drugs from the market (e.g. terfenadine and terodiline).

2. The potential of compounds to cause TDP was evaluated by monitoring their effects on MAPD in dog. Four compounds known to increase QT interval and cause TDP were investigated: terfenadine, terodiline, cisapride and E4031. On the basis that only free drug in the systemic circulation will elicit a pharmacological response target, free concentrations in plasma were selected to mimic the free drug exposures in man. Infusion regimens were designed that rapidly achieved and maintained target-free concentrations of these drugs in plasma and data on the relationship between free concentration and changes in MAPD were obtained for these compounds.

3. These data indicate that the free ED₅₀ in plasma for terfenadine (1.9 nM), terodiline (76 nM), cisapride (11 nM) and E4031 (1.9 nM) closely correlate with the free concentration in man causing QT effects. For compounds that have shown TDP in the clinic (terfenadine, terodiline, cisapride) there is little differentiation between the dog ED₅₀ and the efficacious free plasma concentrations in man (<10-fold) reflecting their limited safety margins. These data underline the need to maximize the therapeutic ratio with respect to TDP in potential development candidates and the importance of using free drug concentrations in pharmacokinetic/pharmacodynamic studies.

Introduction

Evaluation of potential adverse pharmacology associated with new chemical entities is important in the design of molecules in drug discovery. It is therefore becoming increasingly important to evaluate and determine potential side-effect issues with compounds early in the drug discovery programme. This allows rational design with respect to such adverse pharmacology in order to maximize the therapeutic ratio of potential development candidates. Increasingly *in vitro* tests are used as the preliminary methodology to evaluate potential side-effects in drug discovery. These *in vitro* assays provide a high throughput method of evaluating compounds. However, there remains a need to validate *in vitro* assays by confirming their *in vivo* relevance.

* Author for correspondence; e-mail: rob_webster@sandwich.pfizer.com

Torsades de pointes (TDP) is an example of one such adverse pharmacology. TDP is a form of ventricular tachycardia that is associated with excessive prolongation of the QT interval. This excessive prolongation of the QT interval is reflected in a delayed myocardial repolarization and a prolongation of action potential duration (Salata *et al.* 1995). TDP is found in many clinical settings, is most often drug induced and can be fatal if the correct medical action is not taken (Monahan *et al.* 1990). It has therefore been proposed that the ability of a compound to induce TDP should be evaluated both *in vitro* and *in vivo* prior to first in man studies (Davis 1998).

A primary *in vitro* screen allows evaluation of a relatively large number of compounds. In this screen the ability of compounds to block HERG potassium currents is assessed (Leishman *et al.* 2000a, b). HERG is an *ether-a-go-go*-related gene that when expressed in *Xenopus* oocytes and HEK293 cells has properties similar to the cardiac rapidly activated delayed-rectifier K^+ current (I_{kr}). Drugs that block either I_{kr} or HERG have been shown to cause prolongation of QT and TDP. Thus activity against HERG potassium current can be used to evaluate the potential of a compound to increase QT interval and cause TDP (Mohammad *et al.* 1997). An *in vivo* model was required actually to assess the ability of a compound to cause QT prolongation on a functioning heart. These data would also allow comparisons of the *in vivo* plasma concentrations at which any significant adverse pharmacology occurred with those required for efficacy, allowing estimations of therapeutic ratios.

In order to develop the above experimental sequence, compounds known to cause prolongation of the QT interval and TDP were evaluated in the *in vitro* and *in vivo* models. The four compounds were E4031 (*N*-(4-[(1-[2-(6-methyl-2-pyridyl)ethyl]-4-piperidyl)-carbonyl] phenyl) methanesulphonamide), cisapride, terfenadine and terodiline (figure 1). Cisapride, terfenadine and terodiline have all been shown to cause QT prolongation and TDP in the clinical setting (MacConnell and Stanners 1991, Hartigan-Go *et al.* 1996, Mohammad *et al.* 1997). E4031 as a class III anti-arrhythmic agent was used as a positive control. It is a potent I_{kr} channel blocker, causing QT prolongation as part of its pharmacology (Vaughan Williams 1970, Katoh *et al.* 1990). Dog is widely used in drug discovery and development to evaluate pharmacokinetics, metabolism and toxicology (Wright *et al.* 1987) and has also been used in the study of TDP (Gras and Llenas 1999).

It is generally considered that drug bound to protein, either in plasma or tissue, is not available to interact with the receptor (Sjoqvist 1985). Therefore, the protein binding of a molecule in plasma can cloud the relationship between plasma concentrations and efficacy. This is especially the case when different compounds are being compared or when a compound is compared across species where protein binding differences may exist (Smith 1997). Therefore, free plasma concentration data were used in the design of the current studies and to compare the data generated for the different compounds in the dog experiments.

In order to evaluate adequately the pharmacodynamic response in dog, several determinations at a given plasma concentration were required. This required that the plasma concentration remained relatively constant for the duration of this testing (~15 min). However, for more detailed examinations longer periods of sustained, constant, plasma concentrations could be required and it was anticipated that this could be up to 2 h. In order to provide this concentration profile,

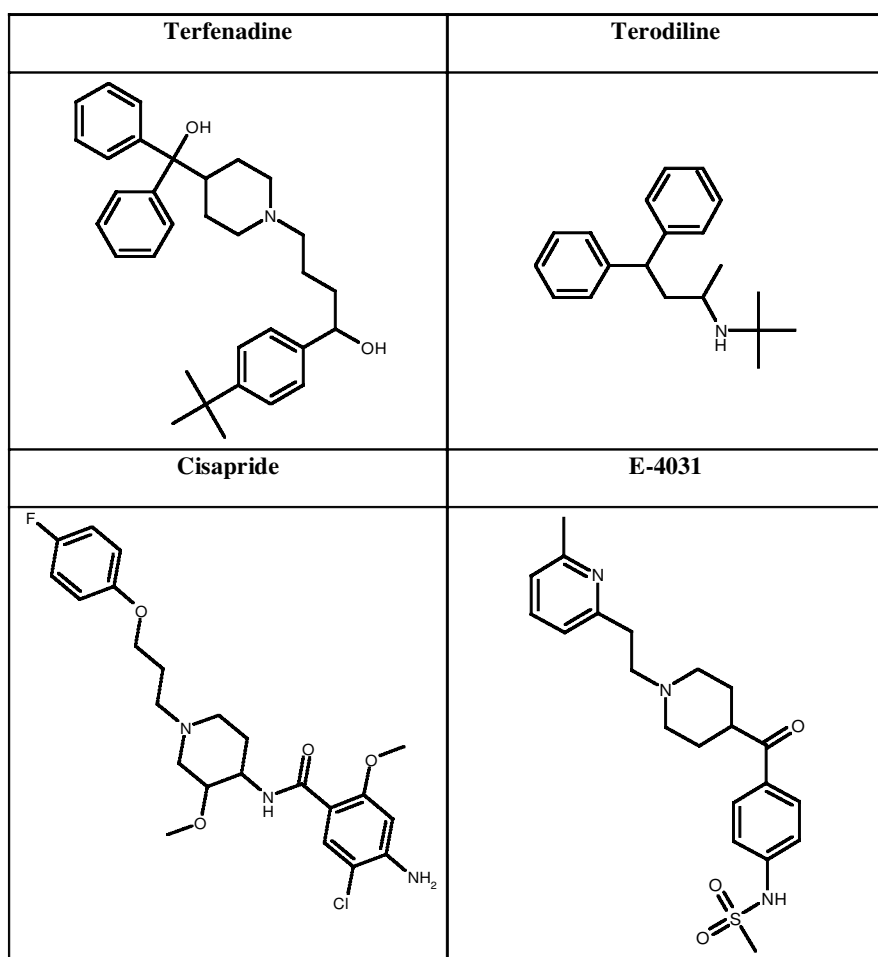


Figure 1. Structures of terfenadine, terodiline, cisapride and E-4031.

an initial i.v. bolus dose followed by a constant rate infusion was employed. This mode of administration would allow these studies to provide equivalent data for each compound regardless of the pharmacokinetics of the molecules. This method of administration also minimizes the amount of compound required and lends itself to routine use in evaluating early development drug candidates where compound supply is often limited.

Target free plasma concentrations for terfenadine, terodiline, cisapride and E4031 were determined using the available clinical data with reference to free plasma concentrations associated with therapy and those at which TDP had been observed. Where possible, target-free concentrations were overlapped to aid in comparison. This approach led to essentially five free plasma concentration targets, each 4-fold apart: 0.7, 3, 12, 48 and 192 nM. The low or high target concentration was omitted depending on the potency of the compound. A sequential bolus/infusion regimen was administered to dog in order to evaluate all the target-free plasma concentrations in a single experiment. The infusion regimen was not evaluated in dog pharmacokinetics studies prior to use in

pharmacological investigations. Therefore, the concentrations of drug in plasma were evaluated alongside the pharmacological measurements using a specific assay developed and validated for these compounds.

The present paper describes the work carried out to establish an appropriate dosing regimen to allow the development and optimization of the methodology required to evaluate the effects of these compounds on MAPD *in vivo* and the subsequent monitoring of plasma concentrations. The relationship between the pharmacodynamic effects and free drug concentrations is also discussed and compared with available *in vitro* and *in vivo* data.

Materials and methods

Dog ECG studies

Male beagle dogs (weight range 11.4–14.9 kg) were anaesthetized with i.v. propofol (Rapinivet 10 mg ml⁻¹) at a dose level of ~5 mg kg⁻¹, following premedication with 10 mg diazepam. Thereafter, anaesthesia was maintained with isoflurane (1–2%) via an N₂O/O₂ mixture in the ratio of 2:1. The animals respired spontaneously. The left femoral vein was cannulated for the administration of compounds and the left femoral artery for the measurement of arterial blood pressure (ABP). The right femoral artery was cannulated with a Millar transducer, which was passed via the aorta into the left ventricle for the measurement of left ventricular pressure. Monophasic action potentials (MAP) from the endocardial surface of the right ventricle were recorded from a 7 F Franz MAP/pacing catheter inserted into the right ventricle via the right jugular vein. The resultant signal was amplified by a DC-coupled preamplifier (EP Technologies). Lead II ECG was recorded throughout the experiment. A bipolar pacing catheter was passed into the right atrium via the right femoral vein and its position verified by observing the resultant electrogram. A further bipolar electrode was passed down the right carotid artery and positioned near a cusp of the aortic valve to record the His bundle potential via a Grass high-performance AC amplifier.

Core temperature was maintained at ~37°C by means of an Harvard thermocirculator and water blanket. The core temperature was verified by means of a Swan Ganz catheter with an intact thermistor connected to a Gould cardiac index computer and passed down the oesophagus. All transducers and recording electrodes were connected to HSE preamplifiers. Data were collected and analysed using a Po-ne-mah digital data acquisition system. Hard copy chart output of the MAP, ECG and His bundle potential was obtained using either a Gould TA11 or Po-ne-mah chart recorder.

Following preparatory surgery, there was 30 min of stabilization. MAP was recorded continuously throughout the experiment and logged by the data acquisition system as the means from complete cardiac cycles every 10 s. The time to 100% repolarization of MAP (MAPD) was subsequently analysed from the hard copy output using a Graphtec digitizing pad. The effects of the compounds and vehicle were monitored both during normal sinus rhythm and atrial pacing. The cycle length of paced frequencies was controlled by a Digitimer (model D4030), which was connected to a Digitimer-isolated stimulator. The heart was then paced at 10% above threshold voltage and a pulse width of 2 ms via the bipolar catheter inserted into the right atrium. Representative values of paced (150, 200 and 225 bpm from the right atrium) and unpaced recordings were taken at ~10-min intervals during the control period and subsequently at ~5 and 15 min after each dose. A period of between 30 and 60 s was allowed before any measurements were taken to allow for sufficient equilibration following a change in pacing frequency. Arterial blood samples (2 ml) were taken throughout the experiment. These blood samples were centrifuged for 5 min at room temperature ($g = 1500$) and the plasma removed using a pipette. The plasma was frozen and stored for subsequent analysis of drug content. For each compound four to six dogs were used to evaluate the effect of drug on MAPD.

Compounds were formulated in either 0.9% saline (E4031) or 30% β -hydroxy propyl cyclodextrin dissolved in 0.1 M HCl (terfenadine, terodiline and cisapride) at a concentration of 5 mg ml⁻¹. Both vehicles had been shown to have no marked effect of MAPD in dog.

All studies involving animals were carried out in accordance with the requirements of UK national legislation and conducted under necessary authorities.

Plasma assays for terfenadine, terodiline, cisapride and E4031

Terfenadine was quantified in plasma samples using a hplc assay with ultraviolet (UV) detection. Terfenadine was isolated from 1 ml aliquots of dog plasma using a 100 mg C2 solid-phase extraction cartridges (Isolute). The extraction cartridges were activated using 1 ml methanol followed by 1 ml water. The samples were added to the extraction cartridge and allowed to drip through under gravity. The extraction cartridges were washed with 1 ml water followed by 1 ml water/methanol (1:1). The

compound was eluted into tapered tubes using 1 ml methanol. The methanol was evaporated to dryness under nitrogen (37°C), resuspended in 200 µl mobile phase and injected on to the hplc system. Samples were chromatographed on a Hypersil C18 hplc column (Shandon 5 µm, 250 × 4.6 mm) using a mobile phase consisting of water (65%), acetonitrile (35%) and 0.1% trifluoroacetic acid. The flow rate was 1.5 ml min⁻¹. Terfenadine had a retention time of 9 min and was detected by UV absorbance at 220 nm.

Terodiline was extracted from 0.2 ml aliquots of dog plasma diluted with water (1 ml) using ethyl acetate (2 ml). The samples were mixed and centrifuged to separate the phases. The ethyl acetate was removed and evaporated to dryness under a stream of nitrogen (37°C). The samples were reconstituted in 300 µl 2 mM ammonium acetate dissolved in methanol/water (70:30) and 180 µl was injected onto the hplc system for quantification. Terodiline was chromatographed using a Hypersil HS100 C18 hplc column (5 µm, 50 × 4.6 mm; Shandon) and a mobile phase consisting of 2 mM ammonium acetate dissolved in methanol/water (90:10) adjusted to pH 4.0 with glacial acetic acid (flow rate = 1 ml min⁻¹). Terodiline had a retention time of 1.7 min and was detected using a Sciex API 2000 triple quadrupole mass spectrometer with a turbospray interface. The mass spectrometer was functioning in positive-ion multiple reaction monitoring mode. Of the eluent from the hplc column, 2% was split and directed into the mass spectrometer source using an AcurateTM flow splitter. The transition 282 to 167 was used to detect the compound as this was the major transition observed in the mass spectrometer.

Cisapride was extracted from basified plasma samples using *tert*-butyl methyl ether (TBME). Dog plasma samples (100 µl) were basified with 100 µl 1 M sodium hydroxide and 2 ml TBME added. The samples were mixed by inversion for 5 min and centrifuged to separate the phases. The TBME was removed by aspiration and placed in to a 96 deep-well block (Porvair). The samples were evaporated to dryness under a stream of nitrogen (37°C). The dried extract was resuspended in 200 µl 2 mM ammonium acetate dissolved in methanol/water (70:30) and 180 µl injected onto the same hplc-MS-based analytical system used for terodiline. Cisapride had a retention time of 1.2 min and was detected using the transition 466 to 184 as this was the major transition observed in the mass spectrometer.

E4031 was extracted from 0.5 ml aliquots of plasma using a C2 end-capped solid-phase extraction cartridges (Isolute). The extraction cartridges were activated using 1 ml methanol followed by 1 ml water. Samples were basified using 0.1 M sodium hydroxide (0.5 ml) added to the extraction cartridges and allowed to drip through under gravity. Each extraction cartridge was washed with distilled water and E4031 eluted using methanol containing 1% trifluoroacetic acid. The resultant extracts were evaporated to dryness under a stream of nitrogen (37°C) and then resuspended in 100 µl mobile phase prior to injecting 80 µl on to the hplc system.

The hplc system used to evaluate E4031 consisted of a Hypersil BDS C18 hplc column (5 µm, 150 × 4.6 mm; Shandon) and a mobile phase consisting of water (85%) and acetonitrile (15%) containing 0.1% trifluoroacetic acid. The mobile phase flow rate was 1 ml min⁻¹. E4031 had a retention time of 12 min and was detected by UV absorbance at 270 nm.

Performance monitoring of assays

The inaccuracy and cumulative variation of all assays used in these studies were evaluated by analysis of replicate quality control samples at the bottom, middle and top of the assay range in plasma ($n = 5$ samples/concentration).

In all assays quality control samples were used to determine the performance of the assay on a day-to-day basis. Quality control samples were within 20% of the expected values at the low end of the calibration curve, whilst at all other points they were within 15% of the expected values. All samples where concentrations exceeded the range of the calibration curve were diluted with control dog plasma and re assayed.

In vitro plasma protein-binding determinations

Plasma protein-binding values were determined for terfenadine and E4031 in pooled male beagle dog plasma ($n = 3$ different dogs) by equilibrium dialysis using a semi-micro-Teflon dialysis cell (Spectrum Medical Industries, Inc.). Cellulose dialysis membranes with a molecular weight cut-off of 4000–6000 kD (Spectrapor) were used. Literature plasma protein-binding data were available for terodiline (86%; Hallen *et al.* 1990) and cisapride (95%; Veereman-Wauter *et al.* 1991) in the dog; hence, these were not determined.

Terfenadine and E4031 were added to plasma at nominal concentrations of 5 and 2 µM respectively. This plasma (1 ml/cell) was then added to one side of the dialysis apparatus and dialysed against Krebs buffer (pH 7.4, 1 ml). The dialysis cells were slowly rotated (20 rpm) at room temperature for at least 12 h. After this period of dialysis plasma and buffer samples (0.5 ml) were removed from the apparatus and diluted with the opposite phase (0.5 ml of either plasma or buffer). These samples were analysed using the appropriate assay methodology detailed above for plasma. Spiked buffer was also dialysed against unspiked buffer to ensure that equilibrium had occurred during the course of the experiment. Concentrations in both buffer samples following dialysis had to be essentially equal for the dialysis experiment to be considered valid. Plasma protein-binding values were determined using the following equation:

$$\text{Plasma protein binding (\%)} = 100 - \left(\frac{\text{buffer concentration}}{\text{plasma concentration}} \times 100 \right).$$

Infusion regimen design

Literature pharmacokinetic data were used wherever available to design the infusion regimen for terfenadine, terodiline, cisapride and E4031. Where data were not available in the literature, values were determined in house.

The pharmacokinetic of terfenadine in dog were estimated from available data in man and rat. Terfenadine is highly cleared in both rat ($254 \text{ ml min}^{-1} \text{ kg}^{-1}$; Ohtani *et al.* 1997) and man (Garteiz *et al.* 1982) with clearance at least equal to liver blood flow. It was therefore assumed that clearance in dog would approximate to liver blood flow. Experimental data suggest that liver blood flows in the male beagle dog (Pfizer strain) are $\sim 40\text{--}50 \text{ ml min}^{-1} \text{ kg}^{-1}$. The volume of distribution was estimated using that determined in rat (4.71 kg^{-1} ; Ohtani *et al.* 1997) and correcting for differences in plasma protein binding using the method of Obach *et al.* (1997). The volume of distribution in dog was therefore expected to be $\sim 2.0\text{--}2.5 \text{ kg}^{-1}$. The plasma protein binding of terfenadine in dog plasma was determined as detailed above.

The pharmacokinetics of terodiline following i.v. administration have been reported in dog (Hallen *et al.* 1990). Terodiline is rapidly cleared from its systemic circulation following i.v. administration (0.5 mg kg^{-1}) with a value approaching liver blood flow ($40 \text{ ml min}^{-1} \text{ kg}^{-1}$). Volume of distribution of terodiline is 71 kg^{-1} and the terminal half-life is 3 h. However, the pharmacokinetics of this compound are non-linear with clearance reduced at higher doses (15 mg kg^{-1}). Clearance at 0.5 mg kg^{-1} was used to estimate infusion regimen for these studies. The mean unbound fraction ($n = 3$) of terodiline in dog serum is $14 \pm 1.1\%$ (Hallen *et al.* 1990).

The i.v. pharmacokinetics of cisapride have been evaluated in dog and the compound has a half-life of 4.8 h. Cisapride has a low-to-moderate clearance in dog ($5.3 \text{ ml min}^{-1} \text{ kg}^{-1}$) and a volume of distribution of 11 kg^{-1} (Michiels *et al.* 1987). The plasma protein binding of cisapride was determined in dog plasma at 95% (Veereman-Wauter *et al.* 1991).

E4031 has been studied in the mongrel dog using two infusion regimens, namely a $10 \mu\text{g kg}^{-1}$ bolus followed by a $1 \mu\text{g kg}^{-1} \text{ min}^{-1}$ maintenance infusion and a $100 \mu\text{g kg}^{-1}$ bolus followed by a $10 \mu\text{g kg}^{-1} \text{ min}^{-1}$ infusion (Katoh *et al.* 1990). In these later studies total plasma concentrations of E4031 were 83 ± 40 and $818 \pm 372 \text{ nM}$ at time points at least 30 min post-bolus dosing, respectively. These infusion data were used to extrapolate the dosing regimen to be used in the present studies simply by scaling the infusion doses to match the required concentrations. The plasma protein binding of E4031 in dog plasma was determined as detailed above.

Five free plasma concentration targets, each 4-fold apart (0.7, 3, 12, 48 and 192 nM) were used in the present studies and each concentration was achieved in the same experiment using sequential bolus/infusion regimen.

Initial loading bolus doses for terfenadine, terodiline and cisapride were determined by multiplying the target concentration in plasma by the volume of distribution. Given the drugs are unlikely to reach equilibrium rapidly with all tissues into which they can distribute an initial volume of 50% that reported at steady-state was used. The maintenance infusion rates for the same compounds were estimated assuming that the infusion dose was equal to the total plasma concentration required multiplied by the estimated clearance. Total plasma concentrations were determined by correcting the target-free plasma concentration for the extent of plasma protein binding in dog.

These values were then refined using an in-house modelling program developed in Microsoft ExcelTM finally to arrive at the dosing regimen. Owing to the limited pharmacokinetic data available from the literature and some pharmacokinetic parameters in the dog having to be estimated, a simple non-compartmental model was chosen for refinement of the infusion regimen. The concentrations in plasma following administration of the initial loading bolus were modelled using equation 1, whilst, the infusion portion of the dosing regimen was modelled using equation 2. The sum of these two profiles provides the theoretical plasma concentration profile. Details of the infusion regimen designed from the above data and using this methodology are summarized in table 2.

$$\text{Concentration in plasma} = C_0 e^{-K_{el}t}, \quad (1)$$

where C_0 is concentration at the end of infusion.

$$\text{Concentration in plasma} = \frac{\text{infusion rate}}{V_d K_{el}} (1 - e^{-K_{el}t}), \quad (2)$$

where K_{el} is the elimination rate constant and V_d is the volume of distribution.

Pharmacokinetic and pharmacodynamic modelling

The relationship between free plasma concentrations and percentage increase in MAPD (data from animals with a paced heart rate of 150 bpm) for terfenadine, terodiline, cisapride and E4031 was

evaluated using WinNonLin (v.2.1 Pharsight Corporation). Free plasma concentrations were estimated by multiplying the total plasma concentration of drug by the free fraction in plasma. In doing this it is assumed that plasma protein binding is constant across the entire concentration range studied. $E_{\max}$ and EC_{50} were determined using the simple $E_{\max}$ model (model number 101, see equation 3) in WinNonLin. Where plasma concentrations were below the limits of detection in the assay the free concentrations at the lower dose were estimated from those determined at the high doses, assuming a linear relationship between dose and plasma concentration.

$$\text{Effect} = \frac{(E_{\max} \times C)}{(C + EC_{50})}$$

(3)

$E_{\max}$ is maximum effect, EC_{50} is the drug concentration required to produce 50% of the drug induced maximal effect and C is concentration.

Results

Assay performance

The performance of the specific plasma assays developed for terfenadine, terodiline, cisapride and E4031 were evaluated at the bottom, middle and top of the assay ranges. A series of quality control samples was prepared and quantified against the calibration curve for each compound. The inaccuracy and cumulative variation values obtained during the validation of these assays are summarized in table 1. Inaccuracy and cumulative variations were in all cases <20% at the lowest concentration and <15% at both the middle and top of the assay range.

Plasma protein-binding data

The plasma protein-binding values, determined by equilibrium dialysis, of terfenadine and E4031 in dog plasma were 98 ± 1 and $69 \pm 3\%$, respectively. These values were determined at plasma concentrations of 5 and 2 μM respectively.

Table 1. Performance of the assays developed to determine the concentrations of terfenadine, terodiline, cisapride and E4031 in plasma.

Compound	Expected concentration (nm)	Inaccuracy (%)	Cumulative variation (%)
Terfenadine	42	8.9	14
	424	1.6	8
	1059	1.3	2
Terodiline	36	−10.5	9
	356	−8.6	2
	712	−14.6	2
Cisapride	11	9.0	7
	536	−2.1	7
	1073	−3.5	12
E4031	42	17.0	7
	421	3.5	4
	2105	11.0	15

$$\text{Inaccuracy (\%)} = \frac{\text{mean concentration} - \text{expected concentration}}{\text{expected concentration}} \times 100$$

(n = 5).

$$\text{Co-efficient of variation (\%)} = \frac{\text{standard deviation}}{\text{mean concentration}} \times 100$$

(n = 5).

Table 2. Intravenous dosing regimen designed to maintain free plasma concentrations of terfenadine (Terf), terodiline (Terod), cisapride (Cis) and E4031 at the concentration detailed.

Target-free plasma concentration (nM)	Loading bolus dose* (µg kg ⁻¹)				Maintenance infusion dose (µg h ⁻¹ kg ⁻¹)			
	Terf	Terod	Cis	E4031	Terf	Terod	Cis	E4031
0.7	40	–	–	8	64	–	–	2.9
3	160	40	40	32	256	14	13	12
12	640	160	160	128	1024	54	51	46
48	2560	640	640	512	4096	218	204	184
192	–	2560	2560	–	–	870	819	–

* Loading bolus administered over 5 min.
–, Dose not tested as it was considered outside the relevant range.

Free concentrations in plasma following infusion and effects on MAPD

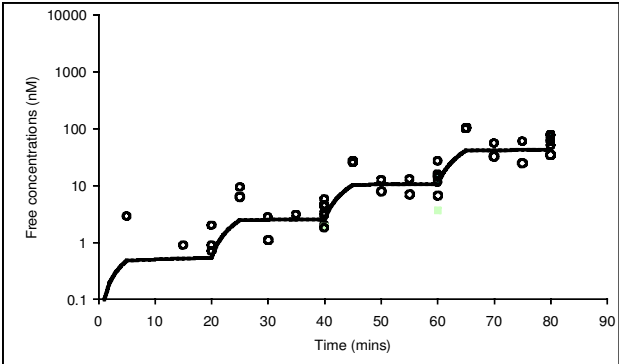
The free plasma concentration achieved in the dog during infusion studies to evaluate the effects of terfenadine, terodiline, cisapride and E4031 on MAPD are summarized in table 3 and figure 2. At low doses of E4031 plasma concentrations were below the limit of quantification of the analytical method. Based on a linear increase at higher doses these were estimated by extrapolation. The relationships between free plasma concentrations of each compound and MAPD are summarized in figure 3 and table 4. The data obtained from this study were also analysed using WinNonLin (simple E_{max} model). The E_{max} and EC_{50} obtained for these compounds using this program are summarized in table 5. The maximum change in MAPD (E_{max}) elicited by the four compounds was in the range 14–27%. The EC_{50} for terfenadine, terodiline, cisapride and E4031 were 1.9, 76, 11.5 and 1.9 nM, respectively. In all studies four to six dogs were used to evaluate the effects of these compounds on MAPD.

Table 3. Free plasma concentrations, target and achieved (*at the end of the maintenance infusion) following intravenous bolus and maintenance infusion administration of terfenadine, terodiline, cisapride and E4031.

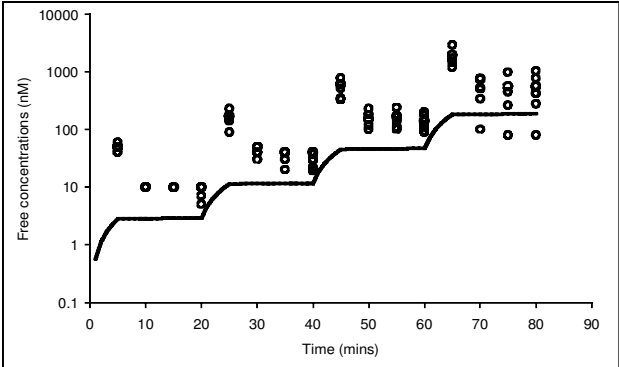
Free concentration in plasma at the end of the maintenance infusion (nM)				
Expected	Achieved (mean ± SD)			
	Terfenadine	Terodiline	Cisapride	E4031
0.7	<0.6	–	–	<13
3	3.2 ± 1.4 (n = 9)	8.1 ± 2.4 (n = 7)	2.0 ± 0.2 (n = 5)	<13
12	13.8 ± 6.2 (n = 10)	29.6 ± 8.7 (n = 11)	7.1 ± 1.8 (n = 5)	19 ± 2.1 (n = 3)
48	64.7 ± 15.4 (n = 9)	127.2 ± 37.2 (n = 11)	24.2 (n = 2)	42 ± 30 (n = 4)
192	–	521.1 ± 273.9 (n = 9)	148 (n = 1)	–

* Free plasma concentrations in the dog were estimated by multiplying the total plasma concentrations by the free fraction in plasma (0.02, 0.14, 0.05 and 0.31 for terfenadine, terodiline, cisapride and E4031, respectively – see text)
–, Dose not tested as it was considered outside the relevant range.

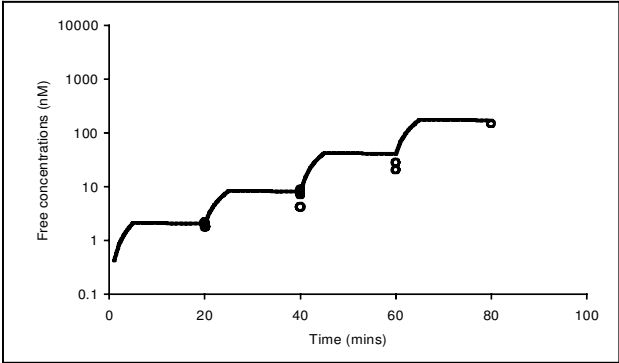
Terfenadine



Terodiline



Cisapride



E4031

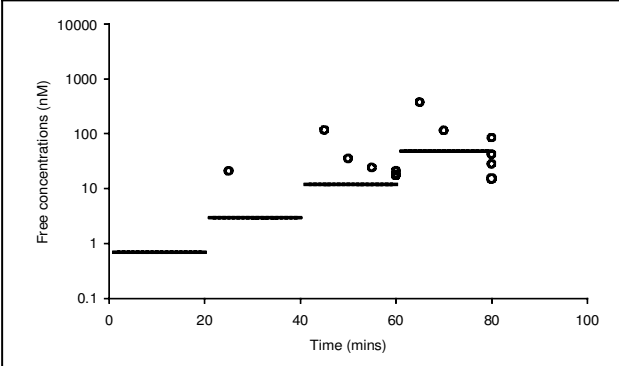


Figure 2. Predicted and actual free plasma concentration data for terfenadine, terodiline, cisapride and E-4031 following i.v. bolus and maintenance infusion administration.

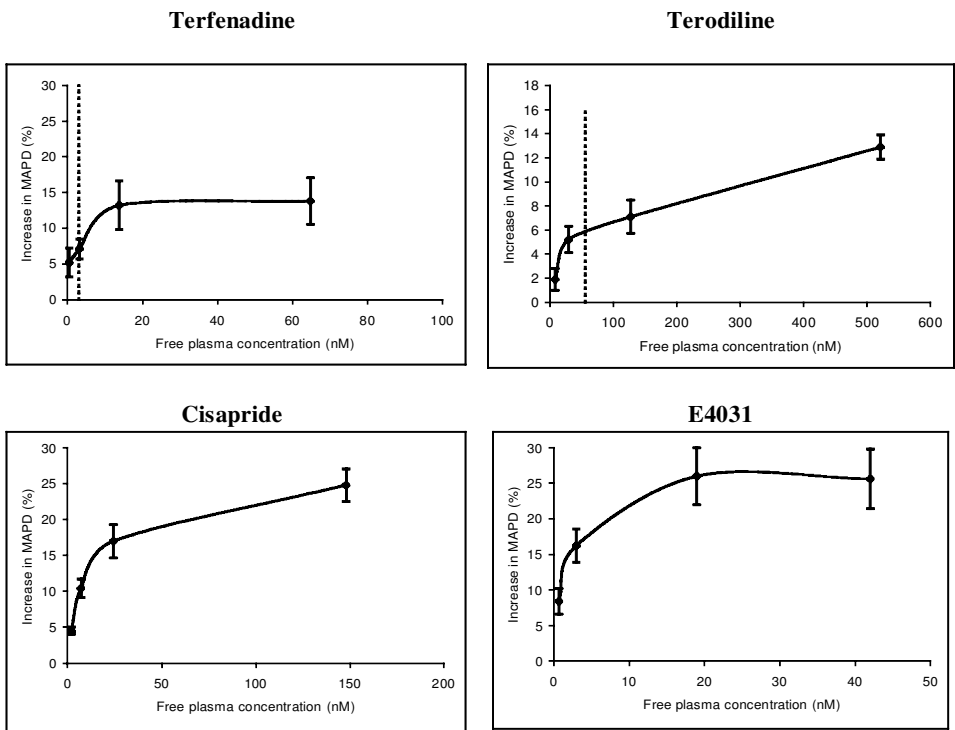


Figure 3. Relationship between free plasma concentrations of terfenadine, terodiline, cisapride and E-4031, and increase in monophasic action potential duration (MAPD, mean $\pm$ SEM). Lowest reported free plasma concentrations of terfenadine and terodiline causing TDP in man are shown as dashed lines. No clinical data were available for cisapride and E4031.

Table 4. Increase in monophasic action potential duration following intravenous bolus and maintenance infusion administration of terfenadine, terodiline, cisapride and E4031.

Compound	Mean free plasma concentration (nM)	Increase in MAPD* (% , mean $\pm$ SEM)
Terfenadine	<0.6	5.2 $\pm$ 2.0
	3.2	7.1 $\pm$ 1.4
	13.8	13.2 $\pm$ 3.4
	64.7	13.8 $\pm$ 3.3
Terodiline	8.1	1.9 $\pm$ 0.9
	29.6	5.2 $\pm$ 1.1
	127.2	7.1 $\pm$ 1.4
	521.1	12.9 $\pm$ 1.0
Cisapride	2.0	4.5 $\pm$ 0.5
	7.1	10.4 $\pm$ 1.3
	24.2	17.0 $\pm$ 2.3
	148	24.8 $\pm$ 2.3
E4031	0.7 ¹	8.4 $\pm$ 1.8
	3 ¹	16.2 $\pm$ 2.3
	19	26.0 $\pm$ 4.0
	42	25.6 $\pm$ 4.2

* Data from Leishman *et al.* (2000a).

¹ Target concentration.

n = four to six dogs per compound.

Table 5. Pharmacokinetic/pharmacodynamic values obtained for terfenadine, terodiline, cisapride and E4031; comparison with IC₂₀ obtained in the HERG *in vitro* assay.

	Terfenadine	Terodiline	Cisapride	E4031
Free $E_{\max}$ (% change in MAPD, mean $\pm$ SEM)	14.1 $\pm$ 1.9	14.0 $\pm$ 2.5	26.3 $\pm$ 1.0	27.3 $\pm$ 1.0
Free EC ₅₀ (nM, mean $\pm$ SEM)	1.9 $\pm$ 1.2	76.0 $\pm$ 44.4	11.5 $\pm$ 1.4	1.9 $\pm$ 0.7
Concentration required to increase MAPD by 10% (nM)	3	140	8	<1
Inhibition of HERG current IC ₂₀ (nM)	18	80	3	5
Clinical data				
Free concentrations associated with the efficacious dose in man (nM)	0.3	8–12	3–5	n.d.
Free concentrations associated with TDP in man (nM)	3–4	56–88	n.d.	n.d.

n.d., No data available in the literature.
Data are derived from four to six dogs/compound.

Discussion

Terfenadine

Terfenadine was a widely used non-sedating antihistamine. Clinically, it has been shown to produce cardiac arrhythmias and TDP in a small number of subjects. Following oral administration of 60mg (in keeping with the clinically effective dose) of [¹⁴C]-terfenadine to healthy males the plasma AUC of terfenadine is 11.9 $\pm$ 6.4 ng h ml⁻¹ whilst the AUC of total radioactivity is 351 $\pm$ 43 ng h ml⁻¹. These data indicate that little of the terfenadine administered actually reaches the systemic circulation unchanged following oral administration (Garteiz *et al.* 1982). It has been demonstrated that terfenadine is largely metabolized to terfenadine carboxylate. This conversion is carried out in man by CYP3A4 (Woosley *et al.* 1993). It is this metabolite that is responsible for the antihistamine activity seen when terfenadine is taken orally (Ohtani *et al.* 1997). Terfenadine carboxylate does not evoke ECG disturbances, unlike terfenadine itself where such ECG disturbances occur at relatively low plasma concentrations (Woosley *et al.* 1993). Therefore, under normal circumstances and at the appropriate doses (60–180 mg p.o.) average peak terfenadine plasma concentrations range between 3 and 9 nM and are observed 1–2 h post-dose (Salata *et al.* 1995). The plasma protein binding of terfenadine has been determined in human plasma at 97% (McTavish *et al.* 1990). Therefore, at therapeutic doses, free concentrations of terfenadine in plasma are expected to be low (~0.3 nM). This concentration is insufficient to cause cardiac arrhythmia or TDP. However, following overdose or if terfenadine is taken along with a cytochrome P450 inhibitor, for example

ketoconazole, systemic exposure is increased (MacConnell and Stanners 1991). It is under these circumstances that cardiac arrhythmia and TDP can occur. For example, following an effective overdose, a plasma concentration of unchanged terfenadine has been reported of 91 nM (free plasma concentration = 3 nM; Davies *et al.* 1989). In addition a plasma terfenadine concentration of 121 nM (free plasma concentration = 4 nM) has been reported in a patient receiving the co-medicant ketoconazole (Monahan *et al.* 1990). In both cases ECG disturbances were reported and involved prolongation of the QT interval.

Clinical data indicate that at therapeutic doses the free plasma concentration of terfenadine is ~0.3 nM, whilst TDP and QT prolongation has been observed at free concentrations of 3–4 nM. Therefore, the infusion regimen designed to evaluate the effects of terfenadine on MAPD in dog were targeted at achieving these free plasma concentrations. Free concentrations 4- and 16-fold higher than those required to bring about QT effects were also examined. The target-free plasma concentrations of terfenadine in these dog studies were therefore 0.7, 3, 12 and 48 nM.

Terodiline

Terodiline is an anticholinergic agent and calcium antagonist used in the treatment of motor urge incontinence. The drug was marketed as a racemate, *R*(+) terodiline possesses antimuscarinic activity whereas the *S*(-) enantiomer has the calcium antagonist properties. In 1991 this drug was withdrawn by its manufacturers after it was linked with serious cardiac arrhythmias. Investigations in patients have shown that a relationship exists between plasma exposure of terodiline and QT prolongation, with a 20-ms increase in QT interval being reported at plasma concentration of between 700 and 1100 nM. Efficacious concentrations of terodiline have been reported to be ~100–150 nM (Hartigan-Go *et al.* 1996). A protein-binding of 92% for terodiline has been reported in human serum (Hallen *et al.* 1995). Therefore, in man the free concentrations required to increase QT interval by 20 ms were ~56–88 nM. Efficacious free concentrations of terodiline in plasma are expected to be ~8–12 nM. Also TDP has been reported in a patient with free plasma concentrations of ~800 nM. The systemic clearance of terodiline is low ($1\text{--}2\text{ ml min}^{-1}\text{ kg}^{-1}$) in man and the compound has a moderate volume of distribution = 5.81 l kg^{-1} (Karlen *et al.* 1982, Hallen *et al.* 1993). It has been reported that a delayed elimination of terodiline occurred in a patient who was a poor metabolizer of debrisoquine (Hallen *et al.* 1993). This polymorphism may be responsible for some of the incidences of TDP reported in patients (due to the increased systemic exposure); however, the link between CYP2D6 polymorphism and TDP is not definitive. Again increased QT interval is often associated with overdose of terodiline. In the above reports racemic terodiline has been quantified; however, the *R*(+) enantiomer of terodiline has been associated with QT prolongation whilst the *S*(-) enantiomer has no effect on QT (Hartigan-Go *et al.* 1996). However, in man at steady-state it has been shown that *R*(+) and *S*(-) terodiline plasma concentrations are virtually equivalent, indicating that both compounds are cleared at a similar rate from the systemic circulation (Hallen *et al.* 1995). No dog data are available to confirm that the clearance rates from plasma of these enantiomers are equivalent in this species. In the absence of these data it has

been assumed that the ratio of enantiomers in dog plasma is unity when the dog and human data have been compared.

Comparison of the clinical data for terodiline and terfenadine indicates that higher free terodiline concentrations are required for both efficacy and to induce increases in QT interval and induce TDP than for terfenadine. Therefore, the free concentration targets in dog were modified with the experiment terminating at a 4-fold higher free concentration than those used in the terfenadine study. This higher free plasma concentration being in line with the terodiline free concentration reported to cause QT prolongation in man. Therefore, the target-free plasma concentrations of terodiline were 3, 12, 48 and 192 nM.

Cisapride

Cisapride is a benzamide-derived prokinetic agent that stimulates motility throughout the entire length of the gastrointestinal tract. This is believed to be due to increased acetylcholine release from the myenteric plexus (Veereman-Wauters *et al.* 1991, Michalets and Williams 2000). Cisapride is effective in the treatment of gastro-oesophageal reflux, dyspepsia, gastroparesis and constipation. As of December 1999 cisapride had been associated with 341 reports of heart rhythm abnormalities (Michalets and Williams 2000). There have been reports of 34 cases of TDP, 23 cases of long QT syndrome and four reported cases of death (Mohammad *et al.* 1997). These arrhythmias are generally associated with high dose (>10 mg every 6 h in adults) or co-medication with other drugs that prolong QT interval such as the tricyclic antidepressant doxepin. Also cisapride is extensively metabolized by CYP3A4 to form norcisapride and co-medication with CYP3A4 inhibitors such as ketoconazole increases the risk of QT prolongation and TDP (Michalets and Williams 2000). It has been suggested that the therapeutic plasma concentration for cisapride in man is 120–170 nM (Mohammad *et al.* 1997). Cisapride has a plasma protein-binding in man of 97.5% (Veereman-Wauters *et al.* 1991). Therefore, estimated efficacious free concentration in man is ~3–5 nM. Based on these efficacious free concentrations of cisapride in man the target-free concentration range in this study was 3–192 nM.

E4031

E4031 is a class III anti-arrhythmic agent that has been reported to prolong action potential duration, QT interval and the effective refractory period without effecting other electrophysiological parameters (Imamura *et al.* 1995). The compound has been shown to block the I_{kr} channel (Kasama *et al.* 1998). E4031 has anti-arrhythmic properties; however, it can also be potentially pro-arrhythmic due to excessive blockage of the I_{kr} channel. For this agent the correct balance between these responses must be achieved in the treatment of cardiac cases (Davies 1998).

Little clinical data were available to guide the choice of target-free plasma concentrations of E4031 for use in these dog studies. It was assumed that as this compound is a potent inhibitor of the I_{kr} channel, it would produce QT changes, prolong MAPD and cause TDP at low free plasma concentration. Therefore, the target-free concentrations in plasma were based on those used for terfenadine, i.e. 0.7, 3, 12 and 48 nM. The infusion regimen estimated to achieve these concentrations is detailed in table 2.

Despite the crude methodology employed to estimate the loading and maintenance infusion doses used in these studies, the concentrations achieved at the end of the maintenance infusion are broadly in keeping with those predicted for terfenadine, cisapride and E4031 (table 3). However, when the same methodology was used to extrapolate the regimen for terodiline, the free plasma concentrations achieved are ~ 2 – 3 -fold in excess of those estimated. These data indicate that the clearance of terodiline is lower in the beagle dog (Pfizer colony) used in this experiment than those reported previously in this same dog strain (Hallen *et al.* 1990). In the earlier pharmacokinetic studies the plasma clearance of terodiline was reduced from 40 to $25 \text{ ml min}^{-1} \text{ kg}^{-1}$ as dose increased from 0.5 to 15 mg kg^{-1} . At 15 mg kg^{-1} the range of clearances obtained in the dog varied markedly (range 12 – $46 \text{ ml min}^{-1} \text{ kg}^{-1}$, $n = 3$ dogs/dose). However, the total doses administered in the current studies are $<15 \text{ mg kg}^{-1}$ and there is no evidence of non-linearity in the plasma concentration data obtained in the studies presented here. Therefore, the reason for these clearance differences remains unclear but could indicate a difference between the two beagle populations.

For all the compounds investigated the concentrations in plasma increase in a linear manner with dose (table 3), indicating that clearance is constant across the dose range studied. As marked changes in plasma protein binding would be expected to effect the plasma clearance of these molecules, these data suggest that plasma protein binding is relatively constant across the dose range studied. These observations support the use of a single value for plasma protein binding, determined for each compound, in estimating the dog plasma-free concentrations.

For all the compounds evaluated the loading bolus dose appears to have been too large with concentrations about two to seven times higher than those targeted at the end of the bolus period. However, this concentration is only transient and levels rapidly approach target levels. These target-free concentrations are then maintained for the duration of the experiment. This concentration overshoot is probably due to insufficient time for the bolus dose to distribute into its total volume of distribution. A longer loading infusion period may offset this issue or alternatively the bolus dose could be further reduced. However, this would increase the duration of the experiment. More advanced modelling would be possible if sufficient data were available to characterize the initial distribution phase for any given drug, which could reduce the initial peak in plasma concentration.

Inclusion of routine plasma sampling and analysis of the concentrations for each compound studied has allowed us to overcome inaccuracy in the initial estimates. These data also allowed determination of the plasma concentration at the same time as pharmacodynamic data were generated. The terodiline data show the importance of plasma profiling during the course of the experiment. Despite the availability of literature pharmacokinetic data for this compound the free concentrations in plasma were clearly different from those estimated. The plasma concentration data obtained in this initial studies could be used to redesign future studies, should this have been found necessary. When plasma concentrations cannot be determined at all doses, due to limited assay sensitivity, data from the higher doses can be used to estimate free plasma exposure at lower doses assuming linearity. This was important for studies with E4031 that caused QT prolongation at low free plasma concentrations, which were below the limit of detection of the assay used in these experiments. The high dose data were used to confirm that the

compound was behaving as predicted and that the concentrations at the end of the E4031 infusions increased in a linear manner with dose.

In these dog experiments MAPD was evaluated as opposed to QT interval. QT interval is dependent on heart rate and several methods have been developed to 'correct' it for differences in heart rate. However, these corrected QT intervals (QTc) have drawbacks (Gralinski 2000). In order to overcome the rate-dependent nature of QT determinations the heart rates of the animals used in these experiments were controlled by ablating the atrioventricular node and pacing using a pace maker (heart rate paced at 150 bpm). However, under these experimental condition SQT interval cannot be determined using surface electrodes. Therefore, MAPD was recorded from the right ventricle. MAPD represents the sum of the ventricular depolarization and repolarization, as does the electrocardiographic QT interval, therefore MAPD is a suitable surrogate for QT in dog (Gralinski 2000).

Comparison of the free plasma concentration achieved in dog with percentage increases in MAPD (figure 3) demonstrates that increased free concentrations of terfenadine, terodiline, cisapride and E4031 in plasma give rise to increased MAPD. These data are in keeping with literature reports indicating that these compounds can increase QT interval and cause TDP man (see above).

A simple $E_{\max}$ model was chosen to analyse the pharmacokinetic and dynamic data obtained from these studies. A sigmoidal $E_{\max}$ model (e.g. the Hill model) was considered. However, as limited pharmacokinetic/pharmacodynamic data were obtained below the EC_{50} , it was felt inappropriate to use such a model as the plateau portion of this curve could not be adequately define.

Comparison of the concentration–effect relationships summarized in figure 3 illustrates that there are two distinct types of profile: first, those obtained for terfenadine and E4031, which present with a profile that approximates to that expected for a simple $E_{\max}$ concentration–effect relationship. The concentration–effect profiles obtained for both terodiline and cisapride appear more complex showing a rapid initial increase in MAPD, followed by a more gradual increase in MAPD with concentration. For both compounds it is not clear whether $E_{\max}$ has actually been achieved. These data may indicate that both terodiline and cisapride are acting on more than one receptor. If this is the case a more complex composite $E_{\max}$ model may be the most appropriate methodology for analysing these data. However, given the study design it is not clear whether this represents a genuine observation or simply experimental variability. Further work would be required to evaluate this observation fully. In the light of these uncertainties it was decided to apply the simple $E_{\max}$ model to all of the data generated in these studies.

The $E_{\max}$ and ED_{50} obtained using the free plasma concentration and percentage increase in MAPD data generated in dog for terfenadine, terodiline, cisapride and E4031 are summarized in table 5. These data indicate that for all compounds the maximum percentage increase in MAPD ($E_{\max}$) was between 14 and 27%. Both terfenadine and E4031 have similar free ED_{50} in plasma (~ 2 nM), cisapride's ED_{50} is ~ 5 -fold higher (11 nM), whilst that of terodiline is 35-fold higher (76 nM). When the unbound plasma concentrations of these compound required to produce a 10% increase in MAPD in dog are considered, a similar ranking of compounds is seen (E4031, <1 nM; terfenadine, 3 nM; cisapride, 8 nM; terodiline, 140 nM). However, evaluation of the pharmacodynamic data in this manner indicates that E4031 produces increases in MAPD at a significantly lower free concentration than terfenadine. The mismatches observed between the EC_{50} and the free concentra-

tion required to increase MAPD by 10%, for example that seen for E4031, are probably due to the sparse data available at low concentrations (i.e. at $<EC_{50}$). Without these data accurate determination of the gradient of the line is difficult. In future studies analysis of lower drug concentrations should be considered. Overall, the data produced using either method of interpretation indicate that an excellent correlation exists between the free concentrations in the dog required to effect MAPD and the clinical data summarized in this paper (table 5). For example, terfenadine increases MAPD with a free ED_{50} in dog plasma of 1.9 nM and causes a 10% change in MAPD in the dog at free concentrations of 3 nM. In man TDP and QT prolongation have been reported at free concentrations of 3–4 nM (Davies *et al.* 1989, Monahan *et al.* 1990).

Comparison of the free concentrations of terfenadine, terodiline and cisapride required to effect MAPD in the dog (also shown to prolong QT interval and cause TDP in man) and the efficacious concentrations clearly demonstrates that these compounds have very small therapeutic ratios. Based on these data the therapeutic ratios for these three compounds range from ~ 2 –10-fold. Given variability in pharmacokinetics in man such small therapeutic ratios especially with respect to TDP are clearly unacceptable.

The ability of these compounds to produce TDP has also been evaluated *in vitro* by determining their abilities to inhibit HERG potassium currents. In order to carry out this comparison the ability of the compounds to inhibit 20% of the HERG potassium current (IC_{20}) was selected. This value was selected as a 50% inhibition of HERG potassium currents was felt to be too extreme an *in vitro* endpoint, whilst 10% inhibition was felt not to be of sufficient magnitude to be reliably detected by the *in vitro* assay. The data from these studies are summarized in table 5 (Leishman *et al.* 2000b). Whilst there is not a complete correlation between the *in vitro* HERG data and the free concentrations required to cause MAPD effects in the dog, overall the values are of a similar magnitude. These data support the use of free drug concentrations (rather than total drug) to evaluate pharmacological effects *in vivo*.

The use of free plasma concentration data is also especially important if these safety data are to be combined with data from other species in order to estimate the therapeutic ratio with respect to QT prolongation or TDP. This is because plasma protein binding can vary widely between species. For example, terodiline has a plasma free fraction in the dog of 14%, whilst that in man is somewhat lower at 8% (Hallen *et al.* 1990, 1995). However, much larger variations in plasma protein binding have been observed between species. The plasma free fraction of zamilfenacin is 10–20-fold lower in human plasma than that seen in rat and dog (Beaumont *et al.* 1996). Not correcting for differences in plasma protein binding could, under certain circumstances (if plasma protein binding is lower in man than animals), lead to over estimates of therapeutic ratios, potentially increasing the risk to volunteers during first in man studies.

In conclusion, this simple study design using a bolus/infusion regimen, plasma sampling and incorporating the assumption that free drug in the systemic circulation is responsible for pharmacological response has allowed the accurate evaluation of the effects of terfenadine, terodiline, cisapride and E4031 on MAPD. The infusion regimen developed for these studies achieved the target-free plasma concentrations in the dog and data on the relationship between free concentration and MAPD were obtained for these compounds. These data are in keeping with

the *in vivo* human data and demonstrate both the narrow therapeutic ratios of the compounds evaluated and the importance of considering free drug concentrations when relating pharmacokinetics and pharmacodynamics.

References

- BEAUMONT, K. C., CAUSEY, A. G., COATES, P. E. and SMITH, D. A., 1996, Pharmacokinetics and metabolism of zafamfenacin in mouse, rat, dog and man. *Xenobiotica*, **26**, 459–471.
- DAVIES, A. J., HARINDRA, V., MCEWAN, A. and GHOSE, R. R., 1989, Cardiotoxic effect with convulsions in terfenadine over dose. *British Medical Journal*, **298**, 325.
- DAVIS, S. A., 1998, The preclinical assessment of QT interval prolongation: a comparison of *in vitro* and *in vivo* methods. *Human and Experimental Toxicology*, **17**, 677–680.
- GARTZEL, D. A., HOOK, R. J., WALKER, B. J. and OKERHOLM, R. A., 1982, Pharmacokinetics and biotransformation studies of terfenadine in man. *Arzneim.-Forsch./Drug Research*, **32**, 1185–1190.
- GRALINSKI, M. R., 2000, The assessment of potential for QT interval prolongation with new pharmaceuticals. Impact on drug development. *Journal of Pharmacology and Toxicological Methods*, **43**, 91–99.
- GRAS, J. and LLENAS, J., 1999, Effects of H₁ antihistamines on animal models of QTc prolongation. *Drug Safety*, **21**, 39–44.
- HALLÉN, B., GABREILSSON, J., NYAMBATI, S., JOHANSSON, A., LARSSON, E. and GUILBAUD, O., 1995, Concomitant single-dose and multiple-dose pharmacokinetics of terodiline in man, with a note on its enantiomers and major metabolites. *Pharmacology and Toxicology*, **76**, 171–177.
- HALLÉN, B., GABREILSSON, J., PALMER, L. and EKSTROM, B., 1993, Pharmacokinetics of R(+) terodiline given intravenously and orally to healthy volunteers. *Pharmacology and Toxicology*, **73**, 153–158.
- HALLÉN, B., GRALLS, M., BROTELL, H. and STROMBERG, S., 1990, Pharmacokinetics of terodiline and a major metabolite in dogs with a correlation to a pharmacodynamic effect. *Pharmacology and Toxicology*, **66**, 373–381.
- HARTIGAN-GO, K., BATEMAN, D. N., DALY, A. K. and THOMAS, S. H. L., 1996, Stereoselective cardiotoxic effects of terodiline. *Clinical Pharmacology and Therapeutics*, **60**, 89–98.
- IMAMURA, H., FURUKAWA, Y., NAKANO, H., KASAMA, M., HOYANO, Y. and CHIBA, S., 1995, Selective inhibition by a class III anti-arrhythmic agent, E4031, of the negative chronotropic response to parasympathetic stimulation in anesthetized dogs. *Journal of Pharmacology and Experimental Therapeutics*, **276**, 467–472.
- KARLEN, B., ANDERSSON, K. E., EKMAN, G. and ULMSTEN, U., 1982, Pharmacokinetics of terodiline in human volunteers. *European Journal of Clinical Pharmacology*, **23**, 267–270.
- KASAMA, M., FURUKAWA, Y., OGUCHI, T., HOYANO, Y. and CHIBA, S., 1998, Effects of low temperature on the chronotropic and inotropic responses to zatebradine, E-4031 and verapamil in isolated perfused dog atria. *Japanese Journal of Pharmacology*, **78**, 493–499.
- KATOH, H., OGAWA, S., FURUNO, I., SATO, Y., YOH, S., SAEKI, K. and NAKAMURA, Y., 1990, Electrophysiologic effects of E-4031, a class III antiarrhythmic agent, on re-entrant ventricular arrhythmias in a canine 7-day-old myocardial infarction model. *Journal of Pharmacology and Experimental Therapeutics*, **253**, 1077–1082.
- LEISHMAN, D. J., DOUTHWAITE, M., KIDD, T., MERNER, P., WALKER, D. K., WEST, H. and WALLIS, R. M., 2000a, Effects of E4031, cisapride, terfenadine and terodiline on monophasic action potential duration (MAPD) and incidence of Torsade des pointes (TdP) in the dog. *British Journal of Pharmacology*, **133**, 131.
- LEISHMAN, D. J., HELLIWELL, R., WAKERELL, J. and WALLIS, R. M., 2000b, Effects of E4031, cisapride, terfenadine and terodiline on cardiac repolarisation in canine Purkinje fibre and HERG channels expressed in HEK293 cells. *British Journal of Pharmacology*, **133**, 130.
- MACCONNELL, T. J. and STANNERS, A. J., 1991, Torsades de pointes complicating treatment with terfenadine. *British Medical Journal*, **302**, 1469.
- MCTAVISH, D., GOA, K. L. and FERRILL, M., 1990, Terfenadine: an updated review of its pharmacological properties and therapeutic efficacy. *Drugs*, **39**, 552–574.
- MICHALETS, E. L. and WILLIAMS, C. H., 2000, Drug interactions of cisapride. *Clinical Pharmacokinetics*, **39**, 49–75.
- MICHELIS, M., MONBALIU, J., HENDRIKS, R., GEERTS, R., WOESTENBORGH, R. and HEYKANTS, J., 1987, Pharmacokinetics and tissue distribution of the new gastrokinetic agent cisapride in rat, rabbit and dog. *Arzneim.-Forsch./Drug Research*, **37**, 1159–1167.
- MOHAMMAD, S., ZHOU, Z., GONG, Q. and JANUARY, C. T., 1997, Blockade of the HERG human cardiac K⁺ channel by the gastrointestinal prokinetic agent cisapride. *American Journal of Pharmacology*, **273**, 2538–2538.

- MONAHAN, B. P., FERGUSON, C. L., KILLEAVY, E. S., LLOYD, B. K., TROY, J. and CANTILENA, L. R., 1990, Torsades de pointes occurring in association with terfenadine use. *Journal of the American Medical Association*, **264**, 2788–2790.
- OBACH, R. S., BAXTER, J. G., LISTON, T. E., SILBER, B. M., JONES, B. C., MACINTYRE, F., RANCE, D. J. and WASTALL, P., 1997, The prediction of human pharmacokinetic parameters from preclinical and *in vitro* metabolism data. *Journal of Pharmacology and Experimental Therapeutics*, **283**, 46–58.
- OHTANI, H., KOTAKI, H., SAWADA, Y. and IGA, T., 1997, A comparative pharmacokinetic-pharmacodynamic study of the electrocardiographic effects of epinastine and terfenadine in rats. *Journal of Pharmacokinetics and Pharmacology*, **49**, 458–462.
- SALATA, J. I., JURKIEWICZ, N. K., WALLACE, A. A., STUPIENSKI, R. F., GUINOSSO, P. J. and LYNCH, J. J., 1995, Cardiac electrophysiological actions of the histamine H1-receptor antagonists astemizole and terfenadine compared with chlorpheniramine and pyrilamine. *Circulation Research*, **76**, 110–119.
- SJOQVIST, F., 1985, Blood binding and drug monitoring. In Tillement, J. P. and Lindenlaub, E. (eds), *Protein Binding and Drug Transport* (Stuttgart: F. K. Schattauer), pp. 429–441.
- SMITH, D. A., 1997, Pharmacokinetics and pharmacodynamics in toxicology. *Xenobiotica*, **27**, 513–525.
- VAUGHAN WILLIAMS, E. M., 1970, Classification of anti-arrhythmic drugs. In E. Sandoe and E. Flenstead-Jenson (eds), *Symposium on Cardiac Arrhythmias* (Sodertälte, Sweden: AB Astra), pp. 449–472.
- VEEREMAN-WAUTERS, G., MONBALIU, J., MEULDERMANS, W., WOESTENBORGH, R., VERLINDEN, M., HEYKANTES, J. and RUDOLPH, C. D., 1991, Study of the placental transfer of cisapride in sheep: Plasma levels in the pregnant ewe, the fetus and the lamb. *Drug Metabolism and Disposition*, **19**, 168–172.
- WOOSLEY, R. L., CHEN, Y., FREIMAN, J. P. and GILLIS, R. A., 1993, Mechanism of the cardiotoxic actions of terfenadine. *Journal of the American Medical Association*, **269**, 1532–1536.
- WRIGHT, A., RAVAL, P., EDEN, R. J. and OWEN, D. A. A., 1987, Histamine H1-receptor antagonist activity assessed in conscious dogs. *Journal of Pharmacological Methods*, **18**, 123–129.

Pharmacokinetics and metabolism of a selective PDE5 inhibitor (UK-343,664) in rat and dog

D. K. WALKER*†, K. C. BEAUMONT†, P. COMBY‡, K. M. EVANS†, J. I. GEDGE†, R. C. HALLIDAY†, S. J. ROFFEY† and P. A. WRIGHT†

† Department of Drug Metabolism, Pfizer Global Research and Development, Sandwich CT13 9NJ, UK

‡ Centre de Recherche Pfizer, BP 159, 37401 Amboise Cedex, France

Received 23 January 2001

1. UK-343,664 is a novel potent and selective PDE5 inhibitor. Plasma clearances in the male and female rat were high (120 and $54 \text{ ml min}^{-1} \text{ kg}^{-1}$), giving rise to short elimination half-lives (0.2 and 0.3 h respectively). Lower clearance in dog ($14 \text{ ml min}^{-1} \text{ kg}^{-1}$) was the primary factor resulting in a longer elimination half-life (3.7 h). The higher clearance in rat than dog was in agreement with *in vitro* metabolism rates in hepatic microsomes.

2. The volume of distribution was lower in rat ($1.3\text{--}2.1 \text{ l kg}^{-1}$) compared with dog (4.6 l kg^{-1}) probably due to increased plasma protein binding in rat (96 versus 81% in dog).

3. Oral bioavailabilities were 2 , 12 and 70% in the male and female rat and dog respectively. $T_{\text{max}} \leq 0.5 \text{ h}$ in all animals.

4. In multiple oral dose studies, increased systemic exposure was seen with increasing dose up to doses of 200 mg kg^{-1} in rat and 150 mg kg^{-1} in dog. A marked super-proportional increase in the male rat indicated a capacity-limited clearance at high doses.

5. At the maximal dose of 200 mg kg^{-1} in the female rat, no clinical signs were observed after 14 days of treatment. Only minimal signs were recorded in the male rat and dog at the highest dose levels investigated.

6. After single oral or intravenous doses of $[^{14}\text{C}]$ -UK-343,664, the majority of radioactivity was excreted in the faeces of both species.

7. UK-343,664 was extensively metabolized in both rat and dog. The major primary pathways in dog involved piperazine *N*-deethylation and loss of a two carbon fragment from the piperazine ring (*N,N'*-de-ethylation). More extensive metabolism in the rat included additional notable metabolites arising from hydroxylation and lactamization of the piperazine ring, which were only minor metabolites in the dog.

Introduction

UK-343,664 (3-ethyl-5-{5-[(4-ethylpiperazino)sulphonyl]-2-propoxyphenyl}-2-(2-pyridylmethyl)-6,7-dihydro-2H-pyrazolo[4,3-d]pyrimidin-7-one; figure 1) is a novel potent and selective inhibitor of the human cGMP-specific phosphodiesterase type 5 enzyme (PDE5) found in human corpus cavernosum and other tissues (Wallis *et al.* 1999). PDE5 inhibitors have been effectively used in the treatment of male erectile dysfunction (Boolell *et al.* 1996). The compound is relatively lipophilic ($\log D_{7.4} = 3.1$) with a weakly basic centre in the piperazine tertiary amine ($pK_a = 6.9$) and is highly soluble at acidic pH ($> 20 \text{ mg ml}^{-1}$ at pH 4.5).

* Author for correspondence; e-mail: don_walker@sandwich.pfizer.com

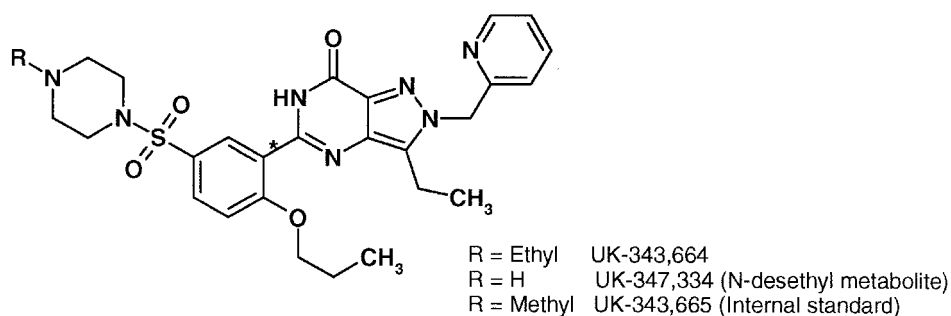


Figure 1. Structure of UK-343,664 (*position of [^{14}C] label) the internal standard for analysis (UK-343,665) and the *N*-desethyl metabolite (UK-347,334).

Here, pharmacokinetic studies have been conducted with UK-343,664 in rat and dog, the species used in toxicological evaluation, as part of the preclinical evaluation of the compound. In addition, radiolabelled compound has been administered to the same species in order to investigate its disposition and metabolic fate.

Materials and methods

Chemicals

UK-343,664 citrate salt, internal standard, UK-343,665 (methyl piperazine analogue of UK-343,664) and UK-347,334 (*N*-desethyl metabolite of UK-343,664) were synthesized at Pfizer Global Research and Development (Sandwich, UK). The chemical structures of these compounds are shown in figure 1. [^{14}C]-UK-343,664 citrate salt was prepared by Amersham International (Cardiff, UK) with a radiochemical purity > 99% (by thin layer chromatography and confirmed by mass spectrometry and HPLC) and a specific activity of $24.5 \mu\text{Ci mg}^{-1}$. The chemical purity of all compounds was $\geq 95\%$.

Administration of single intravenous and oral doses to animals

Intravenous and oral doses of the citrate salt of UK-343,664 for administration to rat and dog were freshly prepared in a vehicle of 5% DMSO and 95% saline at active drug concentrations of 0.5–5.0 mg ml^{-1} . Dose levels for pharmacokinetic studies were selected based on pharmacological activity, whilst those with radioactive compound were in line with doses used in toxicology. Two male beagle dogs (Pfizer colony 12–15 kg) received UK-343,664 by intravenous infusion (for 15 min) into a saphenous vein at 0.5 mg kg^{-1} . The same animals received oral solution doses via gavage tube at 1.0 mg kg^{-1} . Oral doses were administered 7 days after the intravenous doses. Blood samples were collected from temporary indwelling saphenous vein catheters up to 5 h post-dose and thereafter by venepuncture of the cephalic veins. Urine samples (0–7 h) were collected from both dogs after intravenous and oral administration by catheterization of the bladder. One male ($\sim 14 \text{ kg}$) and one female ($\sim 12 \text{ kg}$) dog received [^{14}C]-UK-343,664 as single intravenous (1 mg kg^{-1}) and oral (10 mg kg^{-1}) doses given 3 weeks apart. Doses contained $\sim 100 \mu\text{Ci}$ radioactivity. Animals were housed individually in stainless steel cages (Modular Development Company Ltd) designed for separate collection of urine and faeces. The experimental facilities were maintained at $18.5 \pm 2^\circ\text{C}$ with $55 \pm 10\%$ humidity and a 12-h light/dark cycle. Dogs received $\sim 400 \text{ g}$ Teklad dog maintenance diet (Harlan) per day and water *ad libitum*.

Five male and five female Sprague–Dawley rats ($\sim 250 \text{ g}$; Charles River, Manston, UK) were surgically prepared with a jugular vein catheter at least 2 days prior to dose administration. Two male and two female animals received single intravenous doses into the caudal vein (1 mg kg^{-1}). Three male and three female animals received single oral doses by gavage tube (5 mg kg^{-1}). Blood samples ($175 \mu\text{l}$) were collected from the jugular vein catheter and the cannula flushed with heparinized saline after each sample. All blood samples were collected into lithium heparin tubes, mixed and centrifuged. Following centrifugation, plasma samples were transferred to glass tubes and stored frozen at approximately -20°C prior to analysis. Doses of [^{14}C]-UK-343,664 were administered as single intravenous (1 mg kg^{-1}) and oral (10 mg kg^{-1}) doses to two male and two female rats for each route. Intravenous and oral doses contained ~ 10 and $20 \mu\text{Ci}$ radioactivity respectively. The animals were individually housed in plastic

metabolism cages designed for the separate collection of urine and faeces. All rats received food (rat and mouse maintenance diet no. 1, Special Diet Services) and water *ad libitum* throughout the studies. The experimental facility was maintained at 19–21°C and 50 ± 20% humidity with a 12-h dark/light cycle.

Multiple oral dose pharmacokinetics in rat and dog

Within the toxicology programme for UK-343,664, rat and dog received multiple daily oral doses of the compound for 14 days. Toxicokinetic data were obtained from groups of five male and five female rats receiving oral doses of 10, 45 and 200 mg kg⁻¹ UK-343,664 and from groups of three male and three female dogs receiving oral doses of 15, 50 and 150 mg kg⁻¹ UK-343,664. The doses for dog were prepared as aqueous solutions containing methylcellulose (0.5% w/v) and Tween 80 (0.1% w/v) and administered at 1 ml kg⁻¹ by oral gavage. Blood samples (2.5 ml) were collected by venepuncture of the jugular vein into lithium heparin tubes at 1, 3, 5, 7 and 24 h after dosing on days 1 and 12 of the study. The doses for rat were prepared as aqueous solutions in pure water acidified with 1 M HCl, adjusted to pH 3–3.5 with 1 M NaOH and administered at 10 ml kg⁻¹ by oral gavage. Blood samples (0.5 ml) were collected by venepuncture of the orbital sinus into lithium heparin tubes at 1, 4, 7 and 24 h after dosing on days 1 and 10 of the study. The blood samples from rat and dog were stored on ice until centrifuged, after which plasma samples were transferred to plastic tubes and stored frozen at approximately -20°C prior to analysis.

Observations of animals during toxicological studies

Standard toxicological observations were performed within the toxicity studies in both species. Rats and dogs were regularly observed for clinical signs throughout the studies. Food intake and body weights were recorded during the course of the experiment. In dog, blood pressure and ECG recordings were performed before the start of the study and during the first and second weeks prior to and ~1.5 h after dose administration.

Analysis of UK-343,664 in plasma samples

The concentrations of UK-343,664 were determined in rat and dog plasma using a solid-phase extraction method followed by LC-MS-MS quantification. The method involved the addition of internal standard (50 ng UK-343,665) to 0.5 ml plasma samples which had previously been appropriately diluted with control plasma (obtained from the same species). The plasma samples were diluted with 0.5 ml water and applied to activated solid-phase extraction cartridges (C18 Bond Elut, Analytichem International) and the cartridge washed with 10% acetonitrile in water. Analytes were eluted with 1 ml methanol, the solution evaporated to dryness under a nitrogen stream and the residue redissolved in 200 µl 2 mM ammonium acetate buffer, pH 4.0, in methanol/water (30:70 v/v). Samples (180 µl) were injected onto the hplc column (Hypersil C18, 50 × 4.6 mm) with a mobile phase consisting of 2 mM ammonium acetate, pH 4.0, in methanol/water (90:10 v/v) at 1 ml min⁻¹. Detection was by multiple reaction monitoring for the transitions *m/z* 566 to 346 (UK-343,664) and *m/z* 552 to 346 (UK-343,665) using a Sciex API III+ triple quadrupole mass spectrometer (Perkin Elmer, Sciex, Ontario, Canada) in a positive atmospheric pressure chemical ionization mode. On this system the retention times of UK-343,664 and internal standard were ~2 min. The limit of detection was 1 ng ml⁻¹. In order to satisfy acceptance criteria, analysis of quality control samples provided results within 20% of the correct value at the lower end of the calibration range and within 15% of the correct value at the middle and top of the calibration range (100 ng ml⁻¹). Dog urine was analysed by 1:100 dilution with 2 mM ammonium acetate buffer, pH 4.0, in methanol/water (30:70 v/v), followed by direct injection into the LC-MS-MS system described above.

For the analysis of toxicokinetic samples, a solid-phase extraction method followed by hplc with UV detection of parent compound and metabolite was utilized. This method was specifically developed for the toxicokinetic analysis. Appropriately diluted rat or dog plasma samples (0.1–0.3 ml) were mixed with 0.5 ml water and internal standard (UK-343,665, 200 ng). The dog samples were applied to activated solid-phase extraction cartridges (C18 Bond Elut) and the cartridge washed with 1 ml water followed by 2 × 1 ml acetonitrile/water (30/70 v/v). The rat samples were applied to activated solid-phase extraction cartridges (CN Bond Elut) and the resin washed with 1 ml methanol/water (10/90 v/v). Analytes were eluted with 1 ml methanol and evaporated to dryness. The residues were dissolved in 0.3 ml of the hplc mobile phase. Samples (100 µl) were injected onto the hplc column (Hichrom RPB, 25 × 0.46 cm) with a mobile phase comprising of acetonitrile/0.05 M potassium phosphate, pH 7.4 (42/58 v/v) at 1 ml min⁻¹. Detection was by UV absorption at 225 nm. The limit of determination of this validated assay for both parent compound and *N*-desethyl metabolite, UK-347,334, was 13 ng ml⁻¹ in dog plasma and 20 ng ml⁻¹ in rat plasma.

Pharmacokinetic analysis of data

Terminal elimination rate constant (*k_{el}*) was determined by linear regression of the log plasma concentrations. The area under the plasma concentration versus time curve (AUC) was calculated to the last time point by the linear trapezoidal method. AUC_∞ was calculated by extrapolation to infinity with

k_{el} . Clearance (Cl) was calculated by the relationship $\text{dose}/\text{AUC}_{\infty}$ and the volume of distribution was calculated by the relationship Cl/k_{el} . Oral bioavailability (F) was calculated from the ratio of dose-normalized AUC after oral and intravenous doses.

Plasma protein binding

Samples of control rat and dog plasma containing UK-343,664 at a concentration of $1 \mu\text{g ml}^{-1}$ were dialysed (Spectrapor 1 dialysis membrane 6000–8000 molecular weight cut-off; Spectrum Medical Industries) against isotonic Krebs–Ringer bicarbonate buffer, pH 7.4 (1 ml) for 4 h at 37°C in a rotating dialyser (Dianorm, MSE). For rat plasma, five replicate determinations were performed on a pooled sample of male rat plasma. For dog plasma, triplicate determinations were performed on five individual dog plasma samples. Following dialysis, plasma and buffer samples were analysed by solid-phase extraction and LC-MS-MS for quantitation of UK-343,664.

Liver microsomal rate determinations

Rat and dog liver microsomal incubations (12 ml final volume) were carried out at $1 \mu\text{M}$ UK-343,664 and $0.5 \mu\text{M}$ cytochrome P450. All incubations were performed in triplicate. Microsomes were prepared according to Remmer *et al.* (1966) and stored at -80°C . The concentrations of protein and cytochrome P450 were determined by standard methods (Lowry *et al.* 1951, Omura and Sato 1964). The incubation volume was made up of 50 mM Tris-HCl (pH 7.4), 5 mM MgCl_2 and $5 \mu\text{M}$ MnCl_2 . The reducing equivalents required by cytochrome P450 were provided by NADPH (1 mM), which was regenerated *in situ* using an isocitric acid (5 mM) and isocitrate dehydrogenase (1 unit ml^{-1}) system. Aliquots (1 ml) were taken over 60 min and samples were extracted with 4 ml methyl *tert*-butyl ether containing internal standard ($10 \mu\text{l}$, 0.1 mg ml^{-1}). Samples were chromatographed on a 25 cm Hichrom RPB column with a mobile phase of 5 mM octanesulphonic acid +2 mM KH_2PO_4 , pH 3/acetonitrile (60:40 v/v) at a flow rate of 1 ml min^{-1} . Detection was by UV absorbance at 230 nm.

Analysis of radioactivity

Radioactivity in samples of plasma ($\sim 0.2 \text{ ml}$) and urine ($\sim 1.0 \text{ ml}$) was measured by liquid scintillation counting in 4 ml Starscint (Packard Instruments Ltd). Daily faeces samples were homogenized with an equal volume of water prior to analysis. Radioactivity in triplicate weighed homogenized faeces samples ($\sim 0.5 \text{ g}$) and whole blood ($\sim 0.25 \text{ g}$) was determined by liquid scintillation counting of evolved $^{14}\text{CO}_2$ after combustion of the samples in an Oximate Sample Oxidizer (Packard Instruments Ltd).

Analysis of faeces for metabolites

Composite samples of faecal homogenate following intravenous (0–48 h) and oral (0–72 h) doses to individual dog and pooled samples from the male and female rat (0–24 h) after intravenous and oral doses were analysed. Faecal homogenates were sequentially extracted with 25 ml aliquots of methanol, followed by methanol/0.1 M Tris buffer, pH 6 (24/1 v/v), followed by methanol/0.1 M Tris buffer, pH 9 (23/2 v/v). The extracts were combined, evaporated to dryness under nitrogen and resuspended in initial mobile phase (2 ml) for injection onto the hplc. Each sample was injected onto a gradient hplc system comprising a $25 \times 0.775 \text{ cm}$ RPB column (Hichrom, Reading, UK) eluted by a gradient of 0–100% methanol in 0.1 M aqueous ammonium acetate, pH 5, over 30 min at a flow rate of 1 ml min^{-1} . An in-line radioactivity monitor with data analysis performed using LAURA (Ramona, Lablogic Ltd) was employed to monitor the eluate. Radioactive components were manually collected and analysed by mass spectrometry.

Metabolite identification

Metabolite characterization was performed initially by the comparison of retention times on the gradient hplc system with authentic standards. To confirm metabolite identities, or to identify components for which no reference standard was available, mass spectral analysis was performed. The radioactive components were analysed by direct infusion ($5 \mu\text{l min}^{-1}$, nebulizer gas pressure 40 psi) into an API III plus mass spectrometer (Perkin Elmer Sciex, Toronto, Canada) fitted with an IonSpray interface utilized in positive-ion mode.

Results

Single dose pharmacokinetics in rat and dog

Plasma concentration versus time profiles obtained after single intravenous and oral doses in the male and female rat and dog are shown in figure 2. Pharmacokinetic parameters after intravenous administration are shown in table 1. Follow-

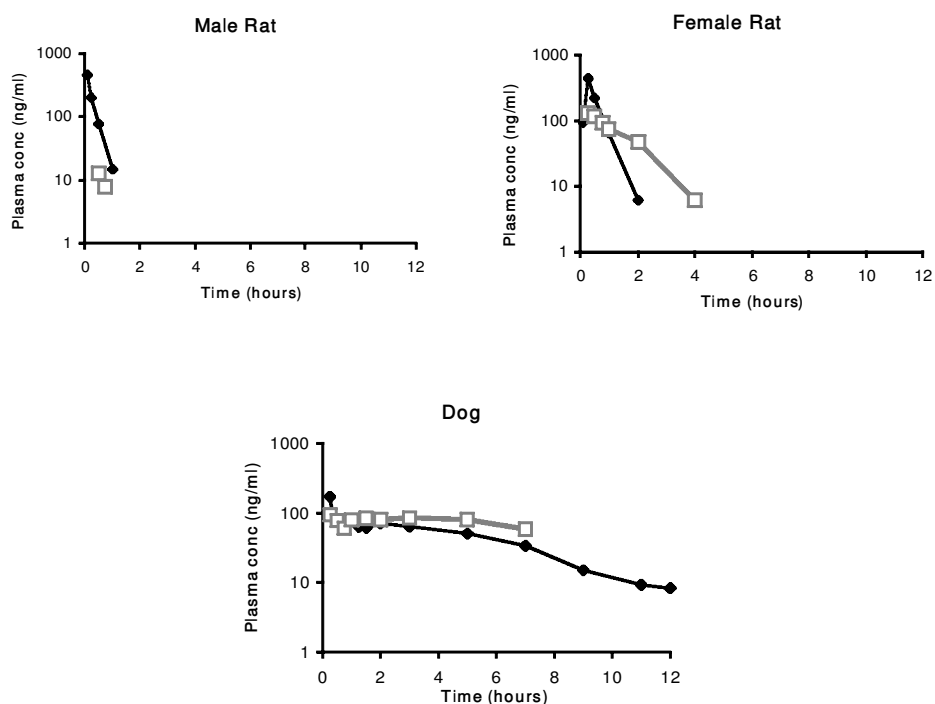


Figure 2. Mean plasma concentrations of UK-343,664 in the male and female rat and dog after single intravenous ($\blacklozenge$) and oral ($\square$) doses of UK-343,664. Administered intravenous doses were 1 mg kg^{-1} in the male and female rat and 0.5 mg kg^{-1} in dog. Administered oral doses were 5 mg kg^{-1} in the male and female rat and 1 mg kg^{-1} in dog. Intravenous data are the average from two male and two female rats and two male dogs. Oral data are the means from three male and three female rats and two male dogs.

ing single intravenous doses to rat, plasma clearance was high in both male and female animals ($>50\%$ liver blood flow), but highest in males with 120 and $54 \text{ ml min}^{-1} \text{ kg}^{-1}$ ($n=2$) for male and female animals respectively. The volume of distribution was similar in both sexes at 2.1 and 1.3 l kg^{-1} for the male and female respectively. The high plasma clearance resulted in short elimination half-lives of 0.2 and 0.3 h , with plasma concentrations falling below the detection limit of the assay after 1 h in males and after 2 h in females. The clearance in dog was markedly lower than that observed in rat with a plasma clearance of $14 \text{ ml min}^{-1} \text{ kg}^{-1}$ ($n=2$) approximating to $\sim 30\%$ liver blood flow. The volume of distribution was also higher in dog with 4.6 l kg^{-1} . The reduced clearance and increased volume of distribution relative to rat resulted in a markedly longer elimination half-life of 3.7 h . Only low levels of drug were detected in urine collected up to 7 h post-intravenous doses to dog, amounting to $2\text{--}3\%$ of the administered dose.

Oral pharmacokinetic parameters in rat and dog are also shown in table 1. Following single oral (5 mg kg^{-1}) doses to the male and female rat, there were marked sex differences in systemic exposure with a mean $C_{\text{max}} = 17$ and 141 ng ml^{-1} ($n=3$) in male and female animals respectively. In both sexes, time to peak concentration was short with a $T_{\text{max}} = 0.5 \text{ h}$ in males and 0.4 h in females. Oral bioavailability, calculated by comparison of dose-normalized oral and intravenous

Table 1. Pharmacokinetic parameters for UK-343,664 after single intravenous and oral administration to the male and female rat and dog.

Parameter	Male rat (<i>n</i> = 2/3*)	Female rat (<i>n</i> = 2/3*)	Dog (<i>n</i> = 2)
Intravenous dose (mg kg ⁻¹)	1	1	0.5
Elimination half-life (h)	0.2	0.3	3.7
Plasma clearance (ml min ⁻¹ kg ⁻¹)	120	54	14
Volume of distribution (l kg ⁻¹)	2.1	1.3	4.6
Oral dose (mg kg ⁻¹)	5	5	1
<i>C</i> _{max} (ng ml ⁻¹)	17 ± 9.7	141 ± 76	95
<i>T</i> _{max} (h)	0.5 ± 0.3	0.4 ± 0.1	0.25
Elimination half-life (h)	nd	1.0 ± 0.1	3.5
Oral clearance (ml min ⁻¹ kg ⁻¹)	> 1000	422	21
Oral bioavailability (%)	2	12	70

**n* = 2 for intravenous dose; *n* = 3 for oral dose.
nd, Not determined.

Values are mean ± SD where available. Samples or data have been combined for other parameters, thus preventing statistical analysis.

AUC_∞, was 2% in males and 12% in females. Absorption was also rapid in dog with an average *C*_{max} = 95 ng ml⁻¹ at a *T*_{max} = 0.25 h after a 1 mg kg⁻¹ single oral dose (*n* = 2). Oral bioavailability in dog was calculated at 70%.

Multiple dose oral pharmacokinetics in rat and dog

Mean pharmacokinetic parameters obtained within the toxicity studies with UK-343,664 in rat and dog are shown in table 2. Data (not shown) from the male and female dog were similar and therefore were combined. A sex difference was again apparent in rat with higher systemic exposure of parent compound observed in female compared with male animals. In both rat and dog, AUC and *C*_{max} were similar on days 1 and 10 or 1 and 12 respectively. In the male rat, over the dose range 10–200 mg kg⁻¹, there was a superproportional increase in both AUC and *C*_{max} suggesting a capacity-limited clearance at higher doses. A superproportional increase in AUC was also observed in the female rat. In dog, increases in *C*_{max} and AUC were approximately linear up to 50 mg kg⁻¹, however there was little further

Table 2. Mean pharmacokinetic parameters for UK-343,664 and *N*-desethyl metabolite, UK-347,334 after multiple oral doses to the male and female rat and dog.

Species	Dose (mg kg ⁻¹)	Day 1			Days 10/12*			
		<i>C</i> _{max} (µg ml ⁻¹)	AUC _{1-7h} (µg.h ml ⁻¹)		<i>C</i> _{max} (µg ml ⁻¹)	AUC _{1-7h} (µg.h ml ⁻¹)		
Male rat (<i>n</i> = 5)	10	0.01	NC	NC	0.01	NC	NC	NC
	45	0.12	0.5	0.2	0.1	0.05	0.3	0.1
	200	5.11	1.10	22.1	4.9	8.07	1.12	35.9
Female rat (<i>n</i> = 5)	10	0.72	0.04	1.1	NC	0.76	0.05	1.2
	45	5.16	0.41	14.2	1.2	4.57	0.41	12.0
	200	14.39	1.19	69.2	5.8	13.85	1.00	64.8
Dog (<i>n</i> = 6)	15	3.28	0.39	9.5	1.7	2.54	0.29	6.8
	50	6.09	0.84	21.4	4.0	5.97	0.94	20.2
	150	6.30	0.86	22.2	4.0	8.76	1.23	29.1

All values are given as UK-343,664/UK-347,334.
*Day 10 for rat; day 12 for dog.
NC, not calculated (insufficient data).

increase in systemic exposure at the dose of 150 mg kg^{-1} . Systemic exposure to the *N*-desethyl metabolite, UK-347,334 was low in both rat and dog, with relative exposure to the parent compound generally $\leq 25\%$ based on AUC. Plasma concentrations of this metabolite were also similar on both days of the studies on which the pharmacokinetics were monitored. Comparison of male and female rat data for the metabolite shows that at a dose of 200 mg kg^{-1} both sexes were exposed to similar levels of metabolite whilst at a dose of 45 mg kg^{-1} systemic exposure in females was at least 8-fold higher than in males.

Clinical observations during toxicology studies

There was no effect of 14-day dosing of UK-343,664 on any observable clinical sign in rat. No effects were recorded on food consumption by rat at any dose level. In the high dose male rat (200 mg kg^{-1}) there was a small decrease in mean body weight (-6%) at the end of the study, however this was not statistically significant and no effects on body weight were seen in the other treatment groups. In dog, emesis and increased salivation were the only treatment related clinical signs. Increased salivation occurred mainly in the high dose group, during or shortly after dosing and generally disappeared by 3 h. The frequency of emesis increased with increasing dose level and high frequency at the top dose (150 mg kg^{-1}) indicated that this dose was poorly tolerated with respect to this parameter. No effects were observed on food consumption or body weight in dog. No other toxicological findings of note were observed. When compared with predose values, treatment with UK-343,664 caused minimal increases in the heart rate of dog recorded ~ 1.5 h after dosing. The magnitude of these increases was highly variable between animals and showed no clear dose relationship with mean increases of between 9 and 12% across the dose range.

Plasma protein binding

The mean ($\pm$ SD) proportion of UK-343,664 bound to rat plasma proteins at an initial concentration of $1 \mu\text{g ml}^{-1}$ was $95.8 \pm 0.6\%$ ($n = 5$ replicates, pooled plasma). In five separate samples of dog plasma the extent of binding was $80.6 \pm 4.4\%$.

In vitro rates of metabolism

Disappearance half-lives of UK-343,664 in male and female rat liver microsomes were 7 ± 0.6 and 33 ± 3 min respectively. No quantifiable disappearance of parent compound (half-life > 120 min) was observed over the 60-min incubation in dog liver microsomes.

Excretion studies

The recoveries of radioactivity in urine and faeces following intravenous and oral doses of [^{14}C]-UK-343,664 to rat and dog are shown in table 3. In both species, and by either route of administration, the majority of radioactivity was excreted in the faeces, with $< 10\%$ of the dose recovered in urine. Excretion was

Table 3. Excretion of radioactivity (as % administered dose) in urine and faeces of rat and dog after single intravenous and oral administration of [^{14}C]-UK-343,664.

Species and route of administration	Dose (mg kg $^{-1}$)	Urine excretion (0-t)*	Faeces excretion (0-t)*	Total excretion	
				(0-24 h)	(0-t)*
Male rat (n = 2) i.v.	1	2.0	103.6	103.3	105.6
Male rat (n = 2) p.o.	10	0.5	97.3	96.5	97.8
Female rat (n = 2) i.v.	1	2.8	99.1	95.9	101.9
Female rat (n = 2) p.o.	10	2.8	92.3	89.8	95.1
Dog (n = 2) i.v.	1	3.5	83.1	64.6	86.6*
Dog (n = 2) p.o.	10	9.0	74.4	56.2	84.6**

* t = 96 h for rat; 192 h for dog.

** Includes cage washings.

rapid with > 66% of recovered radioactivity present within 24 h after dosing in rat and dog.

Faecal metabolites

With the vast majority of radioactivity recovered in faeces from both rat and dog, metabolite profiling was restricted to faecal homogenate samples. Recovery of faecal radioactivity by solvent extraction was > 85% and > 95% of applied radioactivity was eluted from the gradient hplc system described. Metabolite profiles were therefore assumed to be representative of the major transformation pathways of UK-343,664. Chromatography of faecal extracts provided evidence of extensive metabolism of UK-343,664 with similar profiles obtained after intravenous and oral administration. The major components observed in rat and dog were isolated and analysed by direct infusion into the triple quadrupole mass spectrometer.

Representative metabolite profiles following oral administration to rat and dog are shown in figure 3. On this hplc system, UK-343,664, had a retention time of ~28 min. Radioactive components (figure 3, P) eluting at this retention time and accounting for 21 and 9% respectively of the faecal radioactivity in rat and dog were confirmed as parent compound by API mass spectrometry (parent MH^+ ion, 566). A major component in both rat and dog faecal extracts with a retention time of ~26 min (designated M1), has an equivalent retention time to the *N*-desethyl metabolite, UK-347,334. This structure was confirmed by mass spectrometry (parent MH^+ ion, 538). This metabolite accounted for 36 and 56% respectively of rat and dog faecal radioactivity. A major component in dog faecal extracts, designated M2 (retention time ~25 min), accounted for 22% of the sample radioactivity. Comparison with authentic standard and mass spectral analysis (parent MH^+ ion, 540) confirmed this structure to be the *N,N'*-desethyl metabolite resulting in opening of the piperazine ring. This metabolite was present in rat faecal homogenate as a minor component in fraction M3. The fraction M3 (retention time ~24 min) accounted for 19 and 7% respectively of rat and dog faecal homogenates. Mass spectral analysis (parent MH^+ ion, 512) indicated that this metabolite was the product of *N*-de-ethylation and *N,N'*-de-ethylation resulting in a piperazine ring opened primary amine metabolite. The other major regions in rat faecal homogenate extracts designated M4 (approximate retention time 23 min, 6% of radioactivity) and M5 (~22 min, 10% of radioactivity)

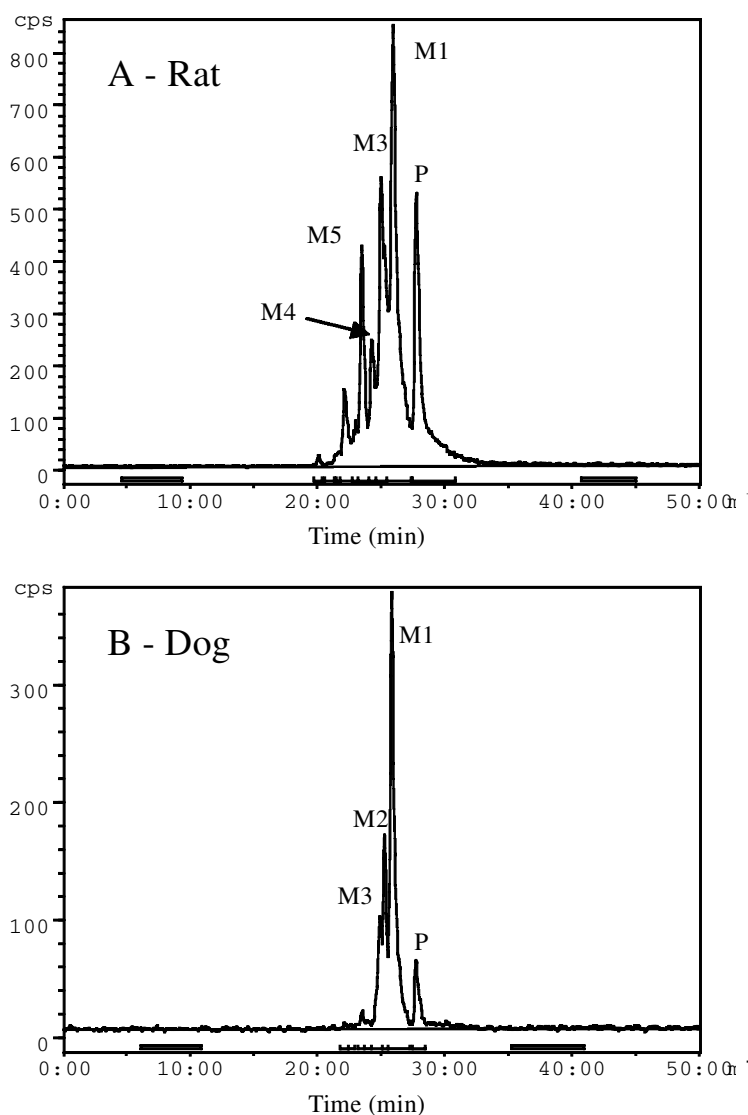


Figure 3. Representative hplc profiles of radioactivity in faecal extracts from the male rat and male dog following oral administration of [^{14}C]-UK-343,664. The gradient hplc system comprised of a 25×0.755 cm RPB column eluted with 0–100% methanol in 0.1 M aqueous ammonium acetate (pH 5) over 30 min at 1 ml min^{-1} . Final conditions were maintained for 20 min.

were also subject to mass spectral analysis. Fraction M4 provided a major parent ion MH^+ of 568. The fragmentation profile obtained was consistent with *N*-de-ethylation of the piperazine ring together with lactamization of this ring and additional hydroxylation of the *O*-propyl side-chain. Fraction M5 provided a major parent ion MH^+ of 554. The fragmentation profile obtained was consistent with *N*-de-ethylation and mono-hydroxylation both occurring in the piperazine ring. A summary of these proposed major metabolic pathways is depicted in figure 4. Rat and dog faecal metabolite profiles were similar after intravenous doses to

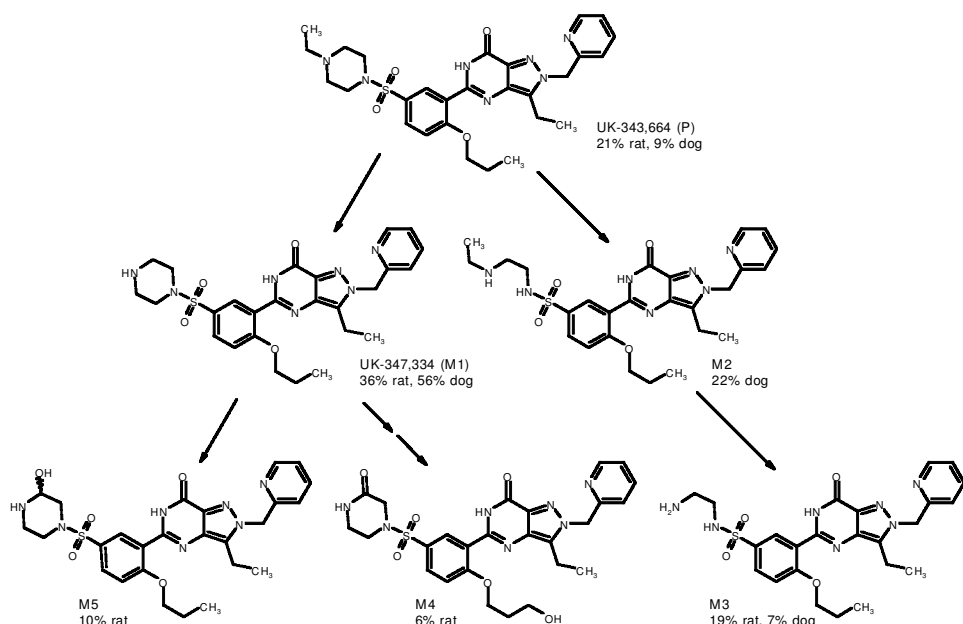


Figure 4. Proposed major metabolic pathways and metabolites of UK-343,664. Percentages of total faecal radioactivity are given for each metabolite detected in rat and dog after oral dosing.

those reported above for the oral route. By the intravenous route, the component corresponding to parent compound accounted for ~10 and 9% of faecal radioactivity in rat and dog respectively. Other components were of similar proportions to those recorded following oral doses.

Circulating metabolites of UK-343,664

Comparison of dog plasma concentrations of total radioactivity and parent compound (measured by specific LC-MS-MS assay) indicated that the majority of radioactivity in the circulation was parent compound. After intravenous and oral administrations the mean AUC_{0–24 h} ratio for parent compound compared with total radioactivity was 92 and 69% respectively ($n=2$ for each route). No further profiling as to the nature of the circulating radioactivity was performed. Radioactivity showed approximately equal distribution between red blood cells and plasma with similar concentrations obtained from whole blood and plasma throughout the time profile. Concentrations of radioactivity in whole, blood and plasma are compared with concentrations of parent compound in plasma following oral doses to dog ($n=2$) in figure 5.

Discussion

In animal pharmacokinetic studies, UK-343,664 exhibited a sex difference in clearance in the rat which was in keeping with the differences observed in the rates of *in vitro* metabolism. Thus, clearance in the male rat was very high at around liver blood flow and somewhat lower in female animals although still $> 50\%$ liver

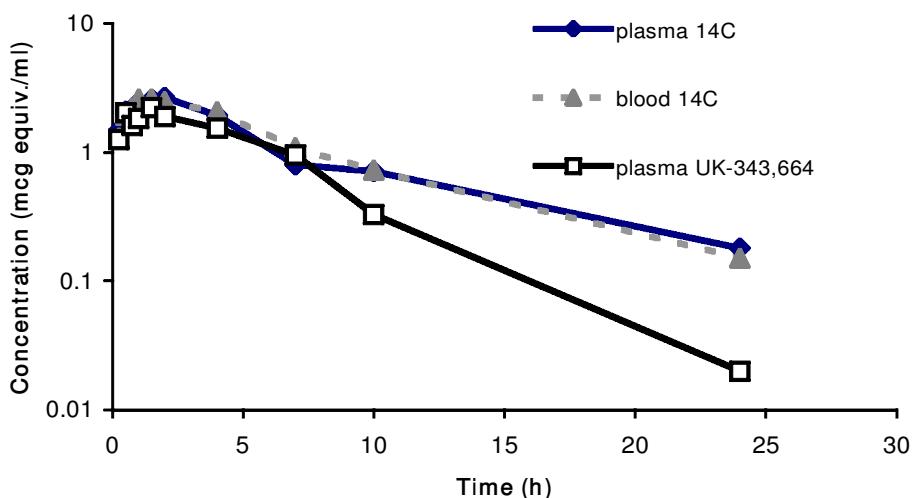


Figure 5. Average concentration-time profiles of plasma and whole blood radioactivity and plasma UK-343,664 following single oral doses of [^{14}C]-UK-343,664 to dog at a dose level of 10 mg kg^{-1} ($n = 2$).

blood flow. Such a sex difference in rat is commonly observed for compounds cleared by cytochrome P450 and is generally unique to rodent species. Indeed a similar sex difference was observed in rat for the structurally related compound sildenafil (Walker *et al.* 1999). Given the relative lipophilicity of UK-343,664 ($\log D_{7.4} = 3.1$), the compound was expected to be predominantly eliminated by metabolism. This was supported by the low urinary clearance recorded in the dog. Markedly reduced *in vitro* metabolism in dog liver microsomes was reflected in considerably lower clearance in the dog compared with rat at around 30% liver blood flow. The volume of distribution of UK-343,664 was higher in the dog than rat (4.6 versus $1.3\text{--}2.1 \text{ kg}^{-1}$), and probably reflected decreased plasma protein binding in the dog (81 versus 96% bound), allowing more drug to partition into tissue. Unbound volumes of distribution in the female rat and dog are very similar (31 and 241 kg^{-1}), which are in keeping with the high cross-species correlation previously observed for this parameter (Obach *et al.* 1997). The relatively large volume of distribution and moderate clearance in the dog provides an elimination half-life of 3.7 h , which is significantly longer than that observed in rat ($0.2\text{--}0.3 \text{ h}$).

Taking all of the available data into consideration, UK-343,664 appears to be well absorbed in animal species. Oral bioavailability of 70% at a dose of 1 mg kg^{-1} in dog is consistent with extensive absorption and an expected first-pass extraction of $\sim 30\%$. Similar levels of parent compound are present in faeces after both oral and intravenous doses of [^{14}C]-UK-343,664 to dog which again would support complete absorption. The high clearance in rat makes it difficult to judge the extent of absorption after oral doses to this species from pharmacokinetic data alone. However, the extent of excretion of parent compound in faeces is slightly higher after oral dosing ($\sim 22\%$) than intravenous dosing ($\sim 10\%$) which would suggest that only a small proportion of the dose ($\sim 15\%$) is not absorbed.

When the linearity of systemic exposure is examined across the entire dose range investigated within both pharmacokinetic and toxicokinetic studies, species

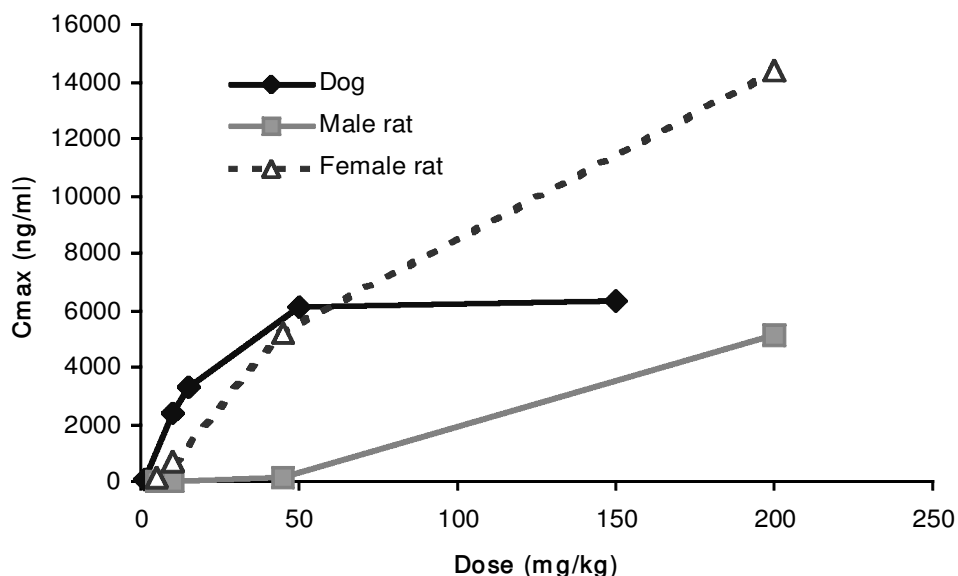


Figure 6. Relationship between $C_{\max}$ and oral dose of UK-343,664 in rat and dog from all studies performed. Values for dog are $n=2$ at doses of 1 and 10 mg kg^{-1} and $n=6$ at 15, 50 and 150 mg kg^{-1} . Values for male and female rat are $n=3$ at 5 mg kg^{-1} and $n=5$ at 10, 45 and 200 mg kg^{-1} .

differences in the dose proportionality are observed (figure 6). In dog, the plasma exposure increases relatively linearly with dose up to 15 mg kg^{-1} , but shows subproportional increases beyond this with virtually no further rise between doses of 50 and 150 mg kg^{-1} . The data for AUC (not shown) in the dog follows a similar pattern to $C_{\max}$. The most likely explanation for such non-linear behaviour would be dissolution rate limited absorption at these very high oral doses although an increased incidence of emesis in high dose animals may also contribute to this observation. The relatively lipophilic nature of UK-343,664 ($\log D_{7.4} = 3.1$) is consistent with the aqueous solubility limiting bioavailability at high doses (Smith *et al.* 1990). $C_{\max}$ data in the female rat show a less marked subproportionality than dog at high doses with a 2.8-fold rise between doses of 45 and 200 mg kg^{-1} (4.4-fold dose increase). This would again support a saturation of the absorption process at high doses, albeit less pronounced than in dog. The AUC data in the female rat actually shows a linear or slightly superproportional increase over the same dose range, suggesting that absorption is simply slowed or there is an additional component of capacity limited clearance coming into effect. The picture in the male rat is different again. Where there is a marked superproportional increase in both $C_{\max}$ (~50-fold) and AUC (~100-fold) between doses of 45 and 200 mg kg^{-1} . The most likely explanation for this would appear to be saturation of the very high clearance in the male rat. Systemic exposure to the *N*-desethyl metabolite of UK-343,664 also increases superproportionally over this dose range suggesting that clearance of this metabolite is also saturable and thus probably mediated by the same enzyme as parent compound.

UK-343,664 shows minimal clinical signs in animals even at the high doses used within the toxicology programme. In rat, the only observed effect was a small

and statistically insignificant decrease in body weight (6%) in the high dose males. Thus despite the higher systemic exposure of both parent and metabolite in female compared with male animals this fails to translate into clinical observations. In dog the only observed effects are emesis and an increase in salivation together with a mild increase in heart rate which did not appear to be dose dependent. The plasma concentrations achieved within the toxicology studies were markedly in excess of those observed in man in clinical studies with UK-343,664. In these clinical studies, UK-343,664 was given as single escalating doses up to a maximum dose of 800 mg. At this top dose in man the mean $C_{\max} = 1.7 \pm 0.5 \mu\text{g ml}^{-1}$, half-life = 4–6 h and $\text{AUC} = 10.6 \pm 3.3 \mu\text{g h ml}^{-1}$ (Abel *et al.* 2001). When compared with the highest exposure in toxicology studies, which was observed in the female rat, $C_{\max}$ and AUC in man at the top dose are ~ 8 and 6-fold lower respectively than the steady-state values in the female rat receiving doses of $200 \text{ mg kg}^{-1} \text{ day}^{-1}$.

The excretion of radioactivity almost exclusively in the faeces of rat and dog indicates that metabolites are efficiently excreted via the bile. Major pathways of metabolism in dog are relatively simple, essentially involving two *N*-dealkylation processes, namely piperazine *N*-de-ethylation resulting in the metabolite UK-347,334 and scission of the piperazine ring by removal of a 2-carbon fragment to produce the *N,N'*-desethyl metabolite M2. M3 is formed by combination of these two biotransformation processes. A similar scission of the piperazine ring has also been observed for the PDE5 inhibitor sildenafil (Walker *et al.* 1999) and the elastase inhibitor L-694,458 (Luffer-Atlas *et al.* 1997). In the rat, additional metabolites are formed by oxidation of the piperazine ring and the *O*-propyl side-chain. Other minor metabolites (not reported here) arise through combinations of these pathways and additional oxidation and cleavage of the piperazine ring. The piperazine ring is clearly the major site of metabolism of this molecule. Comparison of total radioactivity and parent compound concentrations after oral doses to dog indicates that parent compound is the major circulating drug related component accounting for $\sim 70\%$ of plasma radioactivity. Data from the toxicokinetic studies indicate that the *N*-desethyl metabolite, UK-347,334, is present at about one-fifth the concentration of parent compound and thus accounts for a large part of the circulating radioactivity not attributable to parent compound. This metabolite was also only a minor circulating component in rat.

In summary the disposition of UK-343,664 in rat and dog is consistent with its physicochemical properties. The compound is sufficiently lipophilic and soluble to be absorbed from the gastrointestinal tract, although solubility may become limiting at high doses ($> 100 \text{ mg kg}^{-1}$). The lipophilic and basic nature provides a large volume of distribution due to high tissue affinity and it is dependent on clearance by metabolism to more polar molecules that are excreted in the bile.

References

- ABEL, S., BEAUMONT, K. C., CRESPI, C. L., EVE, M. D., FOX, L., HYLAND, R., JONES, B. C., MUIRHEAD, G. J., SMITH, D. A., VENN, R. F. and WALKER, D. K., 2001, Potential role for P-glycoprotein in the non-proportional pharmacokinetics of UK-343,664 in man. *Xenobiotica*, **31**, 595–606.
- BOOLELL, M., GEPI-ATTEE, S., GINGELL, J. C. and ALLEN, M. J., 1996, Sildenafil, a novel effective treatment for male erectile dysfunction. *British Journal of Urology*, **78**, 257–261.
- LOWRY, O. H., ROSEBROUGH, N. M., FARR, A. L. and RANDALL, R. J., 1951, Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry*, **193**, 265–275.

- LUFFER-ATLAS, D., VINCENT, S. H., PAINTER, S. K., ARISON, B. H., STEARNS, R. A. and CHIU, S.H., L., 1997, Orally active inhibitors of human leukocyte elastase. III. Identification and characterization of metabolites of L-694,458 by liquid chromatography-tandem mass spectrometry. *Drug Metabolism and Disposition*, **25**, 940–952.
- OBACH, R. S., BAXTER, J. G., LISTON, T. E., SILBER, M., JONES, B. C., MACINTYRE, F., RANCE, D. J. and WASTALL, P., 1997, The prediction of human pharmacokinetic parameters from preclinical and *in vitro* metabolism data. *Journal of Pharmacology and Experimental Therapeutics*, **283**, 46–58.
- OMURA, T. and SATO, R., 1964, The carbon monoxide-binding pigment of liver microsomes. I. Evidence for its hemoprotein nature. *Journal of Biological Chemistry*, **239**, 2370–2378.
- REMMER, H., SCHENKMAN, J. B., ESTABROOK, R. W., SASAME, H., GILLETTE, J. R., NARASIMHULU, S., COOPER, D. Y. and ROSENTHAL, O., 1966, Drug interactions with hepatic microsomal cytochrome. *Biochemical Pharmacology*, **48**, 2147–2156.
- SMITH, D. A., HUMPHREY, M. J. and CHARUEL, C., 1990, Design of toxicokinetic studies. *Xenobiotica*, **20**, 1187–1199.
- WALKER, D. K., ACKLAND, M. J., JAMES, G. C., MUIRHEAD, G. J., RANCE, D. J., WASTALL, P. and WRIGHT, P. A., 1999, Pharmacokinetics and metabolism of sildenafil in mouse, rat, rabbit, dog and man. *Xenobiotica*, **29**, 297–310.
- WALLIS, R. M., CORBIN, J. D., FRANCIS, S. H. and ELLIS, P., 1999, Tissue distribution of phosphodiesterase families and the effects of sildenafil on tissue cyclic nucleotides, platelet function, and the contractile responses of trabeculae carneae and aortic rings *in vitro*. *American Journal of Cardiology*, **83**, 3C–12C.

Potential role for P-glycoprotein in the non-proportional pharmacokinetics of UK-343,664 in man

S. ABEL[‡], K. C. BEAUMONT[†], C. L. CRESPI[§], M. D. EVE[¶],
L. FOX[§], R. HYLAND[†], B. C. JONES[†], G. J. MUIRHEAD[‡],
D. A. SMITH[†], R. F. VENN[†] and D. K. WALKER^{†*}

[†] Department of Drug Metabolism and [‡] Clinical Sciences, Pfizer Global Research and Development, Sandwich CT13 9NJ, UK

[§] Gentest Corporation, 6 Henshaw Street, Woburn, MA 01801, USA

[¶] Pfizer Clinical Research Unit, Kent and Canterbury Hospital, Ethelbert Road, Canterbury, Kent, UK

Received 9 January 2001

1. UK-343,664 is a potent and specific PDE5 inhibitor. Following single oral doses to human volunteers, it exhibited non-proportional pharmacokinetics over the dose range 30–800 mg. Over this 27-fold dose range, $C_{\max}$ and AUC_t increased 247- and 287-fold respectively. The half-life (4–6 h) was similar at all doses. No systemic exposure was quantifiable at doses <10 mg.

2. UK-343,664 is a lipophilic molecule ($\log D_{7.4} = 3.1$) and as such is expected to be cleared mainly by metabolism. Based on studies with expressed human P450 enzymes it was concluded that the metabolism of UK-343,664 was predominantly mediated by CYP3A4. With a moderate $K_m = 76 \mu\text{M}$ for this enzyme, saturation of first-pass metabolism alone was considered unlikely to account for the non-proportional pharmacokinetics.

3. UK-343,664 showed high affinity for P-glycoprotein *in vitro*, with a $K_m = 7.3 \mu\text{M}$. In transport studies in LLC-PK1 cell monolayers transfected with P-glycoprotein, UK-343,664 showed marked polarized transport which was concentration dependent.

4. The high affinity of UK-343,664 for P-glycoprotein is considered to be the primary source of the non-proportional pharmacokinetic profile observed in man.

Introduction

UK-343,664 (figure 1) is a novel potent and specific inhibitor of the human cGMP-specific phosphodiesterase type 5 enzyme (PDE5) found in human corpus cavernosum and other tissues (Wallis *et al.* 1999). PDE5 inhibitors have been effectively used in the treatment of male erectile dysfunction (Boolell *et al.* 1996). The compound is relatively lipophilic ($\log D_{7.4} = 3.1$) with a weakly basic centre at the piperazine tertiary amine ($pK_a = 6.9$).

The compound was progressed to clinical trials where marked non-proportionality was observed in the pharmacokinetic profile after oral doses. Potential sources of the non-linear behaviour were considered to be due to a saturable process governing the systemic availability of UK-343,664. In addition to the possible role of drug-metabolizing enzymes in this profile, the transport protein P-glycoprotein (PGP) was also considered as a potential source. PGP is a member of the ABC

* Author for correspondence; e-mail: don_walker@sandwich.pfizer.com

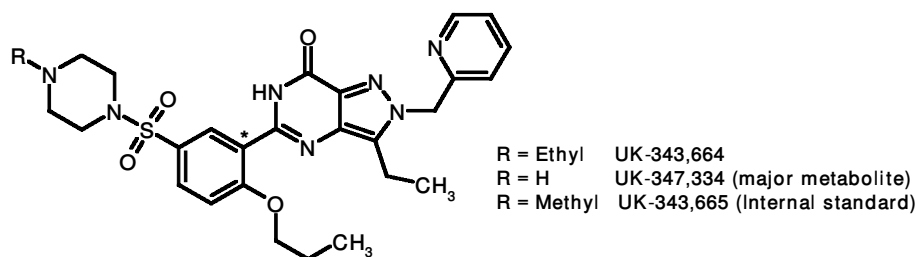


Figure 1. Structure of UK-343,664 (*position of $[^{14}\text{C}]$ label), internal standard for analysis (UK-343,665) and *N*-desethyl metabolite (UK-347,334).

transporter superfamily and is expressed in human liver, intestine and other tissues (Fardel *et al.* 1996). Intestinal PGP is believed to affect the oral bioavailability of drug molecules that are substrates for this transporter (Hunter and Hirst 1997).

This paper describes the pharmacokinetic profile of UK-343,664 in man after single oral doses and the subsequent studies undertaken with an aim to understanding the non-proportional pharmacokinetics that were observed.

Materials and methods

Chemicals

UK-343,664 citrate salt, internal standard, UK-343,665 (methyl piperazine analogue of UK-343,664) and UK-347,334 (*N*-desethyl metabolite of UK-343,664) were synthesized at Pfizer Global Research and Development (Sandwich, UK). The chemical structures of these compounds are shown in figure 1. $[^{14}\text{C}]$ -UK-343,664 citrate salt was prepared by Amersham International (Cardiff, UK) with a radiochemical purity $>99\%$ (by thin-layer chromatography and confirmed by mass spectrometry and HPLC) and a specific activity of $24.5 \mu\text{Ci mg}^{-1}$. $[^3\text{H}]$ -vinblastine sulphate and $[^3\text{H}]$ -verapamil hydrochloride were from Amersham International. All other chemicals were from Sigma Chemical Co. (St Louis, MO, USA). The chemical purity of all compounds was $\geq 95\%$.

Administration to man

Twenty-four male volunteers (age 18–45 years; weight 60–100 kg) provided written informed consent and participated in a double-blind, placebo controlled, four-way crossover dose escalation study in two separate cohorts of 12 subjects. The study was approved by the local ethics committee and was performed at the Pfizer Clinical Research Unit and Kent and Canterbury Hospital. The doses administered were 1, 10, 100 and 400 mg for cohort 1, and 3, 30, 200 and 800 mg for cohort 2. Doses were administered in solution in 0.01 M HCl. Within each group of 12 subjects, nine received active drug and three received placebo. There was a minimum of 7 days between each dose. Samples of whole blood were collected up to 24–48 h post-dose on each study day into heparinized tubes. Following centrifugation, plasma was removed and stored at approximately -20°C in polypropylene tubes prior to analysis.

Analysis of UK-343,664 in plasma and urine samples

The extraction of UK-343,664 from human plasma was performed in an automated manner using a Tecan Genesis Robot. Human plasma samples (1.0 ml) were mixed with 1 ml 1 M chloroacetic acid in methanol/water (10:90 v/v) prior to extraction using activated solid-phase extraction sorbent (Certify, Analytichem International) packed in 96-well format with 50 mg sorbent per well (Kaye *et al.* 1996). Following sample application, the cartridges were washed sequentially with 1 ml water, 1 ml 1 M chloroacetic acid in methanol/water (10:90 v/v) and 1 ml methanol. UK-343,664 and internal standard (UK-343,665) were eluted with 1.5 ml ammonium hydroxide/methanol (5:95 v/v) into a 2 ml, 96 deep-well block. These extracts were retained in the 96-well block, evaporated to dryness under a nitrogen stream and resuspended in 200 μl 2 mM ammonium acetate buffer, pH 4.0, in methanol/water (30:70 v/v). Samples (180 μl) were injected onto the HPLC column (Supelcosil LC-DB-C8, 33×4.6 mm) with a mobile phase consisting of methanol/2 mM ammonium acetate, pH 4.0 (80:20 v/v) at 1 ml min^{-1} . Detection was by multiple reaction monitoring for the transitions m/z 566–346 (UK-343,664) and 552–346 (UK-343,665) using a Sciex API III+ triple quadrupole mass spectrometer (Perkin Elmer,

Sciex, Ontario, Canada) in positive atmospheric pressure chemical ionization mode. On this system the retention times of UK-343,664 and internal standard were both ~ 2 min. The limit of quantification was 1 ng ml^{-1} . In order to satisfy acceptance criteria, analysis of quality control samples provided results within 20% of the correct value at the lower end of the calibration range and within 15% of the correct value at the middle and top of the calibration range (100 ng ml^{-1}).

Urine samples were analysed by liquid-liquid extraction. UK-343,665 was used as an internal standard. Urine samples (1.0 ml) were buffered with 2 ml 0.5 M aqueous sodium phosphate, pH 8.5 (2 ml) containing internal standard (UK-343,665, 50 ng ml^{-1}). The samples were then transferred to 3 ml Chem-Elut tubes. After allowing 5 min for adsorption onto the inert support, samples were extracted with *tert*-butyl methyl ether ($2 \times 4 \text{ ml}$). The extracts were evaporated to dryness in a stream of nitrogen at 40°C and reconstituted in 20/80 acetonitrile/water (100 μl). Extracts were injected (90 μl) into a column-switching HPLC system comprising three columns: a guard cartridge ($10 \times 2.1 \text{ mm}$ Kromasil $5 \mu\text{m}$ C1), an extraction column ($150 \times 3.2 \text{ mm}$ Kromasil $5 \mu\text{m}$ C1) and an analytical column ($250 \times 2.0 \text{ mm}$ Luna $5 \mu\text{m}$ (2) C18). Mobile phase 1 comprising 30/70 acetonitrile/0.02 M ammonium phosphate, pH 2.8, was pumped through the guard cartridge and extraction columns at 0.3 ml min^{-1} . Mobile phase 2 comprising 40/60 acetonitrile/0.02 M ammonium phosphate, pH 8.4, was pumped through the analytical column at 0.35 ml min^{-1} . The eluate from the guard cartridge was heart-cut to the extraction column by placing the two in line with each other, typically between 0.25 and 1.75 min after the injection. The eluate from the extraction column was heart-cut to the analytical column by placing the two in line with each other with mobile phase 1 pumping through both. This was done typically between 8.50 and 11.5 min after the injection. The assay was calibrated over the range 5–500 ng ml^{-1} . Calibration lines were constructed by plotting the peak:height ratio of UK-343,664 to UK-343,665 against the concentration ratio of UK-343,664 to UK-343,665 using linear weighted ($1/y^2$) least-squares regression. Samples expected or found to contain UK-343,664 at a level $>500 \text{ ng ml}^{-1}$ were diluted to within this range with control urine.

Pharmacokinetic analysis of data

The terminal elimination rate constant (k_{el}) was determined by linear regression of the log plasma concentrations. The elimination half-life ($t_{1/2}$) was obtained from: $t_{1/2} = 0.693/k_{\text{el}}$. Area under the plasma concentration versus time curve (AUC_t) was calculated to the last time point by the linear trapezoidal method. AUC_∞ was calculated by extrapolation to infinity with k_{el} . C_{max} and T_{max} were respectively the maximum observed plasma concentration and the first time at which this was observed.

Plasma protein-binding determinations

Samples (1 ml) of control human plasma containing [^{14}C]-UK-343,664 at concentrations of 10, 100 and 1000 ng ml^{-1} were dialysed (Spectropor 1 dialysis membrane 6000–8000 molecular weight cut-off; Spectrum Medical Industries) against isotonic Krebs–Ringer bicarbonate buffer, pH 7.4 (1 ml) for 4 h at 37°C in a rotating dialyser (Dianorm, MSE). Triplicate determinations were performed at each concentration. Following dialysis, concentrations of drug in plasma and buffer were measured by liquid scintillation counting.

Measurement of ATPase activity in PGP containing membranes

Human PGP (GENBANK accession number HUMMDR1) containing membranes were from Gentest Corporation having been prepared according to the method of Sarkadi *et al.* (1992) and stored at approximately -80°C prior to use. ATPase assays were conducted in 96-well microtitre plates using a modification of the methods of Sarkadi *et al.* (1992) and Drueckes *et al.* (1995). Incubations were performed in 0.06 ml containing 40 μg membranes, test compound and 5 mM MgATP, in buffer containing 50 mM Tris-MES (2-[*N*-morpholino]ethanesulphonic acid), 2 mM EGTA, 50 mM KCl, 2 mM dithiothreitol and 5 mM sodium azide at 37°C for 20 min. An incubation containing 100 μM sodium orthovanadate was assayed in parallel. The incubation was stopped by the addition of 30 μl 10% SDS + Antifoam A (a silicone polymer; Sigma). The liberated phosphate was measured by the addition of 200 μl 35 mM ammonium molybdate in 15 mM zinc acetate: 10% ascorbic acid (1:4) and incubated for an additional 20 min at 37°C . The liberated phosphate was quantified against a calibration curve constructed from known concentrations of potassium phosphate. Stimulation of ATPase activity brought about by UK-343,664 was compared with that of the known PGP substrates and inhibitors vinblastine and verapamil respectively (Shapiro and Ling 1998, Wang *et al.* 2000).

Stable PGP expression in LLC-PK₁ cells and transport studies

Human PGP cDNA was introduced into a clonal population of LLC-PK₁ cells in a vector that confers resistance to hygromycin B. LLC-PK₁ cells were propagated in Medium 199 supplemented with 7% foetal calf serum. Transport studies were conducted in 24-well TranswellsTM (Corning Costar). Cells were seeded at 4×10^4 cells per insert and used for transport studies 4–8 days post-

seeding. Transport of [^{14}C]-UK-343,664 was measured in Hanks' balanced salt solution buffered with 10 mM HEPES (pH 7.2) under sink conditions in both the apical to basolateral (A to B) and basolateral to apical (B to A) directions in both PGP-transfected and non-transfected cell monolayers. At least duplicate monolayers were used for each determination. Quantitation of the amount of compound transported was by liquid scintillation counting. Comparison of UK-343,664 transport across these cell monolayers was again made to the known PGP substrates and inhibitors, vinblastine and verapamil respectively.

Metabolism by human liver microsomes and recombinant P450s

The metabolism of [^{14}C]-UK-343,664 (10 μM) was assessed in human liver microsomes (prepared from a pool of eight individual livers) and microsomes from baculovirus cells engineered to express individually one of CYP1A2, CYP2C8, CYP2C9, CYP2C19, CYP2D6 and CYP3A4 as the cells' only P450 (Gentest Corporation). Transplant-quality human liver tissue was from the International Institute for the Advancement of Medicine (Exton, PA, USA). All tissues were procured with informed consent in accordance with the Uniform Anatomical Gift Act (UAGA) USA. The donors ranged in age from 19 to 59 years and included four males and four females. No donor had a known drug history, with the exception of one donor who had been treated for diabetes. No donor had a history of alcohol abuse. Pooled livers were used in order to achieve microsomes more representative of an average human liver. Incubations (1 ml, $n = 3$) were performed at 150 pmol P450 ml $^{-1}$ (0.5 nmol P450 ml $^{-1}$ for human liver microsomes) for 30 min. Samples were extracted with 5 ml ethyl acetate and 2 ml sodium borate buffer, pH 10, and chromatographed on a 250 $\times$ 7.75 mm RPB column (Hichrom), using a methanol/0.1 M ammonium acetate mobile phase gradient elution (0–100% methanol over 15 min, held at 100% for further 5 min). The flow rate was 2 ml min $^{-1}$ and detection was by an inline radiochemical detector (β -Ram, Lablogic Ltd). Activity was calculated in terms of total radiolabelled peak area for metabolites, expressed as a percentage of the total radioactivity for the incubation.

Metabolism of UK-343,664 by recombinant CYP3A4

The formation of UK-347,334 (*N*-desethyl metabolite) from UK-343,664 was characterized in microsomes from baculovirus cells engineered to express CYP3A4 (Gentest Corporation, Woburn, MA, USA). Kinetic studies were performed at 50 pmol CYP3A4 ml $^{-1}$ for 10 min over a substrate concentration range of 1–500 μM ($n = 3$). Samples were extracted with 5 ml ethyl acetate and 2 ml 0.2 M sodium borate buffer, pH 10, plus internal standard (10 μl , 0.02 mg ml $^{-1}$). Samples were chromatographed on a 150 $\times$ 3.2 mm RPB column (Hichrom) with a mobile phase of 10 mM octanesulphonic acid +10 mM KH_2PO_4 , pH 3/methanol (68:32 v/v). Detection was by UV absorbance at 226 nm. The amount of UK-347,334 formed in the incubations was determined by interpolation from standard curves constructed in microsomes from 1 to 1000 ng UK-347,334 (CV = 11.3, 7.1 and 2.9% for concentrations of 2.5, 50 and 1000 ng ml $^{-1}$). Data from the kinetic experiments were analysed by Grafit (v.3.01) by an iterative non-linear least-squares algorithm to obtain K_m .

Results

Plasma assay validation

The analytical method for UK-343,664 in human plasma used mixed-mode solid-phase extraction followed by HPLC-MS-MS using a close analogue of UK-343,664 as an internal standard. This internal standard, UK-343,665 (figure 1) differs only in the substitution of a methyl group for the ethyl group of the parent. The assay for human plasma showed good precision and accuracy over the range 1 to 100 ng ml $^{-1}$. Over three analytical runs, values for inaccuracy were less than 15% at 1, 10 and 100 ng ml $^{-1}$ and values for imprecision were less than 8% at the same concentrations based on replicate analysis of six samples. The compound was also shown to be stable at room temperature, 4°C and –20°C, as well as to three freeze–thaw cycles (data not shown).

Urine assay validation

The analytical method for UK-343,664 in human urine used liquid–liquid extraction on a Chem-Elut cartridge, followed by column-switching HPLC with

UV detection at 230 nm. The same internal standard as for the plasma assay (UK-343,665) was used. This assay also showed good precision and accuracy from 5 to 500 ng ml⁻¹, with imprecision <6% and inaccuracy <3% at levels of 5, 100 and 500 ng ml⁻¹ ($n=6$ replicate analyses at each) for each of three validation batches.

Single dose pharmacokinetics in man

Pharmacokinetic parameters obtained in the clinical pharmacokinetic study with UK-343,664 are shown in table 1. The mean concentration–time profiles following oral doses of 30–800 mg are shown in figure 2. At doses <10 mg, plasma concentrations were below the limit of quantification (<1 ng ml⁻¹) for all subjects. Concentrations remained below this level in four of nine subjects who received the dose of 10 mg; the mean pharmacokinetic parameters quoted at this dose level are for the five subjects in whom plasma concentrations were quantifiable. At doses >10 mg, $C_{\max}$ increased supraproportionally with dose, such that for an 80-fold

Table 1. Pharmacokinetic parameters (mean $\pm$ SD) for UK-343,664 after single oral doses to healthy male volunteers.

Parameter	10 mg ($n=5$)	30 mg ($n=9$)	100 mg ($n=9$)	200 mg ($n=9$)	400 mg ($n=9$)	800 mg ($n=8$)
Elimination half-life (h)	n.d.	n.d.	5.7 $\pm$ 1.0	4.8 $\pm$ 0.6	4.1 $\pm$ 0.7	5.1 $\pm$ 1.7
$C_{\max}$ (ng ml ⁻¹)	1.3 $\pm$ 0.3	7.1 $\pm$ 2.8	78 $\pm$ 42	255 $\pm$ 86	903 $\pm$ 300	1743 $\pm$ 535
$T_{\max}$ (h)	3.5 $\pm$ 1.7	1.2 $\pm$ 0.5	0.8 $\pm$ 0.4	0.6 $\pm$ 0.2	0.6 $\pm$ 0.3	1.2 $\pm$ 0.7
AUC_t (ng h ml ⁻¹)	5.6 $\pm$ 5.2	36.9 $\pm$ 9.3	281 $\pm$ 116	950 $\pm$ 271	3215 $\pm$ 581	10607 $\pm$ 3335
AUC_{∞} (ng h ml ⁻¹)	n.d.	n.d.	291 $\pm$ 118	965 $\pm$ 275	3246 $\pm$ 584	10624 $\pm$ 3333
Urinary excretion (0–24 h) (% dose)	0.8 $\pm$ 0.3	1.2 $\pm$ 0.4	2.0 $\pm$ 0.7	3.4 $\pm$ 1.4	3.3 $\pm$ 1.0	5.2 $\pm$ 1.1

n.d., Not determined.

Volumes are arithmetic mean $\pm$ SD.

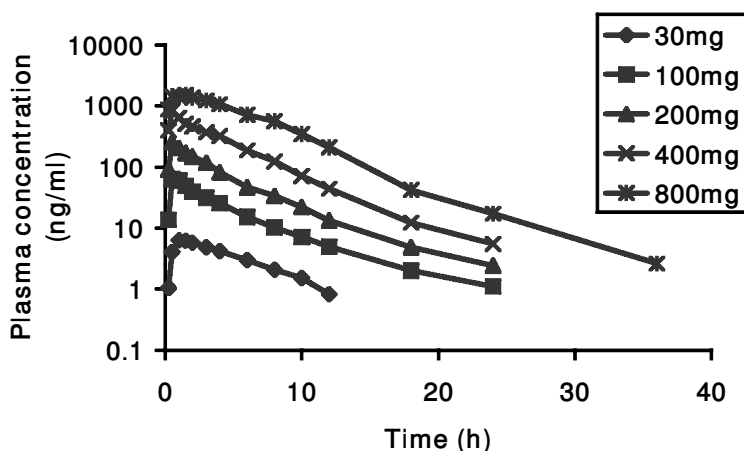


Figure 2. Mean plasma concentrations of UK-343,664 in human male volunteers after single oral doses of UK-343,664. Data are mean concentrations (nine subjects at doses from 30 to 400 mg and eight subjects at 800 mg).

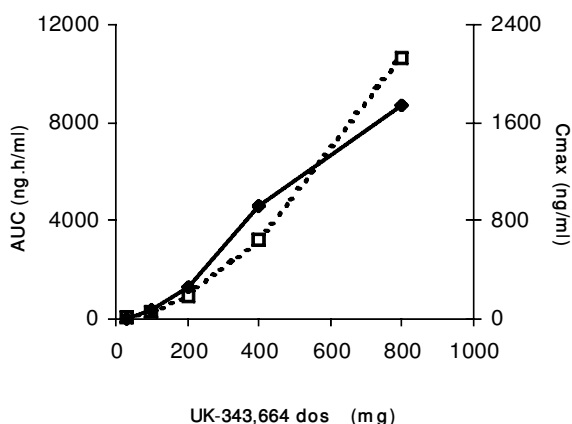


Figure 3. Mean AUC (□) and $C_{\max}$ (◆) from single oral doses of 10, 30, 100, 200, 400 and 800 mg UK-343,664 to a total of 24 male human volunteers (eight to nine subjects exposed at each dose level).

increase in dose (10–800 mg) there was an ~ 1300 -fold rise in this parameter. A similar supraproportional rise was observed in AUC_t , which increased 287-fold over the dose range of 30–800 mg (27-fold). Owing to limited data at doses < 100 mg, AUC_{∞} was not calculated at the 30 mg dose; however, between 100 and 800 mg the mean AUC_{∞} increased 37-fold. This non-proportional relationship between dose and exposure is shown in figure 3. As the dose increased over the range 10–400 mg there was a reduction in $T_{\max}$ from 3.5 to 0.6 h suggesting more rapid absorption at higher doses. However, peak concentrations occurred later at the top dose of 800 mg where $T_{\max} = 1.2$ h. The elimination half-life was similar (mean range 4.1–5.7 h) across the dose range studied. Only low levels of UK-343,664 were excreted in the urine (0–24 h), which increased slightly with increasing dose from $0.8 \pm 0.3\%$ at a dose of 10 mg to $5.2 \pm 1.1\%$ at a dose of 800 mg (table 1).

Plasma protein binding

The binding of UK-343,664 to human plasma proteins remained relatively constant over the concentration range 10–1000 ng ml^{-1} . The mean proportion of drug bound to protein was 75.9 ± 1.9 , 85.0 ± 1.0 and $85.7 \pm 0.4\%$ ($n = 3$) at concentrations of 10, 100 and 1000 ng ml^{-1} respectively.

Activation of ATPase activity in PGP membranes

The liberation of inorganic phosphate in response to PGP stimulation provided a rapid measure of drug affinity for PGP using a simple microtitre-based spectrophotometric assay. The concentration–response relationship for UK-343,664 in this assay is shown in figure 4. The apparent K_m (half maximal response) for UK-343,664 in this assay was $7.3 \mu\text{M}$. In comparison, K_m for the known PGP substrates/inhibitors vinblastine and verapamil was 15 and $17 \mu\text{M}$ respectively (data not shown).

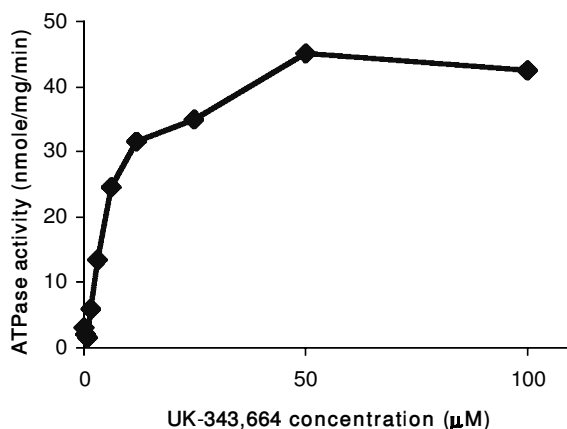


Figure 4. Stimulation of ATPase activity in human PGP containing insect cell membranes in response to addition of varying concentrations of UK-343,664. All determinations were performed in duplicate.

Drug transport across cell monolayers

Polarization of transport of UK-343,664 was compared in PGP transfected and non-transfected cells by examination of the ratio of the rates of basolateral to apical transport (B to A) compared with apical to basolateral transport (A to B). The concentration dependence of these ratios in the two cell lines for UK-343,664 and vinblastine is shown in figure 5. Polarization of the transport of UK-343,664 in PGP transfected cells was most marked at low concentration with a ratio of 5.3 at a concentration of $0.8 \mu\text{M}$. This compared with a ratio of <2 in the non-transfected cells. The extent of polarized transport in PGP transfected cells was greatly diminished at increasing concentrations of UK-343,664, such that this ratio was <1.5 at a concentration of $29 \mu\text{M}$ and virtually indistinguishable from non-transfected cells. In contrast, vinblastine showed markedly greater polarization of transport in the presence of PGP, with a polarization ratio of >30 at the lowest concentration tested ($0.5 \mu\text{M}$). Whilst this ratio also decreased at higher substrate concentration, it remained >4 at the highest concentration tested ($40 \mu\text{M}$) and was always greater than that observed in non-transfected cells. This difference between

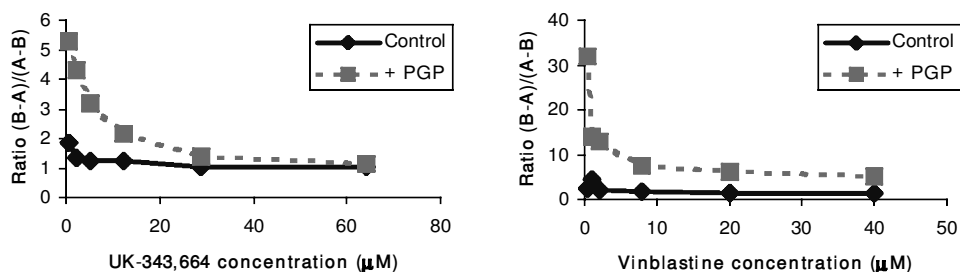


Figure 5. Concentration dependence of the polarization of transport of UK-343,664 and vinblastine across LLC-PK₁ cell monolayers. Transport studies were performed in 24-well plate format with at least duplicate determinations at each concentration. Studies were performed in non-transfected LLC-PK₁ cells (control) and the same cells that had been transfected with human PGP cDNA (+PGP). Polarization ratios are the ratios of the rates of transport in the basolateral to apical direction compared with the apical to basolateral direction.

vinblastine and UK-343,664 appeared to be dependent on the intrinsic permeability of the two compounds. In control cell monolayers, the extent of permeation of UK-343,664 in the apical to basolateral direction over 30 min amounted to between ~ 7 and 9% across the concentration range. In contrast, vinblastine permeation was ~ 10 -fold lower. Of the three compounds examined, verapamil showed the highest intrinsic permeability such that permeation studies were halted before 30 min to prevent 50% permeation and associated loss of sink conditions. This high intrinsic permeability was associated with minimal polarization of transport in PGP containing cell monolayers, such that A to B and B to A transport were virtually identical and minimal differences were observed between control and PGP transfected cells (data not shown).

Metabolism of UK-343,664 by recombinant P450s

CYP3A4 was shown to have the greatest ability of the P450s examined to metabolize [^{14}C]-UK-343,664. Over 30-min incubations, total metabolite formation from $10\text{ }\mu\text{M}$ [^{14}C]-UK-343,664 amounted to 0, 2.6, 1.6, 11.1, 6.0 and 38.0% total radioactivity by cells solely expressing CYP1A2, 2C8, 2C9, 2C19, 2D6 and 3A4 respectively. This contrasted to 20.7% metabolite formation by the pooled human liver microsomal preparation. Additional studies employing CYP probe substrates indicated that CYP3A4 was under expressed compared with human liver, whilst other enzymes were over expressed, therefore indicating that CYP3A4 is a major enzyme in UK-343,664 metabolism. The radiochromatogram of the solvent extract of the recombinant CYP3A4 incubation (figure 6) indicated that

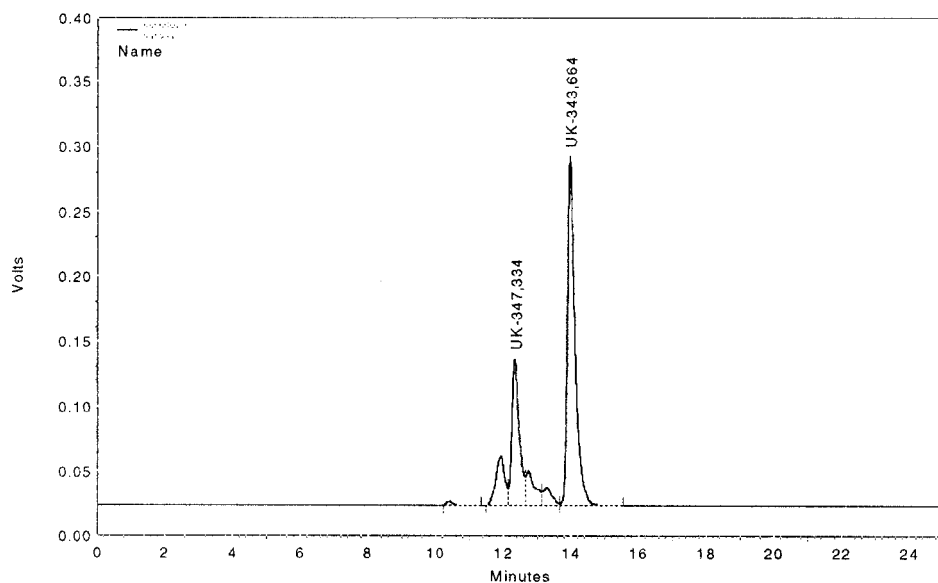


Figure 6. Profile of radioactivity obtained following solvent (ethyl acetate) extraction from recombinant CYP3A4 microsomal preparation ($150\text{ pmol P450 ml}^{-1}$) following 30-min incubation with $10\text{ }\mu\text{M}$ [^{14}C]-UK-343,664. UK-347,334 is the *N*-desethyl metabolite of UK-343,664. The gradient HPLC system comprised of a $250 \times 7.75\text{ mm}$ Hichrom RPB column, eluted with 0–100% methanol in 0.1 M ammonium acetate over 15 min at 2 ml min^{-1} .

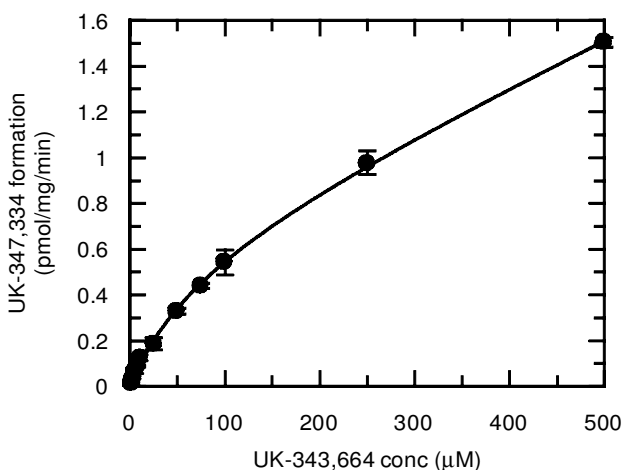


Figure 7. Michaelis–Menten plot of the rate of formation of the *N*-desethyl metabolite of UK-343,664 (UK-347,334) in recombinant CYP3A4 microsomes over a substrate concentration range of 1–500 μM . The data were analysed by the computer program Grafit (v.3.01) using an iterative non-linear least-squares algorithm and provided a $K_m = 76.2 \mu\text{M}$. Data are mean $\pm$ SD of $n = 3$ determinations.

the *N*-desethyl metabolite (UK-347,334) was the major product based on co-chromatography with authentic standard. A similar metabolite profile was observed in the extract from the pooled human liver microsome incubation (data not shown).

Michaelis–Menten kinetics of UK-343,664 N-de-ethylation

The rate of formation of the *N*-desethyl metabolite of UK-343,664 (UK-347,334) by recombinant CYP3A4 was linear over 10 min and up to a P450 concentration of 250 pmol ml^{-1} . The extent of formation of this metabolite by CYP3A4 over a substrate concentration range 1–500 μM is shown in figure 7. Analysis of the data using the computer program Grafit provided a $K_m = 76 \mu\text{M}$.

Discussion

Over the relatively extensive oral dose range of UK-343,664 which was investigated (10–800 mg), non-proportionality is not surprising for a drug which is cleared predominantly by metabolism. Given the relative lipophilic nature of UK-343,664 ($\log D_{7.4} = 3.1$), the compound was expected to be predominantly eliminated by metabolism. This was supported by the low renal clearance observed at all dose levels. Based on the moderate $K_m = 76 \mu\text{M}$ for CYP3A4, extensive first-pass metabolism of UK-343,664 was not expected at low doses. Most noticeable therefore in the case of UK-343,664 was the pharmacokinetic profile at the lower end of this dose range. The very low exposure and shape of the plasma concentration–time curve, with $T_{\max}$ occurring at 3.5 h after the 10 mg dose compared with 0.6–1.2 h at higher doses, indicated the gut was acting as a barrier to absorption and contributing or causing the non-proportionality. At the initial low doses studied, it was unexpected that the exposure would be as low as observed, with

oral clearance at the 30 mg dose being $\sim 150 \text{ ml min}^{-1} \text{ kg}^{-1}$. The concentration independence of UK-343,664 plasma protein binding across the range of concentrations encountered in the clinical studies indicates that protein binding is not a contributing factor to the non-proportional pharmacokinetics.

In light of the clinical data with UK-343,664, further studies were performed in an attempt to elucidate the source of the non-proportional behaviour. Increasingly the transporter protein PGP has been implicated in limiting the absorption of drug molecules. This protein has been identified as limiting the oral bioavailability of cyclosporine and is second only to hepatic CYP3A4 in accounting for variability in patient exposure (Lown *et al.* 1997). PGP has also been implicated in the incomplete bioavailability of the β -blocker, talinolol (Gramatté *et al.* 1996) and the NK₂ antagonist, UK-224,671 (Beaumont *et al.* 2000). In the case of talinolol, the effects of the PGP inhibitor verapamil on the oral pharmacokinetics of talinolol in the rat have supported the involvement of PGP, by significantly reducing oral clearance (Spahn-Langguth *et al.* 1998).

The studies described here indicate that UK-343,664 has a high affinity for PGP and is a substrate for CYP3A4. Both of these proteins are present in the gut wall cell and can have a pronounced effect on the oral bioavailability of their substrates. At low oral doses of UK-343,664, the concentrations of the drug in the gut lumen will be low (probably below the K_m for PGP). Thus, at these doses, PGP will mediate a significant efflux of UK-343,664 back into the gut lumen. This will lead to a delayed absorption of the compound (as evidenced by the delayed $T_{\max}$) and a greater opportunity for the gut wall CYP3A4 to metabolize the compound. This is analogous to the model proposed for increased gut wall metabolism of cyclosporine (Hebert 1997). Consequently, at low oral doses of UK-343,664 the combination of gut wall and hepatic first-pass extraction leads to low systemic exposure. However, as the dose of UK-343,664 increases, the gut lumen concentrations increase above the PGP K_m , which leads to saturation of this transporter. The results of this are that efflux of the compound from the enterocyte is reduced leading to more rapid absorption ($T_{\max}$ reduction) and a reduced gut wall first-pass extraction. Overall, this leads to an increase in systemic exposure and the observed non-proportional increases in $C_{\max}$ and AUC. The ~ 6 -fold increase in the proportion of UK-343,664 excreted as unchanged drug in the urine over the dose range 10–800 mg is also consistent with increased systemically available drug at higher doses.

The extent of PGP mediated efflux would appear to be dependent not only on PGP affinity, but also to the intrinsic permeability properties of a molecule. Hence, whilst vinblastine and verapamil show similar affinity for PGP in the ATPase assay, the permeability of verapamil in the permeability assay is markedly higher and hence overcomes susceptibility to efflux in *in vitro* systems. Thus, in the transport studies described here, vinblastine demonstrated markedly polarized transport due to its poor intrinsic permeability in the apical to basolateral direction but high PGP-mediated transport in the basolateral to apical direction. In contrast, the good intrinsic permeability of verapamil results in similar transport in both directions, as the PGP mediated transport rate does not exceed the passive process. This is reflected in the good absorption observed for verapamil in man (Sandstrom *et al.* 1998, 1999). UK-343,664 is of intermediate intrinsic permeability and hence shows concentration dependence to its susceptibility to PGP-mediated efflux. At low doses, the rate of permeation results in an intracellular

concentration that is insufficient to saturate PGP and a substantial proportion of UK-343,664 is effluxed. However, at high concentrations the proportion effluxed becomes negligible as the transporter becomes saturated.

In conclusion, PGP appears to have a key role in the absorption of UK-343,664. For compounds that are readily permeable across the gastrointestinal membranes, this may well have a limited effect as the rate of passage exceeds the capacity of this transporter. However, for compounds of low and intermediate permeability, affinity for PGP can play a key role in limiting the rate and extent of absorption. It may well be that the poor absorption ascribed to high molecular weight compounds (Lipinski *et al.* 1997) is in part due to PGP limited absorption as well as poor permeation *per se*.

Acknowledgements

The authors thank innumerable colleagues within Pfizer, Sandwich, for discussion on the emerging data with this compound. Particular thanks are due to Christine Tyman for practical contributions to the *in vitro* pharmacokinetic studies and the associated bioanalysis, to Helen Jenkins for plasma protein-binding determinations, to Mark Bayliss and Richard Gage (urine bioanalysis), and to Jackie Luckwell, Pat Wright, Richard Hucker and Ken Saunders (plasma bioanalysis).

References

- BEAUMONT, K., HARPER, A., SMITH, D. and BENNETT, J., 2000, The role of P-glycoprotein in determining the oral absorption and clearance of the NK₂ antagonist, UK-224,671. *European Journal of Pharmaceutical Science*, **12**, 41–50.
- BOOLELL, M., GEPI-ATTEE, S., GINGELL, J. C. and ALLEN, M. J., 1996, Sildenafil, a novel effective treatment for male erectile dysfunction. *British Journal of Urology*, **78**, 257–261.
- DRUECKES, P., SCHINZEL, R. and PALM, D., 1995, Photometric microtiter assay of inorganic phosphate in the presence of acid labile organic phosphates. *Analytical Biochemistry*, **230**, 173–177.
- FARDEL, O., LECUREUR, V. and GUILLOUZO, A., 1996, The P-glycoprotein multidrug transporter. *General Pharmacology*, **27**, 1283–1291.
- GRAMATTE, T., OERTEL, R., TERHAAG, B. and KIRCH, W., 1996, Direct demonstration of small intestinal secretion and site-dependent absorption of the β -blocker talinolol in humans. *Clinical Pharmacology and Therapeutics*, **59**, 541–549.
- HEBERT, M. F., 1997, Contributions of hepatic and intestinal metabolism and P-glycoprotein to cyclosporine and tacrolimus oral drug delivery. *Advanced Drug Delivery Reviews*, **27**, 201–214.
- HUNTER, J. and HIRST, B. H., 1997, Intestinal secretion of drugs. The role of P-glycoprotein and related drug efflux systems in limiting oral drug absorption. *Advanced Drug Delivery Reviews*, **25**, 129–157.
- KAYE, B., HERRON, W. J., MACRAE, P. V., ROBINSON, S., STOPHER, D. A., VENN, R. F. and WILD, W., 1996, Rapid, solid phase extraction technique for the high-throughput assay of darifenacin in human plasma. *Analytical Chemistry*, **68**, 1658–60.
- LIPINSKI, C. A., LOMBARDO, F., DOMINY, B. W. and FEENEY, P. J., 1997, Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. *Advanced Drug Delivery Reviews*, **23**, 3–25.
- LOWN, K. S., MAYO, R. R., LEICHTMAN, A. B., HSIAO, H.-L., TURGEON, D. K., SCHMEIDLIN-REN, P., BROWN, M. B., GUO, W., ROSSI, S. J., BENET, L. Z. and WATKINS, P. B., 1997, Role of intestinal P-glycoprotein (mdr1) in interpatient variation in the oral bioavailability of cyclosporine. *Clinical Pharmacology and Therapeutics*, **62**, 248–260.
- SANDSTROM, R., KARLSSON, A., KNUTSON, L. and LENNERNAS, H., 1998, Jejunal absorption and metabolism of R/S-verapamil in humans. *Pharmaceutical Research*, **15**, 856–862.
- SANDSTROM, R., KNUTSON, T. W., KNUTSON, L., JANSSEN, B. and LENNERNAS, H., 1999, The effect of ketoconazole on the jejunal permeability and CYP3A metabolism of (R/S)-verapamil in humans. *British Journal of Clinical Pharmacology*, **48**, 180–189.

- SARKADI, B., PRICE, E. M., BOUCHER, R. C., GERMANN, U. A. and SCARBOROUGH, G. A., 1992, Expression of the human multidrug resistance cDNA in insect cells generates a high activity drug-stimulated membrane ATPase. *Journal of Biological Chemistry*, **267**, 4854–4858.
- SHAPIRO, A. B. and LING, V., 1998, The mechanism of ATP-dependent multidrug transport by P-glycoprotein. *Acta Physiologica Scandinavica*, **163**, 227–234.
- SPAHN-LANGGUTH, H., BAKTIR, G., RADSCHUWETT, A., OKYAR, A., TERHAAG, B., ADER, P., HANAFY, A. and LANGGUTH, P., 1998, P-glycoprotein transporters and the gastrointestinal tract: Evaluation of the potential *in vivo* relevance of *in vitro* data employing talinolol as model compound. *International Journal of Clinical Pharmacology and Therapeutics*, **36**, 16–24.
- WALLIS, R. M., CORBIN, J. D., FRANCIS, S. H. and ELLIS, P., 1999, Tissue distribution of phosphodiesterase families and the effects of sildenafil on tissue cyclic nucleotides, platelet function, and the contractile responses of trabeculae carneae and aortic rings *in vitro*. *American Journal of Cardiology*, **83**, 3C–12C.
- WANG, E.-J., CASCIANO, C. N., CLEMENT, R. P. and JOHNSON, W. W., 2000, *In vitro* flow cytometry method to quantitatively assess inhibitors of P-glycoprotein. *Drug Metabolism and Disposition*, **28**, 522–528.

Pharmacokinetics of the novel, high-affinity and selective dopamine D3 receptor antagonist SB-277011 in rat, dog and monkey: *in vitro/in vivo* correlation and the role of aldehyde oxidase

N. E. AUSTIN†, S. J. BALDWIN†, L. CUTLER†, N. DEEKS†, P. J. KELLY†, M. NASH†, C. E. SHARDLOW†, G. STEMP‡, K. THEWLIS‡, A. AYRTON† and P. JEFFREY†

† Department of Drug metabolism and Pharmacokinetics, GlaxoSmithKline, The Frythe, Welwyn, Hertfordshire AL6 9AR, UK.

‡ Department of Medicinal Chemistry, GlaxoSmithKline, New Frontiers Science Park, Harlow, Essex, UK.

Received 27 February 2001

1. *In vitro* studies with the selective dopamine D3 receptor antagonist SB-277011 were conducted in liver microsomes and homogenates from rat, dog, cynomolgus monkey and human to correlate the rate of metabolism with the *in vivo* pharmacokinetics of the compound in rat, dog and cynomolgus monkey.

2. In the presence of NADPH, SB-277011 was relatively stable in the presence of liver microsomes from rat, dog, cynomolgus monkey and human with an intrinsic clearance (CL_i) of $< 2 \text{ ml min}^{-1} \text{ g}^{-1}$ liver for all species. In total liver homogenates, SB-277011 was metabolized at a similar rate in rat and dog ($CL_i < 2 \text{ ml min}^{-1} \text{ g}^{-1}$ liver) to that in liver microsomes but in cynomolgus monkey and human ($CL_i = 9.9$ and $45 \text{ ml min}^{-1} \text{ g}^{-1}$ liver, respectively) the intrinsic clearance was ~6- and 35-fold higher, respectively, than that in liver microsomes.

3. In the absence of NADPH, SB-277011 was rapidly cleared in liver homogenates from cynomolgus monkey and human ($CL_i = 7.4$ and $27 \text{ ml min}^{-1} \text{ g}^{-1}$ liver, respectively) demonstrating that a significant pathway of metabolism of this compound was via an NADPH-independent non-microsomal oxidative route. This pathway was sensitive to inhibition with isovanillin suggesting that the enzyme responsible was aldehyde oxidase.

4. The *in vivo* pharmacokinetics showed that the plasma clearance of SB-277011 was low in rat ($20 \text{ ml min}^{-1} \text{ kg}^{-1}$), moderate in dog ($14 \text{ ml min}^{-1} \text{ kg}^{-1}$) and high in cynomolgus monkey ($58 \text{ ml min}^{-1} \text{ kg}^{-1}$), which is consistent with the *in vitro* findings and demonstrated a greater capacity for the monkey to metabolize this compound. The oral bioavailability of SB-277011 in rat, dog and cynomolgus monkey was 35, 43 and 2%, respectively. Given the high clearance of this compound in cynomolgus monkey, the low oral bioavailability is probably as a result of high first-pass elimination, specifically by aldehyde oxidase, rather than poor absorption.

5. The high *in vitro* clearance of SB-277011 in human liver homogenates and the involvement of aldehyde oxidase in the metabolism of SB-277011 indicates that the bioavailability of the compound is likely to be low in human.

Introduction

SB-277011 {*trans-N*-[4-[2-(6-cyano-1,2,3,4-tetrahydroquinolin-2-yl)ethyl]cyclohexyl]-4-quinolininecarboxamide} is a novel, high-affinity and selective

* Author for correspondence; e-mail: nigel_e_austin@gsk.com

dopamine D3 receptor antagonist that may have potential in treating the negative symptoms of schizophrenia (Reavill *et al.* 2000).

The pharmacokinetic evaluation of potential drug candidates in various animal species is an important part of the development process. We now report the *in vitro* metabolism of SB-277011 in rat, dog, cynomolgus monkey and human liver microsomes and liver homogenates. In addition, the *in vitro* metabolism in rat, dog and cynomolgus monkey was compared with the *in vivo* pharmacokinetics of the compound in these three species. It has previously been reported that SB-277011 is a low clearance molecule in the rat ($CL_b = 24 \text{ ml min}^{-1} \text{ kg}^{-1}$) and CNS penetrant with a steady-state brain-blood ratio = 3.6:1 (Reavill *et al.* 2000).

Materials and methods

Chemicals

SB-277011-A (the 'A' denotes the hydrochloride salt) and a single metabolite of SB-277011; SB-369912 {*trans*-*N*-[4-[2-(6-cyano-1,2,3,4-tetrahydroisoquinolin-2-yl)ethyl]cyclohexyl]-2-oxo-1,2-dihydroquinoline-4-carboxamide}, with purities of >98 and >90%, respectively, were obtained from the Medicinal Chemistry Department (SmithKline Beecham, Harlow, UK). The internal standards BRL-32872 (*N*-(dimethoxy-phenyl)-*N*-(3-{[2-(3,4-dimethoxy-phenyl)ethyl]methyl-amino}propyl)-4-nitro-benzamide) and SB-279832 ({*trans*-*N*-[4-[2-(6-cyano-1,2,3,4-tetrahydroisoquinolin-2-yl)ethyl]cyclohexyl]-4-quinolininecarboxamide} were also from the Medicinal Chemistry Department. Their purities were >90%. [^{14}C]SB-277011-A, labelled on the carbonyl group on the amide bond (figure 1), with specific activity $114 \mu\text{Ci mg}^{-1}$ and a radiochemical purity of 96%, was prepared by the Synthetic Isotope Chemistry Unit (SmithKline Beecham). All other chemicals were commercially available and of analytical grade.

Preparation of subcellular liver fractions

Liver homogenates and microsomal fractions were prepared from male rats (Crl:CD (SD); Charles River, Manston, UK), ~200 g body-weight, male beagle dogs (Harlan Interfauna, Cambell Farms Ltd, Huntingdon, UK) ~10 kg body-weight, male cynomolgus monkeys (Shamrock Farms, Smallhole, UK) ~3 kg body-weight and human liver. Human (male and female) liver tissue was obtained from either Vitron, Inc. (Tucson, Arizona, USA), the International Institute for the Advancement of Medicine (Pennsylvania, PA, USA) or the Human Cell Culture Centre (HCCC, Maryland, USA). All human samples were from healthy donors and in all cases, the cause of death was not due to any known biochemical deficiency in the liver. Liver homogenates and microsomal fractions were prepared by differential centrifugation. Fresh liver samples were homogenized to 25% (w/v) in ice-cold 0.25 M sucrose/50 mM Tris-HCl buffer, pH 7.4. Homogenates were prepared from individual liver samples obtained from rat, dog, cynomolgus monkey and human ($n = 5, 3, 4$ and 7 livers, respectively) by centrifugation at 460 g for 5 min. The supernatant was stored at $\sim 4^\circ\text{C}$ prior to *in vitro* incubations. The microsomal fractions were isolated from pooled rats ($n = 10$), dog ($n = 1$), monkey ($n = 1$) and pooled human ($n = 5$) as described by Baldwin *et al.* 1999. Homogenate and microsomal protein concentrations were determined by the method of Smith *et al.* (1985), using the Pierce BCA (bicinchoninic acid) protein assay reagent.

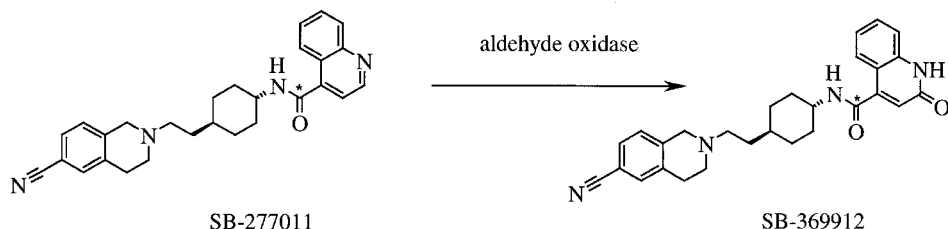


Figure 1. Structure of [^{14}C]SB-277011-A. The 'A' denotes the hydrochloride salt and * denotes the position of the radiolabel.

In vitro studies with subcellular hepatic fractions

SB-277011-A (at a final concentration $0.5\text{ }\mu\text{M}$, added in DMSO, final solvent concentration not exceeding 0.5% (v/v)), was incubated with an NADPH-regenerating system (final concentration 0.5 mM NADP^+ , 5 mM glucose 6-phosphate and 1 U ml^{-1} glucose 6-phosphate dehydrogenase in 2% (w/v) sodium hydrogen carbonate) and homogenate ($\sim 0.004\text{ g liver ml}^{-1}$ incubation) or microsomal protein ($\sim 0.5\text{ mg ml}^{-1}$ incubation) in 50 mM potassium phosphate buffer, pH 7.4, in a final incubation volume of 1 ml . Homogenate incubations were initiated by the addition of liver homogenate after a 5 min pre-incubation period, whereas, microsomal incubations were initiated by the addition of the NADPH-regenerating system. All incubations were performed at 37°C in a shaking water bath. Aliquots ($50\text{ }\mu\text{l}$) were taken from the incubation over 30 min and the reaction terminated by adding $200\text{ }\mu\text{l}$ acetonitrile containing BRL-32872 (50 ng ml^{-1}) as internal standard.

Parallel incubations with cynomolgus monkey liver homogenates were carried out at a higher substrate concentration, $10\text{ }\mu\text{M}$ [^{14}C]SB-277011-A for 60 min at 37°C , in order to determine the metabolic profile of the compound. These incubations were performed in the presence and absence of the aldehyde oxidase inhibitor, isovanillin (at a final concentration $100\text{ }\mu\text{M}$, added in DMSO, final solvent concentration not exceeding 0.5% (v/v)).

Analysis of in vitro samples

After centrifugation, the supernatants of incubations of $0.5\text{ }\mu\text{M}$ SB-277011-A were analysed by HPLC-MS-MS for disappearance of parent compound in order to obtain relative rates of intrinsic clearance. Incubations (unextracted) of $10\text{ }\mu\text{M}$ [^{14}C]SB-277011-A were analysed by radio-HPLC and selected samples were analysed by HPLC-MS and HPLC-MS-MS. Separation was achieved using a Kromasil $5\text{ }\mu\text{m}$ C18 column ($100 \times 3.2\text{ mm}$) and gradient elution of increasing acetonitrile:methanol (1:1 (v/v)) concentration versus 0.05 M ammonium acetate *aq.* at a flow rate of 0.5 ml min^{-1} and a column temperature of 30°C . The gradient started with 4% organic, which was increased linearly to 28% over 14 min, the organic content was then increased linearly to 33% by 48 min after the start of the run and again to 80% after 64 min. The organic content was then increased to 95% over the next 1 min and held at this concentration for a further 5 min. After this the organic content was returned to initial conditions (4%) over a further 1 min. Detection was by a radioflow detector (β -ram, Lablogic Sheffield, UK) with a homogenous liquid cell system and Packard Ultima-Flo scintillation fluid. Data analysis was performed using LAURA software (Lablogic). Mass spectrometry was carried out using a VG Quattro instrument with data processing via a PC running MassLynx v.2.1 (Micromass, Manchester, UK). The ionization mode was electrospray with alternate negative and positive ion using cone voltages of 20 and 25 V, respectively. The scanning mass range in MS mode was m/z $50\text{--}800$. The collision gas used was argon at a pressure of $\sim 5\text{--}9 \times 10^{-4}\text{ mBar}$. The collision energy was 20 eV .

Furthermore, a single metabolite produced from rabbit hepatocyte incubations (NZW, Harlan Porcellus, Shaw's Farm, Bicester, UK) (methodology not shown) was isolated using Waters OASIS HLB 3cc solid-phase extraction cartridges and identified by proton NMR analysis (Analytical Sciences, SmithKline Beecham) and HPLC-MS comparisons to the synthetic standard, SB-369912.

Analysis of in vitro data

The *in vitro* or intrinsic clearance (CL_i) was determined from the first-order elimination rate constant by non-linear regression using Grafit v.3.0 (Leatherbarrow 1992), using the actual volume of the incubation and either assuming 52.5 mg microsomal protein g^{-1} liver (Iwatsubo *et al.* 1997) for microsomal clearances or scaling by a factor of 4 in order to correct for the homogenate preparation which was 25% (w/v). Intrinsic clearances were expressed as $\text{ml min}^{-1}\text{ g}^{-1}$ liver.

Pharmacokinetic studies: dosing and sample collection

Rat. Three male rats (CrI:CD (SD) Charles River), 269–284 g body-weight were used. Each animal undergoing surgery received a subcutaneous injection of carprofen (ZenecarpTM 50 mg ml^{-1} , C-Vet VP) at 7.5 mg kg^{-1} and ampicillin (PenbritinTM 100 mg ml^{-1} ; Beecham Research) at 100 mg kg^{-1} before surgery. Under general anaesthesia using a mixture of isoflurane in oxygen, a cannula was inserted via a femoral vein into the vena cava (for drug administration), hepatic portal vein (for drug administration) and jugular vein (for blood sampling) as described by Griffiths *et al.* (1996). Cannulae were exteriorized at the back of the neck. The animals were placed in jackets with tethers and housed in a plastic-bottom cage in facilities maintained at $19\text{--}23^\circ\text{C}$ and $45\text{--}65\%$ humidity with a 12-h dark/light cycle. Each rat had free access to food (SQC rat and mouse maintenance Diet A, SDS, Witham, UK) and water. Following a postoperative recovery of 6 days, each rat received an intravenous infusion of SB-277011-A at a nominal dose level of $1\text{ mg kg}^{-1}\text{ h}^{-1}$ ($2.280\text{ }\mu\text{mol kg}^{-1}\text{ h}^{-1}$) for 1 h via the hepatic portal vein cannula ($10\text{ ml kg}^{-1}\text{ h}^{-1}$) and then switched for 1 h via the vena cava. SB-277011-A was dissolved in 5% (w/v) glucose *aq.* containing 2% (v/v) DMSO at a concentration of 0.1 mg ml^{-1} (0.228 mM). Following a washout of 2 days, the same rats each received a single

oral (suspension) dose of SB-277011-A at a nominal dose level of 3 mg kg^{-1} ($6.840 \mu\text{mol kg}^{-1}$). SB-277011-A was suspended in 1% (w/v) methylcellulose *aq.* at a concentration of 1.5 mg ml^{-1} (3.420 mM). Serial blood samples were collected from the jugular vein cannula predose and at intervals up to 26 h post-dose. Plasma was separated from red blood cells by centrifugation.

Dog. Three male beagle dogs (weight range 13.2–17.6 kg; Harlan Interfauna, Cambell Farms Ltd, Huntingdon, UK) were used. Each dog was fasted overnight and placed in slings prior to dosing. The dogs were returned to their holding pens immediately after the 8-h sample and fed (Harlan Teklad 9682 Dog Diet, Shaw's Farm, Bicester, UK). The dogs were allowed free access to water except whilst in the slings. For the intravenous phase, a catheter was inserted into a saphenous vein for dose administration and a cephalic vein for blood sampling. SB-277011-A was dissolved in 5% (w/v) glucose *aq.* containing 2% (v/v) DMSO (0.2 mg ml^{-1} ; 0.456 mM) and infused for 2 h at a nominal dose level of 1 mg kg^{-1} ($2.280 \mu\text{mol kg}^{-1}$). Following a recovery of 6 days, the same dogs were dosed orally with SB-277011-A as a single loose filled gelatin capsule at a nominal dose level of 3 mg kg^{-1} ($6.840 \mu\text{mol kg}^{-1}$). Blood samples were collected predose and at intervals up to 36 h post-dose. Plasma was separated from blood cells by centrifugation.

Monkey. Three purpose-bred male cynomolgus monkeys (weight range 2.8–3.9 kg; Shamrock Farms, Smalldole, UK) were used. Studies were ethically reviewed locally and animal care and use was in accordance with the UK Animals (Scientific Procedures) Act. Each monkey was housed socially and fasted overnight prior to dosing. For the intravenous phase, each monkey was held in a primate restraint chair. A catheter was inserted into a saphenous vein for dose administration and a cephalic vein for blood sampling. SB-277011-A was dissolved in 5% (w/v) glucose *aq.* containing 2% (v/v) DMSO (0.2 mg ml^{-1} ; 0.456 mM) and infused for 1 h (5 ml kg^{-1}) at a nominal dose level of 1 mg kg^{-1} ($2.280 \mu\text{mol kg}^{-1}$). Food was made available to the animals ~2 h after the completion of dosing (SDS Mazuri Primate Diet). Following a washout of 1 week, the same monkeys were dosed with SB-277011-A as a single loose-filled gelatin capsule at a nominal dose level of 3 mg kg^{-1} ($6.840 \mu\text{mol kg}^{-1}$). Blood samples were collected predose and at intervals up to 36 h post-dose. Plasma was separated from blood cells by centrifugation.

Analysis of plasma samples

Plasma samples were analysed for SB-277011 using a method based on protein precipitation and LC/MS/MS analysis. Acetonitrile ($200 \mu\text{l}$) containing SB-279832 (200 ng ml^{-1}) as internal standard was added to the samples ($50 \mu\text{l}$). Samples were mixed thoroughly (mechanical shaking for 20 min) and then centrifuged ($14\,500g$ for 15 min at room temperature). The resulting supernatant was transferred to a fresh vial containing ammonium acetate buffer ($50 \mu\text{l}$, 10 mM , $\text{pH } 8.0$). After brief mixing (10 s), samples ($60 \mu\text{l}$) were assayed for SB-277011 concentrations using LC/MS/MS employing positive-ion Turbo Ionspray (Sciex API 300) and a YMC KL-C3 column ($50 \times 4 \text{ mm i.d. } 3 \mu\text{m}$; Hichrom). The mobile phase was 60/40 (v/v) acetonitrile/ammonium acetate buffer (10 mM , $\text{pH } 8.0$) at a flow rate of 1 ml min^{-1} . The lower limit of quantification was 5 ng ml^{-1} ($0.011 \mu\text{M}$) and the assay was linear up to 3000 ng ml^{-1} ($6.840 \mu\text{M}$).

Pharmacokinetic analysis of data

Non-compartmental pharmacokinetic parameters were obtained from the plasma concentration-time curves using WinNonlin Professional v.1.5 (Pharsight Corp., Mountain View, CA, USA). For the rat pharmacokinetic study, an estimate of the bioavailability following intrahepatic portal vein administration (F_{ipv}) was made using the methods of Griffiths *et al.* (1996) and the bioavailability following oral administration (F_{po}) was calculated from the ratio of the area under the plasma concentration versus time curve (AUC) after oral and intravenous doses after normalizing for dose.

Results

In vitro clearance of SB-277011 in the presence of NADPH

In the presence of an NADPH-regenerating system, SB-277011 ($0.5 \mu\text{M}$) was relatively stable in liver microsomes from rat, dog, cynomolgus monkey and human with an intrinsic clearance (CL_i) = 1.4, <0.6, 1.7 and $1.3 \text{ ml min}^{-1} \text{ g}^{-1}$ liver, respectively. However, there was a marked species difference in metabolism of SB-277011 using liver homogenates. Under similar conditions, SB-277011 was metabolized at a similar rate in rat and dog liver homogenates

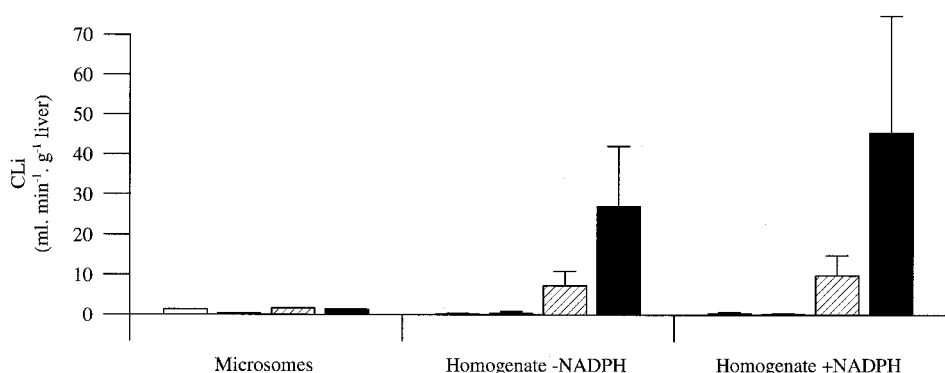


Figure 2. Metabolic stability of SB-277011 in hepatic microsomes and liver homogenates in the presence and absence of an NADPH-regenerating system. Rat (clear bars), dog (spotted bars), cynomolgus monkey (hatched bars) and human (solid bars). Data for hepatic microsomes are from one determination and for liver homogenates are mean $\pm$ SD from three to seven livers.

($CL_i < 0.6 \text{ ml min}^{-1} \text{ g}^{-1} \text{ liver}$) but rapidly metabolized in cynomolgus monkey and human liver homogenates with $CL_i = 9.9 \pm 5.0$ and $45 \pm 29 \text{ ml min}^{-1} \text{ g}^{-1} \text{ liver}$, respectively. The relative CL_i of SB-277011 obtained with liver microsomes and homogenates is given in figure 2.

In vitro clearance of SB-277011 in the absence of NADPH

In the absence of an NADPH-regenerating system, the metabolism of SB-277011 in liver homogenates was similar to that in the presence of NADPH. SB-277011 was relatively stable in rat and dog with $CL_i < 0.6 \text{ ml min}^{-1} \text{ g}^{-1} \text{ liver}$, and rapidly metabolized in cynomolgus monkey and human liver homogenates with $CL_i = 7.4 \pm 3.5$ and $27 \pm 15 \text{ ml min}^{-1} \text{ g}^{-1} \text{ liver}$, respectively (figure 2).

SB-277011 pharmacokinetics

The blood concentration-time profiles of SB-277011 following intravenous infusion and oral administration of SB-277011-A to rat, dog and cynomolgus monkey are shown in figure 3 and the related pharmacokinetic parameters are shown in table 1. In rat, dog and cynomolgus monkey, following intravenous infusion, SB-277011 exhibited apparent terminal phase half-lives of 2.0, 4.2 and 0.7 h, respectively. These were due to the low clearance of the compound in rat, moderate clearance in dog and high clearance in monkey (20 , 14 and $58 \text{ ml min}^{-1} \text{ kg}^{-1}$, respectively) combined with steady-state volumes of distribution greater than total body water (2.9 , 4.5 and 2.9 l kg^{-1} , respectively). The *in vivo* plasma clearance of SB-277011 in rat, dog and cynomolgus monkey was consistent with the *in vitro* findings and demonstrated a greater capacity for the monkey to metabolize the compound.

Following oral administration, the bioavailability of SB-277011 in the rat was 35%. The maximum oral bioavailability (F_{ipv} ; assumes 100% absorption and no pre-absorptive first-pass elimination) was 41% implying that SB-277011 was well absorbed with minimal pre-absorptive first-pass elimination. Oral bioavailability of SB-277011 in dog was 43% and similar to rat. However, oral bioavailability of

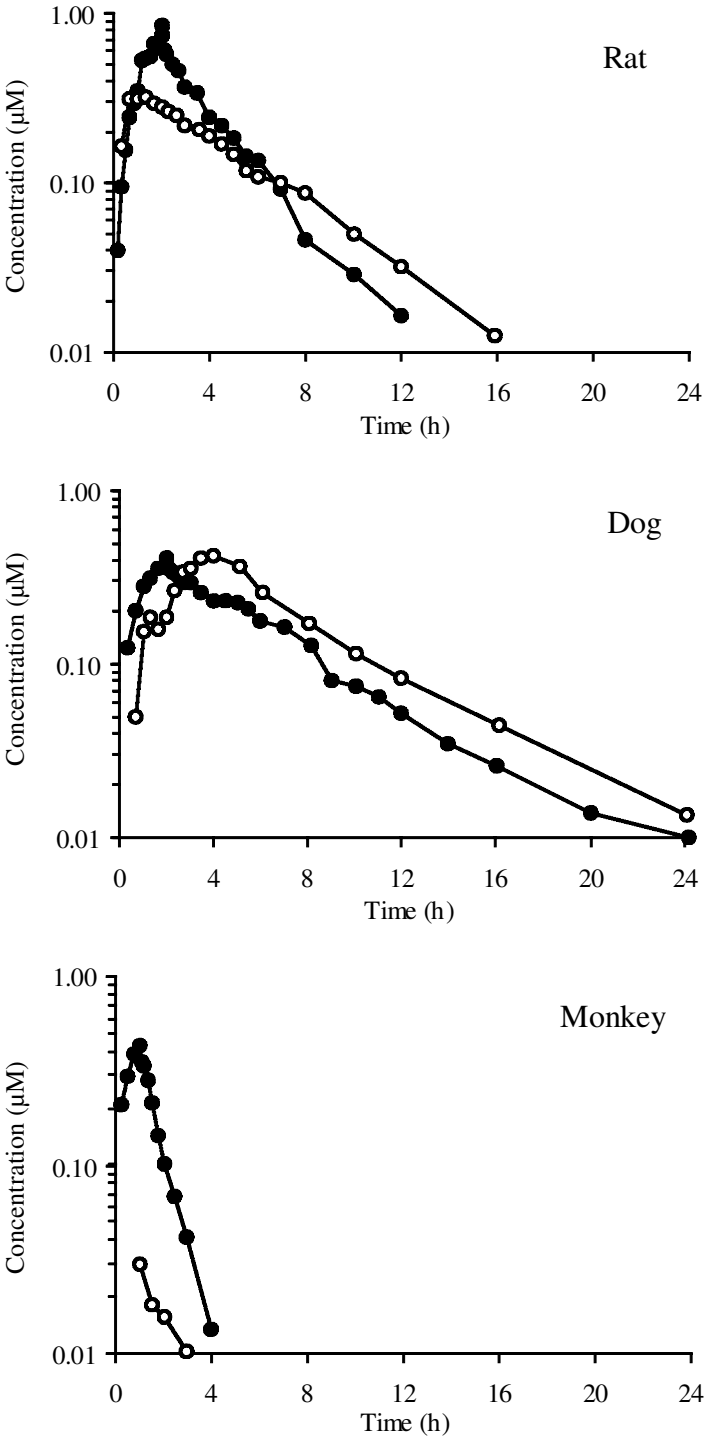


Figure 3. Mean blood concentrations of SB-277011 in rat, dog and monkey following intravenous infusion and oral administration of the hydrochloride salt (SB-277011-A) at dose levels of 1 and 3 mg kg⁻¹ (2.280 and 6.840 μmol kg⁻¹), respectively. Data, intravenous (closed circles) and oral (open circles) are the mean of three animals.

Table 1. Pharmacokinetic parameters for SB-277011 in rat, dog and monkey following intravenous infusion and oral administration of the hydrochloride salt; SB-277011-A.

Parameter	Rat	Dog	Monkey
Intravenous			
Dose (mg kg ⁻¹)	1.0	1.0	1.0
Terminal phase half-life (h)	2.0 ± 0.1	4.2 ± 0.4	0.7 ± 0.1
Plasma clearance (ml min ⁻¹ kg ⁻¹)	20 ± 2	14 ± 2	58 ± 5
Volume of distribution at steady-state (l kg ⁻¹)	2.9 ± 0.3	4.5 ± 0.4	2.9 ± 0.4
AUC _{0-inf} (μMh)	98.3 ± 10.0	147.1 ± 16.2	36.7 ± 5.2
Oral			
Dose (mg kg ⁻¹)	3.0	3.0	3.0
C _{max} (μM)	0.337 ± 0.036	0.467 ± 0.156	0.030 ± 0.012
T _{max} (h) ^a	1.0 (0.7–1.3)	3.5 (2.7–4.0)	1 ^b
Terminal phase half-life (h)	2.9 ± 0.6	4.6 ± 0.5	ND
F _{ipv} , bioavailability following intrahepatic portal vein administration (%)	41 ± 2 ^c	ND	ND
F _{po} , bioavailability following oral administration (%)	35 ± 1 ^c	43 ± 9 ^d	2 ± 1 ^d

ND, not determined.

^a For T_{max}, the median and range are given.

^b All animals.

^c Bioavailability determined following administration as a 1% (w/v) methylcellulose suspension *aq*.

^d Bioavailability determined following administration of bulk material as a single, loose-filled capsule.

Data are the mean ± SD from three animals.

SB-277011 in monkey was low (2%) and probably as a results of high clearance in this species rather than poor absorption.

In vitro metabolism of SB-277011

The major pathway of metabolism following incubation of SB-277011 with cynomolgus monkey and human liver homogenates, without the presence of an NADPH-generating system, was identified as oxidation on the 4-quinoline ring of SB-277011. The identity of this oxidative metabolite was confirmed by proton NMR spectroscopy and HPLC-MS comparisons of the isolated metabolite from rabbit hepatocyte incubations (data not shown), together with co-chromatography with the synthetic standard, as being SB-369912 (figure 1). No significant metabolites were evident following incubation of SB-277011 with rat and dog liver homogenates.

Enzymology involved in SB-277011 metabolism

The oxidative conversion of SB-277011 to SB-369912, produced predominantly in cynomolgus monkey and human liver homogenate incubations, was investigated for the involvement of aldehyde oxidase. At a substrate concentration of 10 μM SB-277011, isovanillin (100 μM) caused complete inhibition of the formation of SB-369912 in cynomolgus monkey liver homogenates. Radiotracers of cynomolgus monkey liver homogenate incubations in the presence and absence of isovanillin are given in figure 4.

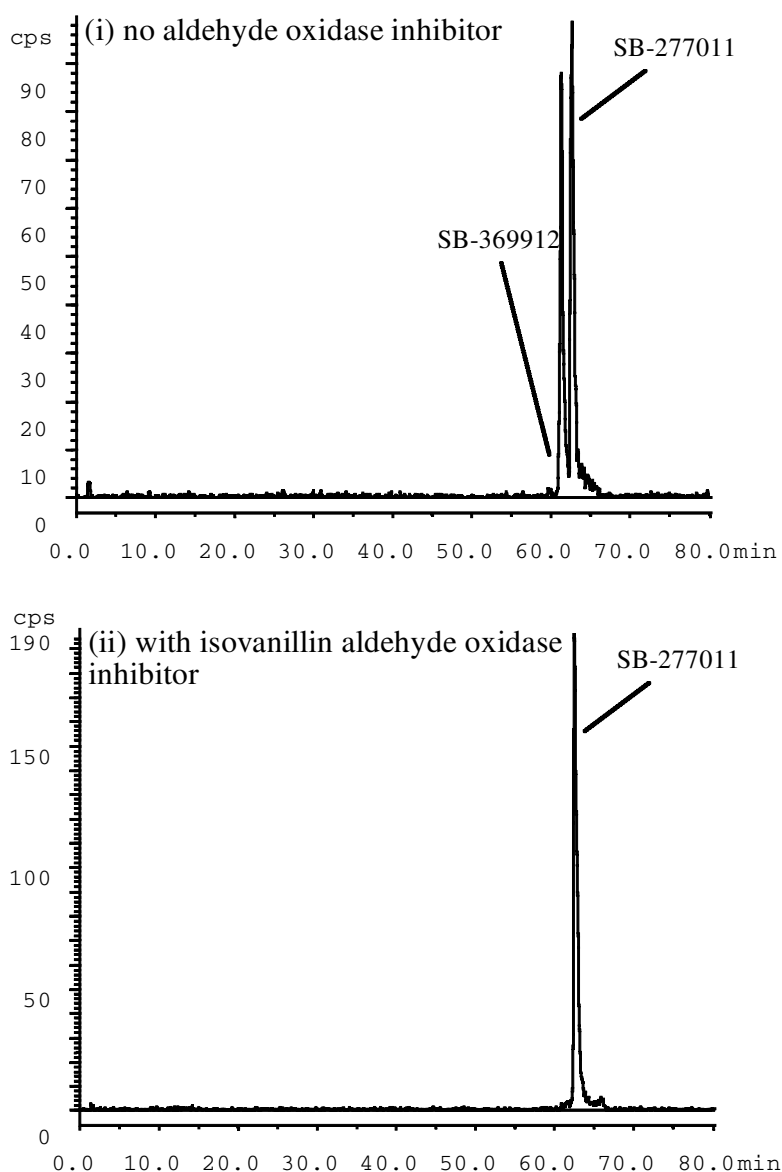


Figure 4. HPLC radiochromatograms of incubations of $[^{14}\text{C}]$ SB-277011-A (10 μM) for 60 min with cynomolgus monkey liver homogenate (i) without isovanillin (aldehyde oxidase inhibitor) and (ii) with isovanillin.

Discussion

The metabolism of the selective dopamine D3 receptor antagonist SB-277011, in the presence of an NADPH-regenerating system with microsomal incubations from rat, dog, cynomolgus monkey and human, appeared relatively stable. However, SB-277011 exhibits species differences in pharmacokinetics following intravenous infusion to rat, dog and cynomolgus monkey which were not predicted from *in vitro* microsomal incubations. SB-277011 has a low clearance in rat, moderate clearance in dog and high clearance in cynomolgus monkey. Following

oral administration, the bioavailability of SB-277011 in the rat and dog was good (~40%) but in cynomolgus monkey oral bioavailability was low (~2%), probably as a result of high first-pass hepatic elimination as opposed to poor absorption.

Since the *in vitro* clearance of SB-277011 in microsomal fractions did not reflect that observed *in vivo*, further investigations were carried out using fresh liver homogenates. In the presence and absence of an NADPH-regenerating system, SB-277011 was rapidly cleared in human and monkey liver homogenate incubations at a rate that was not reflected in hepatic microsomes, where it appeared relatively stable. These experiments would suggest that SB-277011 is metabolized via an NADPH-independent non-microsomal oxidative route and as such a further understanding of the enzymology involved in the metabolism of SB-277011 was required.

The predominant route of metabolism for SB-277011, *in vitro* was demonstrated to be via oxidation on the quinoline ring with the major metabolite being confirmed as SB-369912. An enzyme known to catalyse the oxidation of a wide range of endogenous and exogenous *N*-heterocycles and aldehydes (Beedham 1987a) with different substrate specificity from that of microsomal monooxygenases, such as cytochrome P450 and FMO (Beedham 1985, Yoshihara and Tatsumi 1997), is the cytosolic molybdenum containing hydroxylase enzyme, aldehyde oxidase. Isovanillin is an aldehyde oxidase substrate analogue and can be used to demonstrate the involvement of aldehyde oxidase in the metabolism of compounds (Beedham 1987a). At a concentration of 100 μM , isovanillin caused complete inhibition of the conversion of SB-277011 to SB-369912 in cynomolgus monkey liver homogenate incubations. Taken together, these *in vitro* studies provide strong evidence that aldehyde oxidase is responsible for oxidation of the 4-quinoline ring of SB-277011. The levels of aldehyde oxidase activity apparent in the *in vitro* experiments, namely high in monkey and human liver homogenates and low in rat and dog, also seems to reflect the reported species dependent nature of aldehyde oxidase activity towards different substrates (Stanulovic *et al.* 1971, Krenitsky *et al.* 1974, Beedham 1987a, b, 1998, Beedham *et al.* 1990). As the cynomolgus monkey and human are atypical in this respect, this would suggest that rat and dog are unsuitable for use in predicting the pharmacokinetics of SB-277011 in human.

Thus, based on the combined results from the liver homogenate incubations and pharmacokinetic data obtained from cynomolgus monkey alone, the oral bioavailability of SB-277011 in human is predicted to be low.

Whilst the structure activity relationship of aldehyde oxidase is well established (Beedham 1987a), in our experience not all *N*-heterocyclic containing compounds from the same series as SB-277011 were substrates for aldehyde oxidase suggesting it was not possible to predict whether a compound will be a substrate based on structure alone. Therefore, in the case of SB-277011, as monkey liver homogenates offer the best prospect of measuring aldehyde oxidase activity, potential back-up compounds to SB-277011 were screened using this *in vitro* system prior to *in vivo* pharmacokinetic evaluation.

References

- BALDWIN, S. J., CLARKE, S. E. and CHENERY, R. J., 1999, Characterization of the cytochrome P450 enzymes involved in the *in vitro* metabolism of rosiglitazone. *British Journal of Clinical Pharmacology*, **48**, 424-432.

- BEEDHAM, C., 1985, Molybdenum hydroxylases as drug-metabolizing enzymes. *Drug Metabolism Reviews*, **16**, 119–156.
- BEEDHAM, C., 1987a, Molybdenum hydroxylases: biological distribution and substrate-inhibitor specificity. *Progress in Medicinal Chemistry*, **24**, 85–127.
- BEEDHAM, C., 1987b, Species variation in hepatic aldehyde oxidase activity. *European Journal of Drug Metabolism and Pharmacokinetics*, **12**, 307–310.
- BEEDHAM, C., 1998, Oxidation of carbon via molybdenum hydroxylases. In N. J. Goodman (ed.), *Drug Metabolism: Towards the Next Millennium* (Olmsha: IOS Press), p. 39.
- BEEDHAM, C., BRUCE, S. E., CRITCHLEY, D. J. and RANCE, D. J., 1990, 1-Substituted phthalazines as probes of the substrate-binding site of mammalian molybdenum hydroxylases. *Biochemistry and Pharmacology*, **39**, 1213–1221.
- GRIFFITHS, R., LEWIS, V. A. and JEREY, P., 1996, Models of drug absorption *in situ* and in conscious animals. In R. T. Borchardt *et al.* (eds), *Models for Assessing Drug Absorption and Metabolism* (New York: Plenum).
- IWATSUBO, T., HIROTA, N., OOIE, T., SUZUKI, H., SHIMADA, N., CHIBA, K., ISHIZAKI, T., GREEN, C. E., TYSON, C. A. and SUGIYAMA, Y., 1997, Prediction of *in vivo* drug metabolism in the human liver from *in vitro* metabolism data. *Pharmacology and Therapeutics*, **73**, 147–171.
- KRENITSKY, T. A., TUTTLE, J. V., CATTAN, E. L. and WANG, P., 1974, A comparison of the distribution and electron acceptor specificities of xanthine oxidase and aldehyde oxidase. *Comparative Biochemistry and Physiology*, **49B**, 687–703.
- LEATHERBARROW, R. J., 1992, *Graft 1992*, Version 3.0 (Staines: Erithacus Software).
- REAVILL, C., TAYLOR S. G., WOOD, M. D., ASHMEADE, T., AUSTIN, N. E., AVENELL, K. Y., BOYELD, I., BRANCH, C. L., CLIA, J., COLDWELL, M. C. HADLEY, M. S., HUNTER, A. J., JEFFREY, P., JEWITT, F., JOHNSON, C. N., JONES, D. N. C., MEDHURST A. D., MIDDLEMISS, D. N., NASH, D. J., RILEY, G. J., ROUTLEDGE, C., STEM, G., THEWLIS, K. M., TRAIL, B., VONG, A. K. K. and HAGAN, J. J., 2000, Pharmacological actions of a novel, high-affinity, and selective human dopamine D3 receptor antagonist, SB-277011-A. *Journal of Pharmacology and Experimental Therapeutics*, **294**, 1154–1165.
- SMITH, P. K., KROHN, R. I., HERMANSON, G. T., MALLIA, A. K., GARTNER, F. H., PROVENZANO, M. D., FUJIMOTO, E. K., GOEKE, N. M., OLSON, B. J. and KLENK, C., 1985, Measurement of protein using bicinchoninic acid. *Analytical Biochemistry*, **150**, 76–85.
- STANULOVIC, M. and CHAYKIN, S., 1971, Aldehyde oxidase: catalysis of the oxidation of *N*¹-methylnicotinamide and pyridoxal. *Archives of Biochemistry and Biophysics*, **145**, 27–34.
- YOSHIHARA, S. and TATSUMI, K., 1997, Purification and characterization of hepatic aldehyde oxidase in male and female mice. *Archives of Biochemistry and Biophysics*, **338**, 29–34.

Human liver UDP-glucuronosyltransferase isoforms involved in the glucuronidation of 7-ethyl-10-hydroxycamptothecin

N. HANIOKA^{†‡*}, S. OZAWA[‡], H. JINNO[†],
M. ANDO[†], Y. SAITO[‡] and J. SAWADA[‡]

[†] Division of Environmental Chemistry and [‡] Project Team for Pharmacogenetics, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan

Received 5 February 2001

1. The human liver UDP-glucuronosyltransferase (UGT) isoforms involved in the glucuronidation of 7-ethyl-10-hydroxycamptothecin (SN-38), the active metabolite of irinotecan (CPT-11), have been studied using microsomes from human liver and insect cells expressing human UGTs (1A1, 1A3, 1A4, 1A6, 1A9, 2B7, 2B15).

2. The glucuronidation of SN-38 was catalysed by UGT1A1, UGT1A3, UGT1A6 and UGT1A9 as well as by liver microsomes. Among these UGT isoforms, UGT1A1 showed the highest activity of SN-38 glucuronidation at both low (1 μM) and high (200 μM) substrate concentrations. The ranking in order of activity at low and high substrate concentrations was UGT1A1 > UGT1A9 > UGT1A6 > UGT1A3 and UGT1A1 > UGT1A3 > UGT1A6 $\geq$ UGT1A9, respectively.

3. The enzyme kinetics of SN-38 glucuronidation were examined by means of Lineweaver–Burk analysis. The activity of the glucuronidation in liver microsomes exhibits a monophasic kinetic pattern, with an apparent K_m and $V_{\max}$ of 35.9 μM and 134 $\text{pmol min}^{-1} \text{mg}^{-1}$ protein, respectively. The UGT isoforms involved in SN-38 glucuronidation could be classified into two types: low- K_m types such as UGT1A1 and UGT1A9, and high- K_m types such as UGT1A3 and UGT1A6, in terms of affinity toward substrate. UGT1A1 had the highest $V_{\max}$ followed by UGT1A3. $V_{\max}$ of UGT1A6 and UGT1A9 were approximately 1/9 to 1/12 of that of UGT1A1.

4. The activity of SN-38 glucuronidation by liver microsomes and UGT1A1 was effectively inhibited by bilirubin. Planar and bulky phenols substantially inhibited the SN-38 glucuronidation activity of liver microsomes and UGT1A9, and/or UGT1A6. Although cholic acid derivatives strongly inhibited the activity of SN-38 glucuronidation by UGT1A3, the inhibition profile did not parallel that in liver microsomes.

5. These results demonstrate that at least four UGT1A isoforms are responsible for SN-38 glucuronidation in human livers, and suggest that the role and contribution of each differ substantially.

Introduction

Irinotecan (7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxycamptothecin, CPT-11) (figure 1), a water-soluble chemical derivative of camptothecin with a heterocyclic ring structure, is an alkaloid originally extracted from the Chinese tree *Camptotheca acuminata* (Sawada *et al.* 1991, Slichenmyer *et al.* 1994). CPT-11 is currently registered for use in Japan, Europe and the USA in patients with metastatic colorectal cancer refractory to several first-line therapies including

* Author for correspondence; e-mail: hanioka@nihs.go.jp

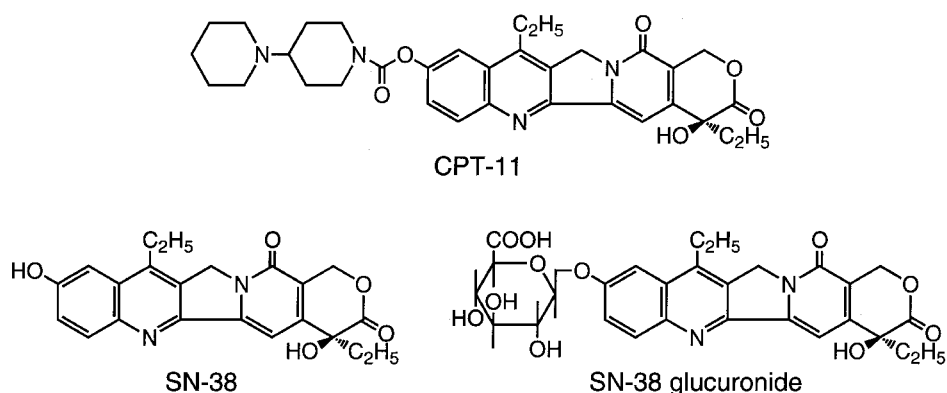


Figure 1. Chemical structures of CPT-11, SN-38 and SN-38 glucuronide.

5-fluorouracil, and it shows clinical activity against several other types of solid tumours (Cunningham *et al.* 1998, Rougier *et al.* 1998).

The biological activity of CPT-11 results from its capacity to bind to the transient cleavable complex formed between DNA and topoisomerase I, a nuclear protein involved in DNA replication, preventing dissociation of the DNA-topoisomerase I complex and thereby inhibiting enzyme activity (Jaxel *et al.* 1989, Creemers *et al.* 1994, Vassal *et al.* 1996). CPT-11 is converted by carboxylesterases in the liver and small intestine to an active metabolite, 7-ethyl-10-hydroxycamptothecin (SN-38) (figure 1), which has a 100–1000-fold higher antitumor activity than the parent CPT-11 (Kawato *et al.* 1991, Rivory *et al.* 1996, Zamboni *et al.* 1998). SN-38 is further conjugated by hepatic UDP-glucuronosyltransferases (UGTs, EC 2.4.1.17) to an inactive β -glucuronide derivative (figure 1), and excreted into bile and urine (Lokiec *et al.* 1995, Haaz *et al.* 1997, Iyer *et al.* 1998). SN-38 glucuronide can be deconjugated to form SN-38 by intestinal bacterial β -glucuronidase (Kaneda *et al.* 1990, Takasuna *et al.* 1996).

It has been reported that one of the major side effects of CPT-11 is severe diarrhoea, and that the toxicity is due to an accumulation of SN-38 in the intestine (Araki *et al.* 1993, Wiseman and Markham 1996). Furthermore, SN-38 glucuronidation rates inversely correlate with the severity of diarrhoea in patients treated with increasing doses of CPT-11 (Gupta *et al.* 1994). These findings suggest that glucuronidation of SN-38 protects against the side-effects of CPT-11. Therefore, the conversion of SN-38 to SN-38 glucuronide by hepatic UGTs is a key pathway in the sequential metabolic pathway of CPT-11. UGTs are endoplasmic reticulum-bound enzymes that catalyse glucuronidation of endobiotic and xenobiotic lipid-soluble compounds into water-soluble and excretable compounds (Dutton 1980, Burchell and Coughtrie 1989). More than 30 hepatic UGT isoforms have been identified from various mammalian species by protein purification or cDNA cloning, and classified into two families of protein termed UGT1 and UGT2 on the basis of primary amino acid sequences (Mackenzie *et al.* 1997, Burchell *et al.* 1998). In humans, UGT1 family isoforms are encoded by one gene that has multiple unique exons located upstream of four common exons. By contrast, UGT2 family isoforms are encoded by separate genes, each comprised of six exons (Clarke and Burchell 1994, Burchell *et al.* 1995, Mackenzie *et al.* 1997).

UGT1A1 has been regarded to be the predominant isoform in SN-38 glucuronidation, since Iyer *et al.* (1998) reported that the formation rate of SN-38 glucuronide is significantly correlated with UGT1A1 activity (bilirubin glucuronidation) in human liver microsomes, and that the level of SN-38 glucuronidation activity is higher in human embryonic kidney cells transfected with UGT1A1 than with UGT1A4 and UGT2B7. UGT1A7, which is expressed only in gastric tissue (Strassburg *et al.* 1997, Tukey and Strassburg 2000), has shown to be capable of glucuronidating SN-38 using a heterologous cell expression system (Ciotti *et al.* 1999). The substrate specificity of mammalian UGTs has been suggested to be broad (Burchell *et al.* 1995, Radomska-Pandya *et al.* 1999, de Wildt *et al.* 1999). Furthermore, the liver is quantitatively the most important site of glucuronidation for the majority of compounds (Dutton 1980, Burchell and Coughtrie 1989). However, little is known about the involvement of hepatic UGT isoforms except for UGT1A1 in the SN-38 glucuronidation in humans.

The purpose of this study was to determine the capability of human liver UGT1A and UGT2B isoforms to glucuronidate SN-38, and to evaluate possible roles of UGT isoforms other than UGT1A1. To achieve this, the kinetics and inhibition of SN-38 glucuronidation were examined in insect cells expressing specific hepatic isoforms of human UGTs as well as in human liver microsomes.

Materials and methods

Materials

SN-38 (Lot 970507R) and SN-38 glucuronide (Lot 970326) were kindly supplied by Yakult Honsha Co. (Tokyo, Japan). The purity ($\geq 99\%$) of each compound was confirmed by analytical HPLC. Bilirubin, thyroxine, 2-hydroxyestriol, hydoxychoic acid, 4-methylumbelliferone (sodium salt), esculetin, imipramine (hydrochloride), 4-nitrophenol and valproic acid (sodium salt) were obtained from Sigma Chemical Co. (St Louis, MO, USA). Lithocholic acid, octyl gallate, naringenin, 2-aminofluorene, paracetamol, 4-propylphenol and 4-hydroxybiphenyl were obtained from Aldrich Chemical Co. (Milwaukee, WI, USA). 2-Hydroxyestradiol, 4-hydroxyestradiol, 2-hydroxyestrone and 4-hydroxyestrone were purchased from ICN Pharmaceuticals (Costa Mesa, CA, USA). Estriol, 17 β -estradiol, estrone, dihydrotestosterone and ethynylestradiol were obtained from Wako Pure Chemical Industries (Osaka, Japan). 7-Hydroxyflavone, 4-*t*-butylphenol and 4-*t*-pentylphenol were from Tokyo Kasei Kogyo Co. (Tokyo, Japan), and 1-naphthol and 2-naphthol were from Nacalai Tesque (Kyoto, Japan). UDP-glucuronic acid (trisodium salt) was a product of Yamasa Shoyu Co. (Choshi, Japan). All other chemicals and HPLC solvents were of the highest quality commercially available. Pooled human liver microsomes from donors (five males, 23–59 years old; five females, 21–61 years old) and human recombinant UGTs (1A1, 1A3, 1A4, 1A6, 1A9, 2B7 and 2B15) expressed in microsomes of insect cells infected with baculovirus were purchased from Gentest (Woburn, MA, USA). The protein contents were as described in the data sheets provided by the manufacturer. The expression levels of the UGT1A isoforms were checked by immunoblot analysis using anti-human UGT1A antibody (Gentest) according to Galijatovic *et al.* (2000). No notable differences in the expression levels among the UGT1A isoforms were observed (data not shown). Therefore, the SN-38 glucuronidation activity of recombinant UGTs was assessed as per microsomal protein of insect cells.

Assay for SN-38 glucuronidation

SN-38 glucuronidation activity was determined by measurement of the rate of formation of SN-38 glucuronide, as described (Hanioka *et al.* 2001) with slight modification. The standard incubation mixture contained 1–200 μ M SN-38, 100 μ g ml⁻¹ microsomal protein of livers or recombinant UGTs, 10 mM MgCl₂ and 5 mM UDP-glucuronic acid in a final volume of 400 μ l 50 mM Tris-HCl buffer (pH 7.4). SN-38 was dissolved in dimethyl sulphoxide/0.05 N NaOH (50:50). After pre-incubation at 37°C for 1 min, the reaction was started by the addition of UDP-glucuronic acid. The mixture was incubated at 37°C for 40 min and the reaction stopped with 100 μ l 10% (w/v) perchloric acid and vortexing. The samples were centrifuged at 12 000 g for 10 min at 4°C, and the supernatant was analysed by HPLC with an Inertsil ODS-80A column (5 μ m, 150 $\times$ 4.6 mm i.d., GL Sciences, Tokyo, Japan). The elution was conducted with a mixture of 50 mM KH₂PO₄ containing 3 mM sodium 1-octanesul-

phonate (pH 2.5)–acetonitrile–methanol (72:22:6 v/v/v) at a flow rate of 1.0 ml min^{-1} under isocratic conditions, and the detection was based on fluorescence intensity at excitation 370 nm and emission 425 nm.

Kinetic analysis

The Michaelis–Menten parameters (apparent K_m and $V_{\max}$) for SN-38 glucuronidation were estimated by analysing Lineweaver–Burk plots using the software EnzymeKinetics v.1.4 (Trinity Software, Campton, NH, USA). The substrate concentration was 1–200 μM .

Inhibition analysis

Thirty compounds (13 endogenous and 17 exogenous UGT substrates) were tested for their inhibitory effects on SN-38 glucuronidation. The compounds were dissolved in dimethyl sulphoxide/0.05 N NaOH (50:50), with the solvent being used as the control. The final concentration of dimethyl sulphoxide in the reaction medium including the solvent for substrate was 1% (v/v). The compound concentrations for IC_{25} and IC_{50} determinations in human liver microsomes were 0.2–100 μM . IC_{25} and IC_{50} were estimated graphically. For the determination of the inhibitory effects of various UGT substrates on recombinant UGTs, the compound concentrations were chosen at IC_{25} – IC_{50} in liver microsomes. The substrate concentration was set at or near the K_m for SN-38 glucuronidation with each enzyme source (40 μM for liver microsomes, 25 μM for UGT1A1, 150 μM for UGT1A3, 100 μM for UGT1A6 and 15 μM for UGT1A9).

Results

General properties of SN-38 glucuronidation

Microsomes of human livers and insect cells expressing human UGTs (1A1, 1A3, 1A4, 1A6, 1A9, 2B7, 2B15) were used to examine SN-38 glucuronidation. The formation of SN-38 glucuronide was linear with respect to a 80-min incubation in the presence of 100 μg microsomal protein ml^{-1} in Hanioka *et al.* (2001). Therefore, a 40-min incubation was routinely used in this study. When UDP-glucuronic acid was omitted from the incubation mixture, no formation of metabolite was detectable for SN-38 glucuronide with any enzyme source (data not shown). Figure 2 shows the activities of SN-38 glucuronidation at low (1 μM) and high (200 μM) substrate concentrations. The specific activities in liver microsomes at low and high substrate concentrations were 3.12 and 98.9

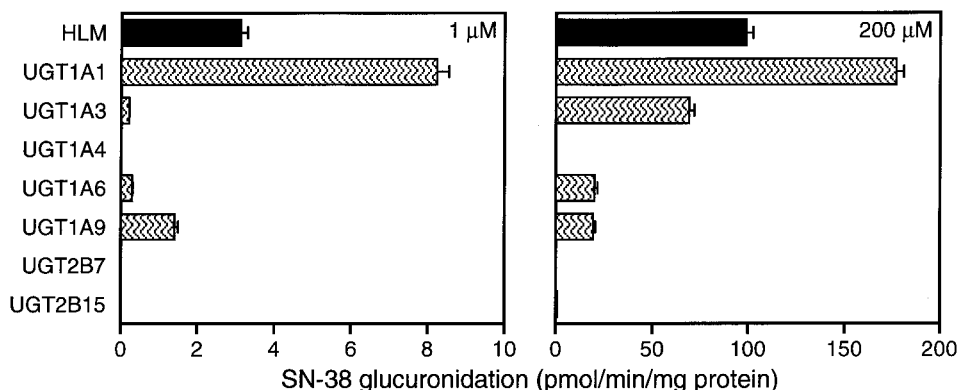


Figure 2. SN-38 glucuronidation activity in microsomes from human livers and insect cells expressing human UGTs at low (1 μM) and high (200 μM) substrate concentrations. Experimental conditions are described in the Materials and methods. Each bar represents the mean $\pm$ SD of three separate experiments performed in duplicate. HLM, human liver microsomes.

pmol min⁻¹ mg⁻¹ protein, respectively. Among the recombinant UGTs examined, UGT1A1 showed the highest activity at both substrate concentrations, the activities being higher than those of liver microsomes. UGT1A3, UGT1A6 and UGT1A9 were also capable of catalysing SN-38 glucuronidation at low and high substrate concentrations. However, the SN-38 glucuronidation activities of UGT1A4, UGT2B7 and UGT2B15 were very low or negligible at both substrate concentrations. The ranking in order of SN-38 glucuronidation activity of recombinant UGTs at low and high substrate concentrations was UGT1A1 > UGT1A9 > UGT1A6 > UGT1A3 and UGT1A1 > UGT1A3 > UGT1A6 ≥ UGT1A9, respectively.

Kinetic analysis of SN-38 glucuronidation

SN-38 glucuronidation in microsomes of human livers and insect cells expressing human UGTs followed Michaelis–Menten kinetics as demonstrated by Lineweaver–Burk plots and the curves were all linear ($r = 0.98$ – 1.00) (data not shown). The apparent kinetic constants for SN-38 glucuronidation are shown in table 1. The activity of SN-38 glucuronidation in liver microsomes exhibited a monophasic kinetic pattern, the K_m , V_{max} and V_{max}/K_m being 35.9 μ M, 134 pmol min⁻¹ mg⁻¹ protein and 3.73 μ l min⁻¹ mg⁻¹ protein, respectively. Of the seven recombinant UGTs examined, UGT1A1 showed the highest V_{max} (256 pmol min⁻¹ mg⁻¹ protein) in catalysing SN-38 glucuronidation with a K_m of 23.8 μ M. V_{max} (132 pmol min⁻¹ mg⁻¹ protein) for UGT1A3 was the next highest, and K_m for UGT1A3 was 6.2-fold of that of UGT1A1. V_{max} of UGT1A6 and UGT1A9 were similar (27.6 and 20.5 pmol min⁻¹ mg⁻¹ protein, respectively). However, K_m differed markedly between these two UGT isoforms, that for UGT1A9 (13.4 μ M) being approximately 1/7 that for UGT1A6. On the other hand, the affinity of UGT2B15 for SN-38 glucuronidation was very low with a K_m of 186 μ M and a V_{max} of 1.63 pmol min⁻¹ mg⁻¹ protein. As a result, the V_{max}/K_m value was substantially < 0.10 μ l min⁻¹ mg⁻¹ protein. Because of the quantitation limit for SN-38 glucuronide, it was not possible to calculate the apparent kinetic constants for SN-38 glucuronidation of UGT1A4 and UGT2B7.

Table 1. Michaelis–Menten parameters for SN-38 glucuronidation in microsomes from human livers and insect cells expressing human UGTs.

Enzyme	K_m (μ M)	(pmol min ⁻¹ mg ⁻¹ protein)	(μ l min ⁻¹ mg ⁻¹ protein)
HLM ^a	35.9 ± 2.6	134 ± 10	3.73
UGT1A1	23.8 ± 2.3	256 ± 19	10.8
UGT1A3	147 ± 12	132 ± 12	0.90
UGT1A ^b		< 0.20	
UGT1A6	97.2 ± 7.9	27.6 ± 2.4	0.28
UGT1A9	13.4 ± 1.3	20.5 ± 1.5	1.53
UGT2B7 ^b		< 0.20	
UGT2B15	186 ± 16	1.63 ± 0.10	< 0.10

Experimental conditions are described in the Materials and methods. Each value represents the mean ± SD of three separate experiments performed in duplicate from Lineweaver–Burk plots.

^a HLM, human liver microsomes.

^b Activities of UGT1A4 and UGT2B7 were lower than determination limit (0.20 pmol min⁻¹ mg⁻¹ protein) at all substrate concentrations.

Inhibition of SN-38 glucuronidation by various UGT substrates

These assays were performed with a substrate concentration at or near the K_m for each enzyme source obtained in the kinetic analysis. The effects of various endogenous and exogenous UGT substrates on SN-38 glucuronidation activity in human liver microsomes are shown in tables 2 and 3 along with the IC_{25} and IC_{50} determined over a broad concentration range for each compound. Also, the inhibitory effects of the compounds at a single concentration in microsomes of

Table 2. IC_{25} and IC_{50} for endogenous UGT substrates for SN-38 glucuronidation activity in human liver microsomes.

Compound	Specificity ^a	IC_{25} (μM)	IC_{50} (μM)
Bilirubin	1A1	0.61 ± 0.12	2.86 ± 0.46
Thyroxine	1A1, 1A9	54.2 ± 3.7	> 100
Estriol	1A1, 1A9, 2B4, 2B7, 2B11	32.3 ± 1.8	> 100
2-Hydroxyestriol	1A1	19.1 ± 2.5	73.1 ± 5.8
17 β -Estradiol	1A1, 1A9	66.9 ± 3.3	> 100
2-Hydroxyestradiol	1A1, 1A3, 1A8, 1A9	7.52 ± 0.84	15.4 ± 1.7
4-Hydroxyestradiol	1A8, 1A9, 2B7	4.79 ± 0.68	10.2 ± 1.1
Estrone	1A3, 1A8, 1A9	> 100	> 100
2-Hydroxyestrone	1A1, 1A3, 1A8, 1A9, 1A10, 2B7	6.11 ± 0.74	11.1 ± 0.9
4-Hydroxyestrone	1A8, 1A9, 1A10, 2B7, 2B11	4.73 ± 0.44	13.5 ± 0.6
Lithocholic acid	1A3, 1A6, 1A9, 2B7	> 100	> 100
Hyodeoxycholic acid	1A3, 1A9, 2B4, 2B7	20.9 ± 1.7	> 100
Dihydrotestosterone	1A8, 1A10, 2B15, 2B17	> 100	> 100

Experimental conditions are described in the Materials and methods. Each value represents the mean $\pm$ SD of three separate experiments performed in duplicate. Control activity was 71.2 ± 3.0 pmol min⁻¹ mg⁻¹ protein.

^a Isoform specificity of human UGTs is from Burchell *et al.* (1995), Radominska-Pandya *et al.* (1999), Tukey and Strassburg (2000) and de Wildt *et al.* (2000).

Table 3. IC_{25} and IC_{50} for exogenous UGT substrates for SN-38 glucuronidation activity in human liver microsomes.

Compound	Specificity ^a	IC_{25} (μM)	IC_{50} (μM)
Ethinylestradiol	1A1, 1A9	17.3 ± 1.0	37.0 ± 2.6
Octyl gallate	1A1, 1A9	6.04 ± 0.82	15.0 ± 1.7
Naringenin	1A1, 1A3, 1A8, 1A9, 2B15	3.89 ± 0.48	10.1 ± 0.9
1-Naphthol	1A1, 1A6, 1A8, 1A9	9.41 ± 0.97	> 100
2-Naphthol	1A1, 1A6, 1A8, 1A9	36.6 ± 4.6	> 100
2-Aminofluorene	1A3, 1A4, 1A9	30.7 ± 2.0	85.3 ± 7.0
7-Hydroxyflavone	1A1, 1A3, 1A9	3.54 ± 0.61	11.1 ± 1.0
4-Methylumbelliferone	1A3, 1A6, 1A8, 1A9, 2B7, 2B15	26.2 ± 2.2	88.3 ± 3.6
Esculetin	1A3, 1A9, 2B15	16.4 ± 1.3	> 100
Imipramine	2A3, 1A4	> 100	> 100
4- <i>t</i> -Butylphenol	1A6, 1A8, 1A9	80.4 ± 3.1	> 100
4-Nitrophenol	1A6, 1A9	67.1 ± 8.8	> 100
Paracetamol	1A6, 1A9	> 100	> 100
4- <i>t</i> -Pentylphenol	1A9	76.3 ± 2.5	> 100
4-Propylphenol	1A9	58.0 ± 4.7	> 100
Valproic acid	2B7	> 100	> 100
4-Hydroxybiphenyl	2B15	12.8 ± 1.4	47.1 ± 4.1

Experimental conditions are described in the Materials and methods. Each value represents the mean $\pm$ SD of three separate experiments performed in duplicate. Control activity was 71.2 ± 3.0 pmol min⁻¹ mg⁻¹ protein.

^a Isoform specificity of human UGTs is from Burchell *et al.* (1995), Radominska-Pandya *et al.* (1999), Tukey and Strassburg (2000) and de Wildt *et al.* (2000).

human livers and insect cells expressing human UGTs such as UGT1A1, UGT1A3, UGT1A6 and UGT1A9 are shown in tables 4 and 5. Bilirubin effectively inhibited SN-38 glucuronidation activities of liver microsomes and UGT1A1 by 42.0 and 60.3% at 2 μM , respectively. However, the inhibitory effect was weak for UGT1A3, UGT1A6 and UGT1A9. The inhibition profiles of SN-

Table 4. Effect of endogenous UGT substrates on SN-38 glucuronidation activity in microsomes from human livers and insect cells expressing human UGTs.

Compound	Residual activity (%)				
	HLM	UGT1A1	UGT1A3	UGT1A6	UGT1A9
Bilirubin (2 μM)	58.0 $\pm$ 4.3	39.7 $\pm$ 1.3	97.9 $\pm$ 8.3	97.6 $\pm$ 2.7	98.0 $\pm$ 4.2
Thyroxine (100 μM)	63.3 $\pm$ 3.2	46.1 $\pm$ 4.0	92.2 $\pm$ 4.6	87.5 $\pm$ 3.1	82.2 $\pm$ 2.1
Estrilol (50 μM)	63.1 $\pm$ 4.2	77.1 $\pm$ 3.1	84.4 $\pm$ 3.6	83.9 $\pm$ 4.3	48.7 $\pm$ 1.0
2-Hydroxyestriol (50 μM)	57.0 $\pm$ 1.3	52.1 $\pm$ 4.0	60.0 $\pm$ 3.6	61.2 $\pm$ 1.6	35.5 $\pm$ 2.3
17 β -Estradiol (100 μM)	65.2 $\pm$ 3.8	54.4 $\pm$ 1.0	103 $\pm$ 6	55.5 $\pm$ 2.9	14.6 $\pm$ 0.8
2-Hydroxyestradiol (10 μM)	60.5 $\pm$ 4.4	48.8 $\pm$ 2.5	82.9 $\pm$ 3.4	86.4 $\pm$ 5.8	60.5 $\pm$ 3.1
4-Hydroxyestradiol (10 μM)	50.3 $\pm$ 2.9	34.4 $\pm$ 2.3	75.5 $\pm$ 2.0	84.2 $\pm$ 6.7	63.1 $\pm$ 1.3
Estrone (100 μM)	91.1 $\pm$ 6.7	95.2 $\pm$ 8.6	88.8 $\pm$ 5.9	89.2 $\pm$ 5.1	73.3 $\pm$ 2.2
2-Hydroxyestrone (10 μM)	52.0 $\pm$ 4.2	42.1 $\pm$ 2.9	87.4 $\pm$ 7.2	80.8 $\pm$ 6.1	81.1 $\pm$ 1.7
4-Hydroxyestrone (10 μM)	56.6 $\pm$ 1.4	38.3 $\pm$ 1.1	72.8 $\pm$ 3.2	81.5 $\pm$ 6.4	69.5 $\pm$ 4.8
Lithocholic acid (10 μM)	91.5 $\pm$ 7.3	59.2 $\pm$ 0.9	16.8 $\pm$ 0.9	7.36 $\pm$ 0.15	22.2 $\pm$ 1.6
Hyodeoxycholic acid (100 μM)	51.4 $\pm$ 3.6	62.1 $\pm$ 3.2	17.4 $\pm$ 0.4	74.1 $\pm$ 5.5	30.5 $\pm$ 2.5
Dihydrotestosterone (100 μM)	147 $\pm$ 10	175 $\pm$ 8	65.3 $\pm$ 4.5	89.1 $\pm$ 8.2	66.0 $\pm$ 1.9

Experimental conditions are described in the Materials and methods. Each value represents the mean $\pm$ SD of three separate experiments performed in duplicate. Control activities (pmol min⁻¹ mg⁻¹ protein) were: HLM (human liver microsomes), 71.2 $\pm$ 3.0; UGT1A1, 141 $\pm$ 7; UGT1A3, 63.4 $\pm$ 4.1; UGT1A6, 14.3 $\pm$ 0.8; and UGT1A9, 10.2 $\pm$ 0.3.

Table 5. Effect of exogenous UGT substrates on SN-38 glucuronidation activity in microsomes from human livers and insect cells expressing human UGTs.

Compound	Residual activity (%)				
	HLM	UGT1A1	UGT1A3	UGT1A6	UGT1A9
Ethynylestradiol (20 μM)	68.9 $\pm$ 2.0	52.7 $\pm$ 1.0	64.6 $\pm$ 4.4	64.1 $\pm$ 1.9	34.2 $\pm$ 2.5
Octyl gallate (10 μM)	58.9 $\pm$ 2.4	31.6 $\pm$ 2.2	82.0 $\pm$ 7.5	93.1 $\pm$ 7.1	75.2 $\pm$ 1.7
Naringenin (10 μM)	50.1 $\pm$ 1.4	34.0 $\pm$ 2.9	96.3 $\pm$ 8.7	96.4 $\pm$ 4.1	68.6 $\pm$ 5.0
1-Naphthol (20 μM)	56.0 $\pm$ 5.0	101 $\pm$ 8	120 $\pm$ 9	70.0 $\pm$ 2.4	13.9 $\pm$ 1.1
2-Naphthol (100 μM)	54.0 $\pm$ 1.9	134 $\pm$ 7	122 $\pm$ 4	55.8 $\pm$ 4.0	14.8 $\pm$ 1.4
2-Aminofluorene (50 μM)	59.3 $\pm$ 5.1	71.9 $\pm$ 5.1	68.4 $\pm$ 5.7	76.0 $\pm$ 2.8	50.9 $\pm$ 3.9
7-Hydroxyflavone (10 μM)	51.8 $\pm$ 2.0	35.1 $\pm$ 1.8	57.8 $\pm$ 4.1	95.0 $\pm$ 7.5	63.9 $\pm$ 3.6
4-Methylumbelliferone (50 μM)	62.3 $\pm$ 4.9	98.6 $\pm$ 7.3	86.9 $\pm$ 1.4	85.6 $\pm$ 7.5	52.7 $\pm$ 2.6
Esculetin (50 μM)	58.3 $\pm$ 4.2	81.8 $\pm$ 5.7	91.6 $\pm$ 6.9	84.8 $\pm$ 6.1	64.5 $\pm$ 4.5
Imipramine (100 μM)	123 $\pm$ 11	110 $\pm$ 9	77.1 $\pm$ 2.0	125 $\pm$ 4	93.6 $\pm$ 6.3
4- <i>t</i> -Butylphenol (100 μM)	70.6 $\pm$ 4.6	121 $\pm$ 4	93.9 $\pm$ 2.1	81.7 $\pm$ 4.9	27.0 $\pm$ 1.6
4-Nitrophenol (100 μM)	66.2 $\pm$ 5.7	105 $\pm$ 6	91.9 $\pm$ 6.9	88.4 $\pm$ 2.4	60.3 $\pm$ 3.9
Paracetamol (100 μM)	95.6 $\pm$ 2.2	98.2 $\pm$ 2.5	108 $\pm$ 9	104 $\pm$ 8	90.6 $\pm$ 4.3
4- <i>t</i> -Pentylphenol (100 μM)	72.7 $\pm$ 3.5	86.9 $\pm$ 6.0	73.2 $\pm$ 1.6	68.9 $\pm$ 2.6	36.3 $\pm$ 3.0
4-Propylphenol (100 μM)	61.2 $\pm$ 2.7	90.1 $\pm$ 7.9	102 $\pm$ 4	85.4 $\pm$ 2.1	40.2 $\pm$ 3.3
Valproic acid (100 μM)	98.7 $\pm$ 5.8	99.3 $\pm$ 5.8	99.6 $\pm$ 1.7	97.9 $\pm$ 7.8	96.3 $\pm$ 4.6
4-Hydroxybiphenyl (20 μM)	64.8 $\pm$ 4.7	68.2 $\pm$ 4.9	102 $\pm$ 8	99.7 $\pm$ 1.6	81.4 $\pm$ 4.9

Experimental conditions are described in the Materials and methods. Each value represents the mean $\pm$ SD of three separate experiments performed in duplicate. Control activities (pmol min⁻¹ mg⁻¹ protein) were: HLM (human liver microsomes), 71.2 $\pm$ 3.0; UGT1A1, 141 $\pm$ 7; UGT1A3, 63.4 $\pm$ 4.1; UGT1A6, 14.3 $\pm$ 0.8; and UGT1A9, 10.2 $\pm$ 0.3.

38 glucuronidation activity for thyroxine, 2-hydroxyestrone, 4-hydroxyestrone, octyl gallate and naringenin were similar to that for bilirubin, although the IC_{25} and IC_{50} were markedly different among the compounds. By contrast, lithocholic acid and hyodeoxycholic acid inhibited the SN-38 glucuronidation activity of UGT1A3, UGT1A6 or UGT1A9 by 69.5–92.6%, the inhibition potency being higher than for liver microsomes and UGT1A1. The activity of SN-38 glucuronidation of UGT1A9 was inhibited >70% by 17β -estradiol, 1-naphthol, 2-naphthol and 4-*t*-butylphenol. 2-Hydroxyestriol, ethynylestradiol, 2-aminofluorene, 4-methylumbelliferone, 4-*t*-pentylphenol and 4-propylphenol also effectively inhibited UGT1A9 rather than other UGT isoforms. The IC_{25} of SN-38 glucuronidation activity in liver microsomes for estrone, imipramine and paracetamol was >100 μ M, and no effective inhibition were observed.

Discussion

Although CPT-11 is a useful anticancer agent for numerous malignancies such as colon and lung cancers (Cunningham *et al.* 1998, Rougier *et al.* 1998), it can cause severe diarrhoea due to an accumulation of SN-38, the active metabolite of CPT-11, in the intestinal tissues (Araki *et al.* 1993, Gupta *et al.* 1994, Wiseman and Markham 1996). It has been reported that SN-38 is efficiently metabolized by UGT1A isoforms such as UGT1A1 and UGT1A7 to an inactive metabolite, SN-38 glucuronide (Iyer *et al.* 1998, Ciotti *et al.* 1999). The substrate specificity of human UGTs is generally broad (Burchell *et al.* 1995, Radomska-Pandya *et al.* 1999, de Wildt *et al.* 1999), and several genetic polymorphisms have been recently identified in the UGT family (Mackenzie *et al.* 1997, Burchell *et al.* 1998, de Wildt *et al.*, 1999, Tukey and Strassburg 2000). Glucuronidation is an important detoxification pathway for SN-38, and information on the role of human liver UGT isoforms in SN-38 glucuronidation is beneficial to medicine. In the current study, the glucuronidation of SN-38 in microsomes of human livers and insect cells expressing human UGTs was investigated using kinetic and inhibition analyses.

A number of approaches have been developed for identification of human UGTs involved in the glucuronidation of endogenous and exogenous compounds *in vitro* (Ritter 2000, Tukey and Strassburg 2000). The metabolism of SN-38 to SN-38 glucuronide by several insect cell microsomes expressing human UGTs as well as human liver microsomes was determined. The UGT isoforms examined in this study are all expressed in human liver (Strassburg *et al.* 1997, Tukey and Strassburg 2000). UGT1A1 showed the highest glucuronidation activity at low and high substrate concentrations among the UGT isoforms examined. UGT1A3, UGT1A6 and UGT1A9 also catalysed SN-38 glucuronidation, however, the formation rates were different between low and high substrate concentrations. The most typical case was that of UGT1A3, whose activity toward SN-38 was 2.67% to that of UGT1A1 at low substrate concentration, but 39.0% at high substrate concentration. On the other hand, the activities for SN-38 glucuronidation of UGT1A4, UGT2B7 and UGT2B15 were very low or negligible at both substrate concentrations. These findings suggest that multiple UGT1A isoforms are involved in the glucuronidation of SN-38 in human liver, and that the affinity toward SN-38 differs among the UGT isoforms.

To clarify the affinity of UGT enzymes for SN-38, we performed kinetic analyses for SN-38 glucuronidation in microsomes of livers and insect cells expressing human UGTs using Lineweaver–Burk plots. The K_m of UGT1A1 for SN-38 glucuronidation was lower than that in liver microsomes, and the V_{max} was the highest among those of other UGT isoforms. The observation that UGT1A1 has both high activity and affinity toward SN-38 glucuronidation agrees with the data of Iyer *et al.* (1998). Not only the V_{max} , but also the K_m for SN-38 glucuronidation of UGT1A3 was relatively high, the affinity of UGT1A3 toward substrate being low compared with that of UGT1A1. UGT1A6 was also involved in SN-38 glucuronidation. However, its V_{max}/K_m was only 2.59% of that of UGT1A1. Although V_{max} of UGT1A9 was lower than those of UGT1A1, UGT1A3 and UGT1A6, the affinity toward substrate was the highest among the UGT isoforms which contribute to SN-38 glucuronidation. UGT2B15 was shown to have a high K_m and low V_{max} for SN-38 glucuronidation, so its contribution may be small. Thus, the UGT isoforms involved in SN-38 glucuronidation could be classified into two types on the basis of affinity toward substrate: low- K_m types such as UGT1A1 and UGT1A9, and high- K_m types such as UGT1A3, UGT1A6 and UGT2B15. Although it is not known why the kinetic pattern for SN-38 glucuronidation in liver microsomes was monophasic, it should be mentioned that the contribution of UGT1A1 was large in the microsomal samples used.

We further performed an inhibition analysis using various endogenous and exogenous UGT substrates to examine about the contribution of UGT1A1, UGT1A3, UGT1A6 and UGT1A9 isoforms to SN-38 glucuronidation. Bilirubin is known to be a specific substrate of UGT1A1 (Burchell *et al.* 1995, Radomska-Pandya *et al.* 1999, Tukey and Strassburg 2000, de Wildt *et al.* 2000). In this study, bilirubin (2 μ M) effectively inhibited SN-38 glucuronidation by recombinant UGT1A1 as well as liver microsomes, but not UGT1A3, UGT1A6 and UGT1A9. Also, SN-38 glucuronidation activities in liver microsomes and recombinant UGT1A1 were selectively inhibited by exogenous UGT1A1 substrates such as octyl gallate and naringenin. Previous studies have shown that UGT1A9 is capable of glucuronidating a wide variety of substrates including estrogens, drugs, phenols and flavonoids (Burchell *et al.* 1995, Radomska-Pandya *et al.* 1999, Tukey and Strassburg 2000, de Wildt *et al.* 2000). The SN-38 glucuronidation activity of recombinant UGT1A9 was extensively inhibited by planar and bulky phenols, as listed in table 5. These compounds also effectively inhibited the activity in liver microsomes and/or UGT1A6, whereas the inhibitory effects on UGT1A1 and UGT1A3 were not so noteworthy. Furthermore, the inhibition profiles for these compounds in UGT1A9 paralleled these in liver microsomes. UGT1A9 has been considered to be an important isoform in the metabolism of drugs such as non-steroidal anti-inflammatory analgesics, diuretics, β -adrenoceptor agonists and COMT inhibitors (Ebner and Burchell 1993, Burchell *et al.* 1995, Albert *et al.* 1999, Lautala *et al.* 2000). Because UGT1A9 is a low- K_m -type isoform, the possibility has to be considered that UGT1A9 as well as UGT1A1 plays an important role in the metabolism of CPT-11. The activity for SN-38 glucuronidation of UGT1A3 and UGT1A6 was extensively inhibited by cholic acid derivatives. However, the inhibition profiles were not reflected in the results in liver microsomes. Although several other compounds also affected the activity for SN-38 glucuronidation of liver microsomes and recombinant UGTs, we

cannot characterize the inhibition profiles. The absolute expression level of each UGT isoform in human livers has not been reported, although tissue-specific expression has been shown using duplex RT-PCR (Strassburg *et al.* 1997, Tukey and Strassburg 2000). The variety of inhibitory effects of UGT substrates on SN-38 glucuronidation may be due to the expression level and ratio of each UGT isoform in livers. As described above, the accumulation of SN-38 in the intestinal tissues has been suggested to be responsible for the diarrhea attributed to CPT-11 treatment (Araki *et al.* 1993, Wiseman and Markham 1996). Also, the expression of UGT1A mRNAs except for UGT1A5 and UGT1A7 has been shown in human intestinal tissues (Ritter 2000, Tukey and Strassburg 2000). Further studies are required to quantify the mRNA and protein levels of each UGT isoform in human hepatic and extrahepatic tissues, and to identify the relationship between CPT-11 adverse effects and UGT isoform expression.

The presence of an extra TA repeat ((TA)₆TAA to (TA)₇TAA) in the promoter region of UGT1A1 has been shown to be associated with Gilbert's syndrome (Bosma *et al.* 1995, Monaghan *et al.* 1996, Burchell and Hume 1999). There appears to be considerable variation in the incidence of Gilbert's syndrome, ranging from 1 to 19% in various group (Monaghan *et al.* 1996, 1997, Ando *et al.* 1998, Sampietro *et al.* 1998, Burchell and Hume 1999). Iyer *et al.* (1999) reported a significant inverse correlation between the number of TA repeats and UGT activity toward SN-38 and bilirubin in liver microsomes. Furthermore, the G71R allele of UGT1A1 has been reported to cause a significant reduction of UGT activity toward bilirubin in Japanese and Asians (Yamamoto *et al.* 1998, Burchell and Hume 1999). In fact, since the association of Gilbert's syndrome and CPT-11 toxicity emerged, the role of UGT1A1 in the glucuronidation of SN-38 has attracted much attention (Iyer *et al.* 1998, 1999, Ciotti *et al.* 1999). Only UGT1A1 has previously been shown to mainly contribute to SN-38 glucuronidation in human liver (Iyer *et al.* 1998). In the present study, the activity for SN-38 glucuronidation was highest with UGT1A1, and noteworthy with other UGT1A isoforms such as UGT1A3, UGT1A6 and UGT1A9. The present findings imply that UGT1A3, UGT1A6 and UGT1A9 rather than UGT1A1 contribute to the glucuronidation of SN-38 in Gilbert's syndrome patients treated with CPT-11.

In conclusion, UGT1A1 mainly catalysed the SN-38 glucuronidation in human livers, though other UGT1A isoforms such as UGT1A3, UGT1A6 and UGT1A9 are also involved. The UGT isoforms involved in SN-38 glucuronidation can be classified into two types on the basis of affinity toward substrate: namely, low- K_m types such as UGT1A1 and UGT1A9, and high- K_m types such as UGT1A3 and UGT1A6. Therefore, we consider that at least four UGT isoforms contribute to the glucuronidation of SN-38, and that the role and contribution of each differ extensively.

Acknowledgements

The authors thank Yakult Honsha Co. for the gift of SN-38 and SN-38G. This study was supported by a grant (MPJ-6) from the Organization for Pharmaceutical Safety and Research of Japan.

References

- ALBERT, C., VALLÉE, M., BEAUDRY, G., BÉLANGER, A. and HUM, D. W., 1999, The monkey and human uridine diphosphate-glucuronosyltransferase UGT1A9, expressed in steroid target tissues, are estrogen-conjugating enzymes. *Endocrinology*, **140**, 3292–3302.
- ANDO, Y., CHIDA, M., NAKAYAMA, K., SAKA, H. and KAMATAKI, T., 1998, The UGT1A1*28 allele is relatively rare in a Japanese population. *Pharmacogenetics*, **8**, 357–360.
- ARAKI, E., ISHIKAWA, M., IIGO, M., KOIDE, T., ITABASHI, M. and HOSHI, A., 1993, Relationship between development of diarrhea and the concentration of SN-38, an active metabolite of CPT-11, in the intestine and the blood plasma of athymic mice following intraperitoneal administration of CPT-11. *Japanese Journal of Cancer Research*, **84**, 697–702.
- BOSMA, P. J., CHOWDHURY, J. R., BAKKER, C., GANTLA, S., DE BOER, A., OOSTRA, B. A., LINDHOUT, D., TYTGAT, G. N., JANSEN, P. L., OUDE ELFERINK, R. P. and CHOWDHURY, N. R., 1995, The genetic basis of the reduced expression of bilirubin UDP-glucuronosyltransferase 1 in Gilbert's syndrome. *New England Journal of Medicine*, **333**, 1171–1175.
- BURCHELL, B., BRIERLEY, C. H., MONAGHAN, G. and CLARKE, D. J., 1998, The structure and function of the UDP-glucuronosyltransferase gene family. *Advances in Pharmacology*, **42**, 335–338.
- BURCHELL, B., BRIERLEY, C. H. and RANCE, D., 1995, Specificity of human UDP-glucuronosyltransferases and xenobiotic glucuronidation. *Life Sciences*, **57**, 1819–1831.
- BURCHELL, B. and COUGHTRIE, M.W., 1989, UDP-glucuronosyltransferases. *Pharmacology and Therapeutics*, **43**, 261–289.
- BURCHELL, B. and HUME, R., 1999, Molecular genetic basis of Gilbert's syndrome. *Journal of Gastroenterology and Hepatology*, **14**, 960–966.
- CIOTTI, M., BASU, N., BRANGI, M. and OWENS, I. S., 1999, Glucuronidation of 7-ethyl-10-hydroxycamptothecin (SN-38) by the human UDP-glucuronosyltransferases encoded at the UGT1 locus. *Biochemical and Biophysical Research Communications*, **260**, 199–202.
- CLARKE, D.J. and BURCHELL, B., 1994, The uridine diphosphate glucuronosyltransferase multigene family: function and regulation. In F. C. Kaufmann (ed.), *Handbook of Experimental Pharmacology*, vol. 112 (Berlin: Springer-Verlag).
- CREEMERS, G. J., LUND, B. and VERWEIJ, J., 1994, Topoisomerase I inhibitors: topotecan and irinotecan. *Cancer Treatment Reviews*, **20**, 73–96.
- CUNNINGHAM, D., PYRHONEN, S., JAMES, R.D., PUNT, C.J., HICKISH, T.F., HEIKKILA, R., JOHANNESEN, T.B., STARKHAMMAR, H., TOPHAM, C.A., AWAD, L., JACQUES, C. and HERAIT, P., 1998, Randomised trial of irinotecan plus supportive care versus supportive care alone after fluorouracil failure for patients with metastatic colorectal cancer. *Lancet*, **352**, 1413–1418.
- DUTTON, G. J., 1980, *Glucuronidation of Drugs and Other Compounds* (Boca Raton: CRC Press).
- EBNER, T. and BURCHELL, B., 1993, Substrate specificities of two stably expressed human liver UDP-glucuronosyltransferases of the UGT1 gene family. *Drug Metabolism and Disposition*, **21**, 50–55.
- GALIJATOVIC, A., WALLE, U. K. and WALLE, T., 2000, Induction of UDP-glucuronosyltransferase by the flavonoids chrysin and quercetin in Caco-2 cells. *Pharmaceutical Research*, **17**, 21–26.
- GUPTA, E., LESTINGI, T.M., MICK, R., RAMIREZ, J., VOKES, E.E. and RATAIN, M.J., 1994, Metabolic fate of irinotecan in humans: correlation of glucuronidation with diarrhea. *Cancer Research*, **54**, 3723–3725.
- HAAZ, M.-C., RIVORY, L. P., JANTET, S., RATANASAVANH, D. and ROBERT, J., 1997, Glucuronidation of SN-38, the active metabolite of irinotecan, by human hepatic microsomes. *Pharmacology and Toxicology*, **80**, 91–95.
- HANIKA, N., JINNO, H., NISHIMURA, T., ANDO, M., OZAWA, S. and SAWADA, J., 2001, High-performance liquid chromatographic assay for glucuronidation activity of 7-ethyl-10-hydroxycamptothecin (SN-38), the active metabolite of irinotecan (CPT-11), in human liver microsomes. *Biomedical Chromatography* (in press).
- IYER, L., HALL, D., DAS, S., MORTELL, M. A., RAMIREZ, J., KIM, S., DI RIENZO, A. and RATAIN, M. J., 1999, Phenotype-genotype correlation of *in vitro* SN-38 (active metabolite of irinotecan) and bilirubin glucuronidation in human liver tissue with UGT1A1 promoter polymorphism. *Clinical Pharmacology and Therapeutics*, **65**, 576–582.
- IYER, L., KING, C. D., WHITTINGTON, P. F., GREEN, M. D., ROY, S. K., TEPHLY, T. R., COMAN, B. L. and RATAIN, M. J., 1998, Genetic predisposition to the metabolism of irinotecan (CPT-11): role of uridine diphosphate glucuronosyltransferase isoform 1A1 in the glucuronidation of its active metabolite (SN-38) in human liver microsomes. *Journal of Clinical Investigation*, **101**, 847–854.
- JAXEL, C., KOHN, K. W., WANI, M. C., WALL, M. E. and POMMIER, Y., 1989, Structure-activity study of the actions of camptothecin derivatives on mammalian topoisomerase I: Evidence for a specific receptor site and a relation to antitumor activity. *Cancer Research*, **49**, 1465–1469.
- KANEDA, N., NAGATA, H., FURUTA, T. and YOKOKURA, T., 1990, Metabolism and pharmacokinetics of the camptothecin analogue CPT-11 in the mouse. *Cancer Research*, **50**, 1715–1720.

- KAWATO, Y., AONUMA, M., HIROTA, Y., KUGA, H. and SATO, K., 1991, Intracellular roles of SN-38, a metabolite of the camptothecin derivative CPT-11, in the antitumor effect of CPT-11. *Cancer Research*, **51**, 4187-4191.
- LAUTALA, P., ETHELL, B. T., TASKINEN, J. and BURCHELL, B., 2000, The specificity of glucuronidation of entacapone and tolcapone by recombinant human udp-glucuronosyltransferases. *Drug Metabolism and Disposition*, **28**, 1385-1389.
- LOKIEC, F., CANAL, P., GAY, C., CHATELUT, E., ARMAND, J. P., ROCHE, H., BUGAT, R., GONÇALVES, E. and MATHIEU-BOUE, A., 1995, Pharmacokinetics of irinotecan and its metabolites in human blood, bile, and urine. *Cancer Chemotherapy and Pharmacology*, **36**, 79-82.
- MACKENZIE, P. I., OWENS, I. S., BURCHELL, B., BOCK, K. W., BAIROCH, A., BELANGER, A., FOURNEL GIGLEUX, S., GREEN, M., HUM, D. W., IYANAGI, T., LANCET, D., LOUISOT, P., MAGDALOU, J., CHOWDHURY, J. R., RITTER, J. K., SCHACHTER, H., TEPHLY, T. R., TIPTON, K. F. and NEBERT, D. W., 1997, The UDP glycosyltransferase gene superfamily: recommended nomenclature update based on evolutionary divergence. *Pharmacogenetics*, **7**, 255-269.
- MONAGHAN, G., FOSTER, B., JURIMA-ROMET, M., HUME, R. and BURCHELL, B., 1997, UGT1*1 genotyping in a Canadian Inuit population. *Pharmacogenetics*, **7**, 153-156.
- MONAGHAN, G., RYAN, M., SEDDON, R., HUME, R. and BURCHELL, B., 1996, Genetic variation in bilirubin UDP-glucuronosyltransferase gene promoter and Gilbert's syndrome. *Lancet*, **347**, 578-581.
- RADOMINSKA-PANDYA, A., CZERNIK, P. J., LITTLE, J. M., BATTAGLIA, E. and MACKENZIE, P. I., 1999, Structural and functional studies of UDP-glucuronosyltransferases. *Drug Metabolism Reviews*, **31**, 817-899.
- RITTER, J. K., 2000, Roles of glucuronidation and UDP-glucuronosyltransferases in xenobiotic bioactivation reactions. *Chemico-Biological Interactions*, **129**, 171-193.
- RIVORY, L. P., BOWLES, M. R., ROBERT, J. and POND, S. M., 1996, Conversion of irinotecan (CPT-11) to its active metabolite, 7-ethyl-10-hydroxycamptothecin (SN-38), by human liver carboxylesterase. *Biochemical Pharmacology*, **52**, 1103-1111.
- ROUGIER, P., VAN CUTSEM, E., BAJETTA, E., NIEDERLE, N., POSSINGER, K., LABIANCA, R., NAVARRO, M., MORANT, R., BLEIBERG, H., WILS, J., AWAD, L., HERAIT, P. and JACQUES, C., 1998, Randomised trial of irinotecan *versus* fluorouracil by continuous infusion after fluorouracil failure in patients with metastatic colorectal cancer. *Lancet*, **352**, 1407-1412.
- SAMPIETRO, M., LUPICA, L., PERRERO, L., ROMANO, R., MOLTENI, V. and FIORELLI, G., 1998, TATA-box mutant in the promoter of the uridine diphosphate glucuronosyltransferase gene in Italian patients with Gilbert's syndrome. *Italian Journal of Gastroenterology and Hepatology*, **30**, 194-198.
- SAWADA, S., OKAJIMA, S., AIYAMA, R., NOKATA, K., FURUTA, T., YOKOKURA, T., SUGINO, E., YAMAGUCHI, K. and MIYASAKA, T., 1991, Synthesis and antitumor activity of 20(S)-camptothecin derivatives: Carbamate-linked, water-soluble derivatives of 7-ethyl-10-hydroxycamptothecin. *Chemical and Pharmaceutical Bulletin*, **39**, 1446-1454.
- SLICHENMYER, W. J., ROWINSKY, E. K., GROCHOW, L. B., KAUFMANN, S. H. and DONEHOWER, R. C., 1994, Camptothecin analogues: Studies from the Johns Hopkins Oncology Center. *Cancer Chemotherapy and Pharmacology*, **34**, 53-57.
- STRASSBURG, C. P., OLDHAFFER, K., MANNS, M. P. and TUKEY, R. H., 1997, Differential expression of the *UGT1A* locus in human liver, biliary, and gastric tissue: Identification of *UGT1A7* and *UGT1A10* transcripts in extrahepatic tissue. *Molecular Pharmacology*, **52**, 212-220.
- TAKASUNA, K., HAGIWARA, T., HIROHASHI, M., KATO, M., NOMURA, M., NAGAI, E., YOKOI, T. and KAMATAKI, T., 1996, Involvement of β -glucuronidase in intestinal microflora in the intestinal toxicity of the antitumor camptothecin derivative irinotecan hydrochloride (CPT-11) in rats. *Cancer Research*, **56**, 3752-3757.
- TUKEY, R. H. and STRASSBURG, C. P., 2000, Human UDP-glucuronosyltransferases: Metabolism, expression, and disease. *Annual Review of Pharmacology and Toxicology*, **40**, 581-616.
- VASSAL, G., TERRIER-LACOMBE, M. J., BISSERY, M. C., VENUAT, A. M., GYERGYAY, F., BENARD, J., MORIZET, J., BOLAND, I., ARDOUIN, P., BRESSAC-DE-PAILLERETS, B. and GOUYETTE, A., 1996, Therapeutic activity of CPT-11, a DNA-topoisomerase I inhibitor, against peripheral primitive neuroectodermal tumour and neuroblastoma xenografts. *British Journal of Cancer*, **74**, 537-545.
- WISEMAN, L. R. and MARKHAM, A., 1996, Irinotecan: A review of its pharmacological properties and clinical efficacy in the management of advanced colorectal cancer. *Drugs*, **52**, 606-623.
- DE WILDT, S. N., KEARNS, G. L., LEEDER, J. S. and VAN DEN ANKER, J. N., 1999, Glucuronidation in humans: Pharmacogenetic and developmental aspects. *Clinical Pharmacokinetics*, **36**, 439-452.
- YAMAMOTO, K., SATO, H., FUJIYAMA, Y., DOIDA, Y. and BAMBA, T., 1998, Contribution of two missense mutations (G71R and Y486D) of the bilirubin UDP glycosyltransferase (UGT1A1) gene to phenotypes of Gilbert's syndrome and Crigler-Najjar syndrome type II. *Biochimica et Biophysica Acta*, **1406**, 267-273.

- ZAMBONI, W. C., HOUGHTON, P. J., THOMPSON, J., CHESHIRE, P. J., HANNA, S. K., RICHMOND, L. B., LOU, X. and STEWART, C. F., 1998, Altered irinotecan and SN-38 disposition after intravenous and oral administration of irinotecan in mice bearing human neuroblastoma xenografts. *Clinical Cancer Research*, **4**, 455–462.

Metabolic activation of 1α -hydroxyvitamin D₃ in human liver microsomes

S. KAMACHI†, K. SUGIMOTO‡, T. YAMASAKI§, N. HIROSE†, H. IDE§ and Y. OHYAMA§

†Product Research Laboratory, Chugai Pharmaceutical Co. Ltd, 3-41-8 Takada, Toshima-ku 171-8545, Japan

‡Pharmacokinetics, Panapharm Laboratories Co. Ltd, Uto, Kumamoto 869-0425, Japan

§Department of Mathematical and Life Sciences, Graduate School of Science, Hiroshima University, Higashi-Hiroshima 739-8526, Japan.

Received 11 January 2001

1. 1α -Hydroxyvitamin D₃ (1α -OH-D₃) is a synthetic prodrug of the active form of vitamin D₃, and requires the hydroxylation at the C-25 position before eliciting its biological activity.

2. 25-Hydroxylation activities for 1α -OH-D₃ were present in both microsomal and mitochondrial fractions of human liver.

3. To determine the P450 enzyme(s) involved in microsomal 25-hydroxylation, 14 P450s (CYP1A1, 1A2, 1B1, 2A6, 2B6, 2C8, 2C9-Arg, 2C9-Cys, 2C19, 2D6-Val, 2D6-Met, 2E1, 3A4, 4A11) were tested for their 25-hydroxylation activity of 1α -OH-D₃. None catalysed the 25-hydroxylation reaction.

4. 1α -OH-D₃ in a high concentration (2.5 ng ml⁻¹) showed small but significant inhibition of the catalytic activities of CYP2C8, 2C9-Cys, 2C19, 2D6-Val and 2E1 for their typical substrates. However, 1α -OH-D₃ in a clinically used low concentration will not significantly affect drug metabolism catalysed by the 14 P450s tested.

5. In summary, the 25-hydroxylation activity of 1α -OH-D₃ that localizes in the microsomal fraction appears to be attributable to a cytochrome P450 other than the microsomal forms tested in this study.

Introduction

1,25-Dihydroxyvitamin D₃ (1,25-(OH)₂D₃) is known to be the active form of vitamin D₃ and mediates biological activities such as enhancing calcium absorption at the intestine and mobilization of bone calcium to maintain the homeostasis of calcium (DeLuca 1988, Jones *et al.* 1998). 1,25-(OH)₂D₃ also regulates cell growth and differentiation, and the immune system (Hewison and O'Riordan 1997).

1,25-(OH)₂D₃ is synthesized from vitamin D₃ by the successive hydroxylation of C-25 in liver and C-1 α in kidney *in vivo* (figure 1). These hydroxylation reactions are catalysed by cytochrome P450 enzymes (CYP) (Okuda *et al.* 1995). 25-Hydroxylases, which catalyse the first step of the biological activation of vitamin D₃, have been purified from rat (Hayashi *et al.* 1986) and pig liver microsomes (Axén *et al.* 1992), and also rat (Masumoto *et al.* 1988) and rabbit liver mitochondria (Dahlbäck and Wikvall 1988). These 25-hydroxylases have been designated as CYP2C11 for the rat microsomal enzyme, CYP2D25 for the pig microsomal enzyme, CYP27A1 for the rat and rabbit mitochondrial enzymes

* Author for correspondence; e-mail: ohyama@sci.hiroshima-u.ac.jp

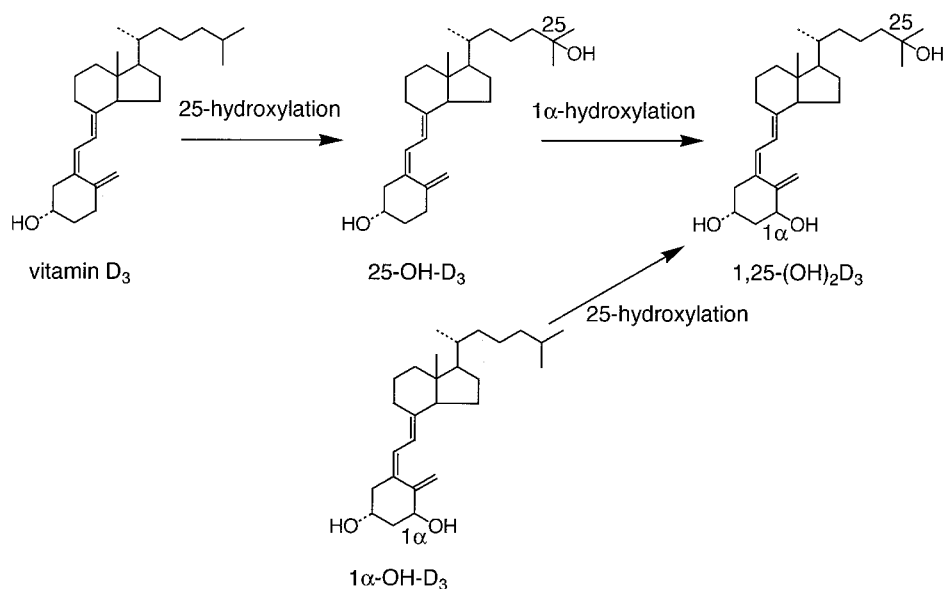


Figure 1. Activation pathways of vitamin D₃ and 1α-OH-D₃. Conversion of vitamin D₃ to 25-OH-D₃ by the 25-hydroxylase is the initial step in the activation process. 25-OH-D₃ is subsequently hydroxylated at the C-1α by 1α-hydroxylase (CYP27B1) in kidney. 1α-OH-D₃ only requires 25-hydroxylation to be converted to the active form of vitamin D₃.

based on the amino acid sequences deduced from their cDNAs (Nelson 2000). Recently, cDNAs of 1α-hydroxylases that catalyse the second step of the activation have also been isolated from mouse, rat and human and named as CYP27B1 (Fu *et al.* 1997a, Shinki *et al.* 1997, St-Arnaud *et al.* 1997, Takeyama *et al.* 1997). The mutations in this gene have been clearly demonstrated to cause vitamin D-dependent rickets type I (Fu *et al.* 1997b, Kitanaka *et al.* 1998).

The activity of CYP27B1 as the genuine 1α-hydroxylase has been well established, whereas the biological role of CYP2C11, 2D25 and 27A1 as 25-hydroxylase remained controversial as the following reasons. CYP2C11 is a rat male-specific P450 and is not expressed in the female rat liver (Hayashi *et al.* 1988). CYP2D25 is expressed not only in the liver, but also in the kidney, and its orthologous gene has not identified in other species (Postlind *et al.* 1997). cDNAs of CYP27A1 have been isolated from rat, rabbit and human and their amino acid sequences are well conserved in vertebrate (Nelson 2000). However, the activity of CYP27A1 for 5β-cholestane-3α,7α,12α-triol 27-hydroxylation that is part of the main pathway of bile acids synthesis is higher than 25-hydroxylation activity for vitamin D₃ by two orders of magnitude (Ohyama *et al.* 1991) and the ablation of CYP27A1 in mouse causes markedly reduced bile acid synthesis without affecting vitamin D₃ metabolism (Rosen *et al.* 1998).

1α-Hydroxyvitamin D₃ (1α-OH-D₃) has been developed as the therapeutic compound for the treatment of the patients suffering a renal disorder causing insufficient 1,25-(OH)₂D₃ production, since it obviates the need of the 1α-hydroxylation step at the kidney. 1α-OH-D₃ is metabolized rapidly to 1,25-(OH)₂D₃ in the liver to elicit its biological function (Fukushima *et al.* 1978, 1980). 1α-OH-D₃ has been clinically used for renal osteodystrophy and hyperpar-

athyroidism. Saarem *et al.* (1984) reported the presence of vitamin D₃ 25-hydroxylase only in mitochondria but not in microsomes in human. On the other hand, Holmberg *et al.* (1986) found the 25-hydroxylase activity both in microsomes and mitochondria.

In the present study, we examined the subcellular localization of the 25-hydroxylase activity in human liver using [³H]-1 α -OH-D₃ with a high radio-specific activity and tested whether major human microsomal P450 enzymes could catalyse 25-hydroxylation. Furthermore, the potential for drug-drug interactions of 1 α -OH-D₃ was also examined since P450s are well known to play a critical role in the metabolic disposition of a variety of drugs and xenobiotics.

Materials and methods

Chemicals and reagents

[22,23-³H]-1 α -OH-D₃ (4.12 TBq mmol⁻¹, radiopurity > 97%) and 1 α -OH-D₃ (purity > 98%) were synthesized at Chugai Pharmaceutical Co. Ltd. (Tokyo, Japan) (Kawase *et al.* 2000). [¹⁻¹⁴C]-lauric acid (1.85 GBq mmol⁻¹) was purchased from Amersham Pharmacia. NADP⁺, glucose 6-phosphate, glucose 6-phosphate dehydrogenase were obtained from Oriental Yeast (Tokyo, Japan). NADPH-P450 reductase was purified according to Yasukochi and Masters (1976). Human liver microsomes (HHM-247, 248, 249; each lot consisted of 10 individual subjects) and mitochondria were obtained from the International Institute for the Advancement of Medicine (PA, USA). Microsomes prepared from the genetically engineered human B-lymphoblastoid cell line (Gentest Co., Woburn, MA, USA) expressing NADPH-P450 reductase with either human CYP1A1, 1A2, 1B1, 2A6, 2B6, 2C8, 2C9-Arg, 2C9-Cys, 2C19, 2D6-Val, 2D6-Met, 2E1, 3A4 or 4A11 were purchased from Daiichi Pure Chemicals Co. (Tokyo, Japan). Anti-human CYP2D6 rabbit antiserum and anti-human CYP2C goat antiserum were from Daiichi Pure Chemicals Co. All other chemicals were of the highest grade commercially available.

Assay of 25-hydroxylation activity with microsomes and mitochondria

Analysis of [³H]-1 α -OH-D₃ metabolites was performed by HPLC as described by Koike *et al.* (1998) with some modifications. Briefly, the assay mixture consisted of 100 mM potassium phosphate (pH 7.4), 3.1 pmol [³H]-1 α -OH-D₃, and a NADPH-generating system (final concentration: NADP⁺, 1.3 mM; glucose 6-phosphate, 3.3 mM; glucose 6-phosphate dehydrogenase 0.4 U ml⁻¹) in a final volume of 450 μ l. The mixture was pre-incubated for 5 min, and the reaction was started by the addition of 50 μ l human liver microsomes (10 mg ml⁻¹) (or microsomes expressing individual P450) and incubated with gentle shaking for 60 min in a 37°C waterbath. The reaction was terminated by 2 ml ethanol and the ethanol soluble fraction was collected by centrifugation. After removing the solvent under reduced pressure, the residue was dissolved in an aliquot of the mobile phase of the following HPLC. The HPLC system (LC-module 1, Waters) connected with a RI detector (FLO-ONE/Beta Model A525, Packard) in series was used for analysis of the metabolites. The column was Lichrospher Si 60 (150 $\times$ 4.6 mm i.d., Kantokagaku, Japan). The mobile phase was hexane:isopropanol = 9:1 (v/v), and flow rate was 1.0 ml min⁻¹. Elution of [³H]-1 α -OH-D₃ metabolites was first monitored at 265 nm. Subsequently, the eluate was automatically mixed with scintillant (ULTIMA-FLO M, Packard) sent by pump at 3.0 ml min⁻¹, and radiolabelled metabolites were detected by a RI detector.

In the case of the analysis of mitochondrial activity, the reaction mixture was supplemented with succinate (final concentration 25 mM) for effective generation of NADPH in the mitochondria.

Assay of 25-hydroxylation activity with reconstituted methods

Human liver microsomes were solubilized with 0.6% cholate and used for the following enzyme assay as described by Ohyama *et al.* (1997). Enzyme activity were reconstituted with a solubilized P450 fraction (0.5 mg protein) and NADPH-P450 reductase (0.4 U for cytochrome C reducing activity) in 490 μ l 100 mM phosphate buffer and measured using 3.1 pmol [³H]-1 α -OH-D₃ as a substrate. The mixture was pre-incubated for 2 min and the reaction was started by the addition of 10 μ l 50 mM NADPH. After a 60-min incubation at 37°C, the reaction was stopped with 4 ml benzene. The benzene extract was dried by flushing with N₂ gas and the residue was dissolved with an aliquot of the mobile phase of HPLC. Analysis condition was as follows: column, Zorbax SIL (250 $\times$ 4.6 mm i.d., DuPont); mobile phase, hexane:isopropanol:methanol = 86/7/7 (v/v), flow rate, 1.0 ml min⁻¹; detection, 265 nm. The eluate from the HPLC was collected every 30 s and mixed with 1.5 ml scintillant and then the

radioactivity of each fraction was measured. For identification of the products, reverse-phase HPLC was also performed using TSK gel ODS-80Ts (150×4.6 mm i.d., Tosoh, Japan) with a two-step linear gradient system (phase A consisted of 50 mM ammonium acetate, pH 6.0: acetonitrile = 80:20 (v/v), phase B was acetonitrile; (A) 80%: (B) 20% at 0 min, (A) 15%: (B) 75% at 30 min, (A) 0%: (B) 100% at 50 min). Detection of metabolites was carried out as described above.

Analysis of inhibition effects of 1α -OH- D_3 on drug oxidation reactions catalysed by P450 isoforms

The experimental conditions were summarized in table 1. The P450 substrates used were based on the supplier's instructions (GENTEST Co.): 7-ethoxyresorufin for CYP1A1, 1A2, 1B1, coumarin for 2A6, 7-ethoxy-4-trifluoro-methylcoumarin for CYP2B6, 5 (or 6)-chloromethyl-fluorescein-diethyether for CYP2C8, diclofenac for CYP2C9, *S*-mephenytoin for CYP2C19, bufuralol for CYP2D6, chlorzoxazone for CYP2E1, testosterone for CYP3A4, and [^{14} C]lauric acid for CYP4A11, and enzyme reactions were carried out as described by Busby *et al.* (1999). The reaction mixture consisted of the NADPH-generating system, P450 expressing microsomes and a specific substrate for each P450 isoform and the enzyme reaction was performed in the absence or presence of cold 1α -OH- D_3 (100, 500 or 2500 pg ml $^{-1}$). After pre-incubation for 5 min, the reactions were initiated by adding the NADPH-generating system. After appropriate incubation times, the metabolites were analysed as shown in table 1.

Results

Subcellular localization of 25-hydroxylation activity in human liver

To examine the subcellular localization of 25-hydroxylase activity in human liver, mitochondrial and microsomal fractions were assayed using [^{3} H]- 1α -OH- D_3 as a substrate. As shown in figure 2, both microsomes and mitochondria produced significant 25-hydroxylation products at comparable levels. The 25-hydroxylation reaction with mitochondria was likely catalysed by CYP27A1, a mitochondrial P450 (Masumoto *et al.* 1988, Guo *et al.* 1993). Microsomal P450 requires specific electron-transferring proteins, NADPH-P450 reductase, for its activity. Mitochondrial P450 activity does not reconstitute with NADPH-P450 reductase (Masumoto *et al.* 1988). Therefore, to eliminate the possibility of the contamination of mitochondria in the microsomal fraction, a reconstitution experiment was carried out. The solubilized fraction of the microsomes produced 1,25-(OH) $_2$ D_3 only when co-incubated with NADPH-P450 reductase (figure 3). Identification of the product was accomplished by comparing its chromatographic properties with authentic 1,25(OH) $_2$ D_3 using two normal-phase and a reverse-phase HPLC systems (figures 2 and 3). These results clearly show that the 25-hydroxylation activity of 1α -OH- D_3 exists in not only mitochondria, but also microsomes, and is consistent with the results of Holmberg *et al.* (1986).

Identification of the P450s catalysing 25-hydroxylation of 1α -OH- D_3

As shown above, human liver microsomes converted 1α -OH- D_3 to 1,25-(OH) $_2$ D_3 . To identify the P450 enzymes catalysing the 1α -OH- D_3 25-hydroxylation reaction, 14 commercially available P450 enzymes (CYP1A1, 1A2, 1B1, 2A6, 2B6, 2C8, 2C9-Cys, 2C9-Arg, 2C19, 2D6-Val, 2D6-Met, 2E1, 3A4, 4A11) were tested. Although the enzyme reaction was performed with highly tritiated 1α -OH- D_3 as a substrate to increase the sensitivity, no 1,25-(OH) $_2$ D_3 was detected, indicating that none of the tested P450 enzymes has the 25-hydroxylase activity (data not shown). Inhibition experiments of 25-hydroxylase activity of human microsomes by anti-human CYP2D6 antiserum and anti-human CYP2C antiserum was also carried out, since CYP2C11 (rat) and CYP2D25 (pig) were

Table 1. Assay conditions for P450 isoforms.

Human P450 isoform	Substrate	Substrate concentration	Microsomes concentration (mg protein ml ⁻¹)	Incubation time (min)	Reaction volume (ml)	Metabolite	Determination method
CYP1A1	7-ethoxyresorufin	4.2 μM	0.1	15	2	resorufin	fluorometry
CYP1A2	7-ethoxyresorufin	4.2 μM	0.25	60	2	resorufin	fluorometry
CYP1B1	7-ethoxyresorufin	4.2 μM	0.5	120	2	resorufin	fluorometry
CYP2A6	coumarin	0.4 mM	0.2	30	0.5	7-hydroxycoumarin	fluorometry
CYP2B6	7-ethoxy-4-trifluoromethylcoumarin	0.1 mM	0.4	60	0.25	7-hydroxy-4-trifluoromethylcoumarin	fluorometry
CYP2C8	5 (or 6)-chloromethylfluorescein-diethylether	0.01 mM	0.4	120	2	5 (or 6)-chloromethylfluorescein	fluorometry
CYP2C9-Arg	diclofenac	0.1 mM	0.4	30	0.25	4'-hydroxydiclofenac	HPLC-UV
CYP2C9-Cys	diclofenac	0.1 mM	2.0	60	0.25	4'-hydroxydiclofenac	HPLC-UV
CYP2C19	S-mephenytoin	0.1 mM	4.0	120	0.2	4'-hydroxymephenytoin	HPLC-UV
CYP2D6-Val	(+)bufuralol	0.1 mM	0.4	10	0.25	1'-hydroxybufuralol	HPLC-fluorometry
CYP2D6-Met	(+)bufuralol	0.1 mM	0.4	10	0.25	1'-hydroxybufuralol	HPLC-fluorometry
CYP2E1	chlorzoxazone	0.5 mM	1.0	60	0.5	6-hydroxychlorzoxazone	HPLC-UV
CYP3A4	testosterone	0.2 mM	0.4	30	0.5	6β-hydroxytestosterone	HPLC-UV
CYP4A11	[¹⁴ C]lauric acid	0.1 mM	0.5	30	0.1	[¹⁴ C]ω-hydroxy lauric acid	HPLC-RI

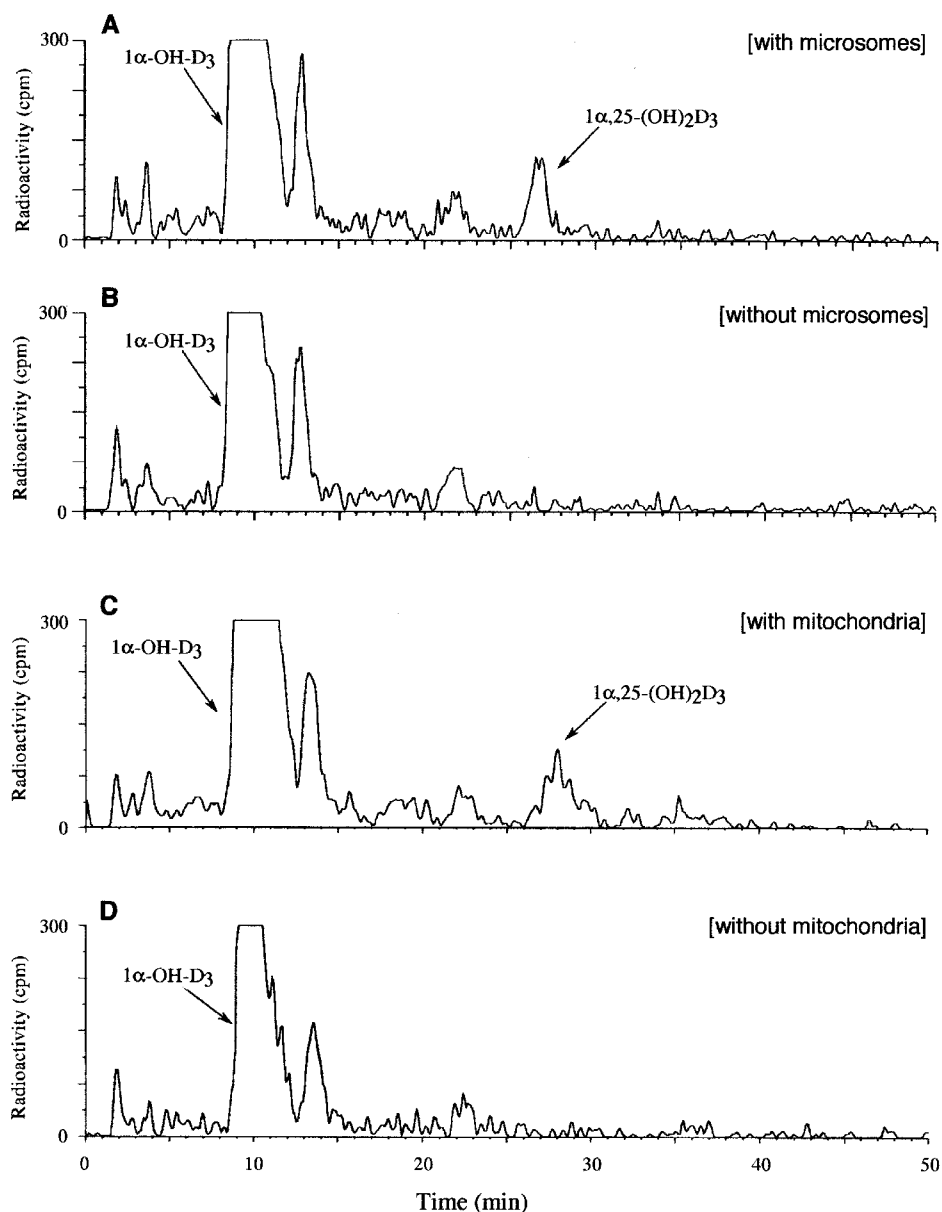


Figure 2. HPLC analysis of metabolites of $1\alpha\text{-OH-D}_3$ with human liver microsomal or mitochondrial fractions. $[^3\text{H}]\text{-}1\alpha\text{-OH-D}_3$ (3.1 pmol) was incubated with microsomes (0.5 mg) or mitochondria (1.0 mg) in the presence of a NADPH-generating system for 60 min at 37°C . The metabolites were analysed by a normal phase HPLC system described in the Materials and methods. Both microsomes (A) and mitochondria (C) produced $1,25\text{-(OH)}_2\text{D}_3$ with about 3% conversion of the substrate. No $1,25\text{-(OH)}_2\text{D}_3$ was detected without microsomes or mitochondria (B, D). The elution positions of authentic $1,25\text{-(OH)}_2\text{D}_3$ and $1\alpha\text{-OH-D}_3$ are indicated. $1,25\text{-(OH)}_2\text{D}_3$ was detected as broad and multiple peaks due to the signal-to-noise (S/N) ratio of direct RI detection system of the HPLC eluate.

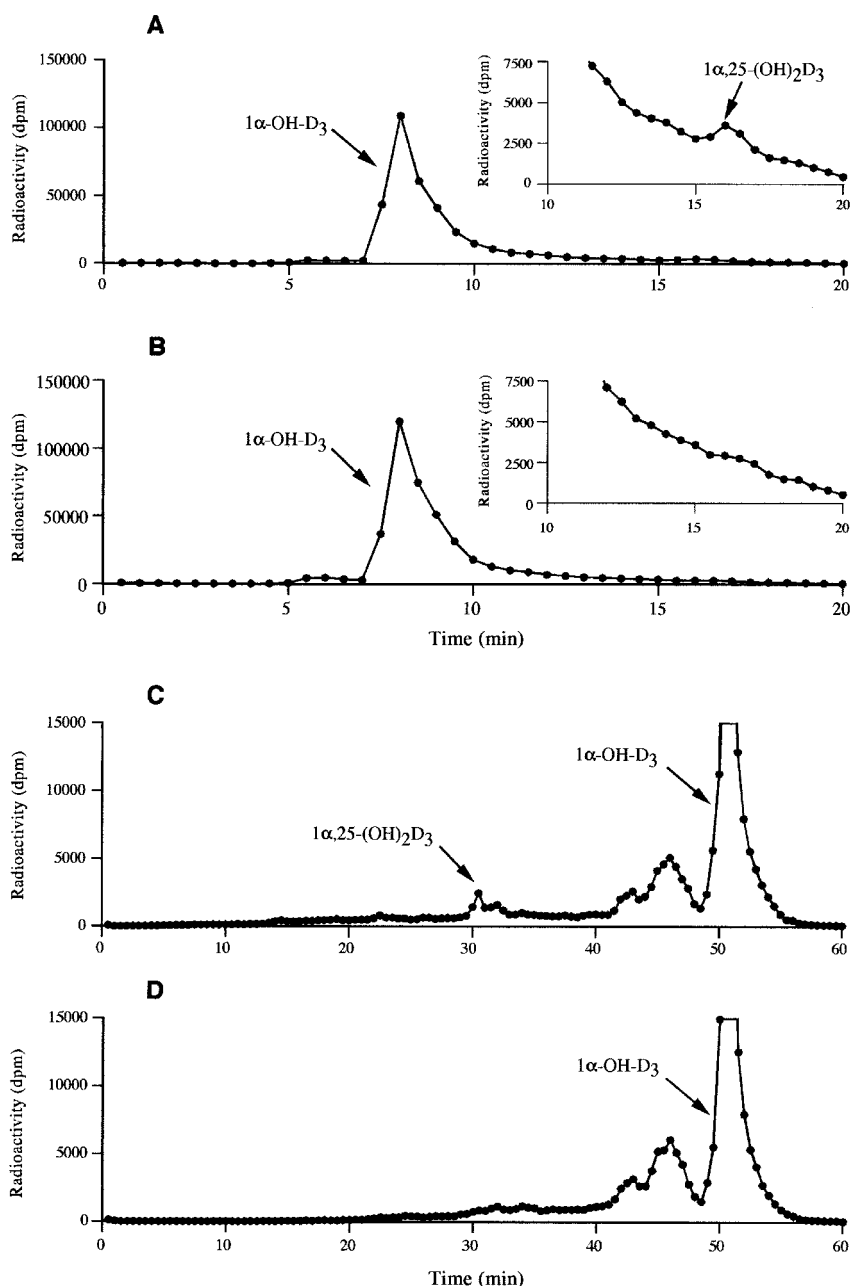


Figure 3. HPLC analysis of metabolites of 1 α -OH-D₃ with solubilized microsomes. Human liver microsomes were solubilized with 0.6% cholate and the enzyme activity was reconstituted with the solubilized fraction (0.5 mg protein) and NADPH-P450 reductase (0.4 U) in 100 mM phosphate buffer. The reaction mixture containing 3.1 pmol [³H]-1 α -OH-D₃ was pre-incubated for 2 min and the reaction was started by the addition of 10 μ l of 50 mM NADPH. After 60-min incubation at 37°C, the reaction mixtures were subjected to normal- and reverse-phase HPLC analysis and the eluate was collected every 30 s. After addition of scintillant, the radioactivity of each fraction was measured by a liquid scintillation counter as described in the Materials and methods. Normal-phase HPLC analysis with (A) or without NADPH-P450 reductase (B). Reverse-phase HPLC analysis with (C) or without NADPH-P450 reductase (D). The elution positions of authentic 1,25-(OH)₂D₃ and 1 α -OH-D₃ are indicated.

previously shown to catalyse the 25-hydroxylation and human P450 enzymes corresponding to CYP2C11 and CYP2D25 would be candidates of 25-hydroxylase. Neither antiserum showed any inhibition (data not shown).

Influences of 1α -OH- D_3 on activities of human P450 isoforms

P450s are involved in metabolism of diverse drugs and xenobiotics. To assess potential *in vivo* drug-drug interactions, the effect of 1α -OH- D_3 on the activities of the recombinant P450 isoforms was investigated by *in vitro* assays. The activity of individual P450 enzymes was measured using a specific substrate with or without 1α -OH- D_3 (100, 500 and 2500 pg ml⁻¹) by the methods summarized in table 1. As shown in figure 4, 1α -OH- D_3 at 100 pg ml⁻¹ had almost no effect on the enzyme activities, except for CYP2C9-Arg. A higher concentration of 1α -OH- D_3 (500 pg ml⁻¹) inhibited the activities of CYP1A1, 1A2, 2C8, 2C9-Arg, 2D6-Val and 4A11 by 10–33%. The highest concentration (2500 pg ml⁻¹) of 1α -OH- D_3 inhibited the activities of CYP1A2, 2C8, 2C9-Cys, 2C9-Arg, 2C19, 2D6-Val, 2E1, 3A4 and 4A11 by 10–50%. The activities of CYP1A1, 1B1, 2A6, 2B6 and 2D6-Met were not affected even at the highest concentration.

Discussion

Vitamin D_3 25-hydroxylation reaction is a prerequisite step for the synthesis of active form of vitamin D_3 , 1,25-(OH) $_2D_3$. Vitamin D_3 25-hydroxylases have been purified from male rat liver microsomes (Hayashi *et al.* 1986), and from rat (Masumoto *et al.* 1988) and rabbit (Dahlbäck and Wikvall 1988) liver mitochondria on the basis of catalytic activities. The rat microsomal 25-hydroxylase has been identified as a rat male-specific P450, CYP2C11 (Hayashi *et al.* 1986) and does not exist in the female (Hayashi *et al.* 1988). Hence, the physiological significance of this enzyme is equivocal. On the other hand, cDNAs for rabbit and rat mitochondrial 25-hydroxylases have been cloned using synthetic oligonucleotide probes and specific antibodies for the purified proteins, respectively, and named as CYP27A1 (Andersson *et al.* 1989, Usui *et al.* 1990). Identification of the orthologous gene in human suggested that CYP27A1 might be a biologically important enzyme. However, subsequent extensive characterization of CYP27A1 has raised the question whether the primary role of CYP27A1 is vitamin D_3 25-hydroxylation or not since CYP27A1 exhibits two orders of magnitude higher turnover number for 27-hydroxylation of 5 β -cholestane-3 α ,7 α ,12 α -triol, an intermediate of bile acid synthesis, than for vitamin D_3 25-hydroxylation (Ohya *et al.* 1991). More recently, it was shown that mice with disrupted CYP27A1 gene showed no significant change of the 25-OH- D_3 concentration in blood, albeit a dramatic change in the composition of bile acids, indicating a major role of CYP27A1 in bile acid synthesis but not in vitamin D 25-hydroxylation (Rosen *et al.* 1998).

The first aim of the present experiments was to examine whether human liver microsomes contain the 25-hydroxylation activity for 1α -OH- D_3 , because conflicting results have been reported. Saarem *et al.* (1984) reported the 25-hydroxylase existed only in mitochondria but not microsomes in human. In contrast, Holmberg *et al.* (1986) found the 25-hydroxylase activity in both microsomes and mitochondria. In the current study, the measurement of the 25-hydroxylase

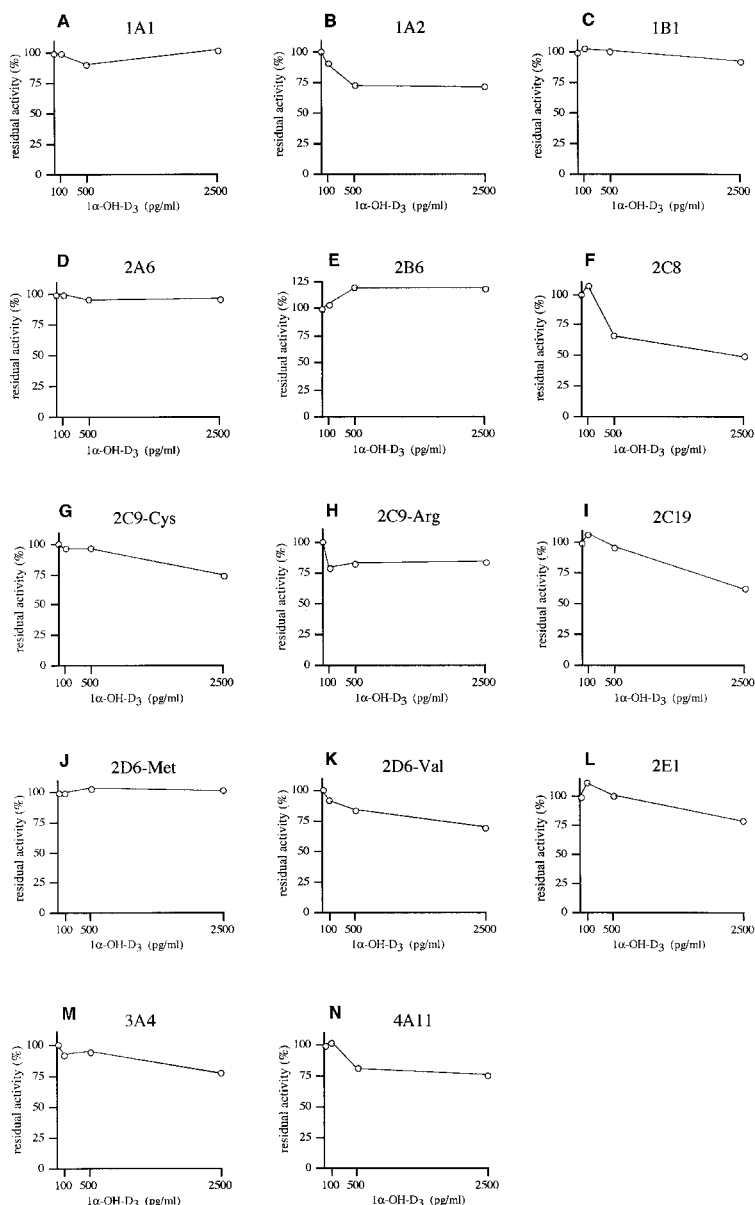


Figure 4. Effect of 1α -OH-D₃ on catalytic activities of various P450s. The substrates and reaction systems were as described in table 1. Each reaction was performed with microsomes expressing individual P450s according to the supplier's instructions (Gentest Co.) and the methods described by Busby *et al.* (1999) in the presence of 100, 500 or 2500 pg ml⁻¹ 1α -OH-D₃. Data were the average of two independent experiments. The substrates and control activities (100%) were (A) 1A1 with 7-ethoxyresorufin, 750 pmol mg⁻¹ min⁻¹; (B) 1A2 with 7-ethoxyresorufin, 130 pmol mg⁻¹ min⁻¹; (C) 1B1 with 7-ethoxyresorufin, 47 pmol mg⁻¹ min⁻¹; (D) 2A6 with coumarin, 1900 pmol mg⁻¹ min⁻¹; (E) 2B6 with 7-ethoxy-4-trifluoro-methylcoumarin, 290 pmol mg⁻¹ min⁻¹; (F) 2C8 with 5 (or 6)-chloromethyl-fluorescein-diethylether, 19 pmol mg⁻¹ min⁻¹; (G) 2C9-Cys with diclofenac, 810 pmol mg⁻¹ min⁻¹; (H) 2C9-Arg with diclofenac, 3380 pmol mg⁻¹ min⁻¹; (I) 2C19 with S-mephenytoin, 64 pmol mg⁻¹ min⁻¹; (J) 2D6-Met with bufuralol, 980 pmol mg⁻¹ min⁻¹; (K) 2D6-Val with bufuralol, 1200 pmol mg⁻¹ min⁻¹; (L) 2E1 with chlorzoxazone, 1850 pmol mg⁻¹ min⁻¹; (M) 3A4 with testosterone, 2240 pmol mg⁻¹ min⁻¹; (N) 4A11 with [¹⁴C]lauric acid, 570 pmol mg⁻¹ min⁻¹.

activity of microsomal and mitochondrial fractions using highly radiolabelled 1α -OH- D_3 revealed that both fractions contained 25-hydroxylase activities at comparable levels. To eliminate the possibility of cross contamination between microsomes and mitochondria, we also examined 25-hydroxylation activity using a reconstituted system comprising microsomal electron transporting protein (NADPH-P450 reductase). Mitochondrial P450 does not use electrons from NADPH-P450 reductase (Masumoto *et al.* 1988). A weak but significant activity was observed for the microsomal fraction with NADPH-P450 reductase but not for that without the reductase. These results indicate that the activity in microsomal fraction was not due to the contamination of mitochondria and that a microsomal P450 with the 25-hydroxylation activity also existed in human liver. These results are consistent with the finding of Holmberg *et al.* (1986).

CYP2C11, CYP2D25, and CYP27A1 identified as 25-hydroxylase are known to exhibit higher hydroxylase activity for 1α -OH- D_3 than vitamin D_3 , a natural substrate, although the reason for this specificity is not clear. Therefore, 1α -OH- D_3 is a suitable substrate to identify 25-hydroxylase due to efficient conversion to the 25-hydroxylated product. We tested 14 commercially available P450 enzymes to identify the P450 catalysing the 25-hydroxylation reaction. However, none showed any activity. Recently, Postlind *et al.* (1997) reported a novel 25-hydroxylase expressed in pig liver microsomes and from comparison of amino acid sequences – it was named as CYP2D25. They suggested the CYP2D family was the most likely candidate for the 25-hydroxylase. CYP2D6, the only P450 enzyme in the human 2D family, did not catalyse 25-hydroxylation and anti-human CYP2D6 antiserum did not inhibit 25-hydroxylation activity of human microsomes (data not shown). The results in the present study suggest the possible involvement of an as yet unidentified minor P450 in the 25-hydroxylation reaction, since the 14 P450s tested in this experiment encompass > 90% of the total liver P450 in content (Guengerich 1995). It is also well known that microsomal fraction contains unidentified inhibitory factors for the 25-hydroxylation reaction (Holmberg 1984). Therefore, the possibility that inhibitory factors might have existed in the commercially available microsomes prepared from the lymphoblastoid cells expressing P450 isoform can not be ruled out. However, these microsomes contained 50–500 pmol P450/mg of protein⁻¹ and the content was considerably higher than that of the individual P450 forms in human liver microsomes. Since a significant 25-hydroxylation activity was detected in human microsomes by our assay methods, the second possibility described above can be eliminated.

P450s are the major enzymes involved in the oxidation of drugs, xenobiotics and endobiotics such as steroids, fat-soluble vitamins. Of xenobiotic and drug metabolism, 90% is likely performed by five P450 species, i.e. CYP1A2, 2C9, 2D6, 2E1, 3A4, although there are considerable variations in the level of the individual forms of P450 (Guengerich 1995). The 1α -OH- D_3 is a prodrug that requires enzymatic activation to be transformed into the active form in the liver. To evaluate potential drug–drug interactions, the inhibition efficiency of 1α -OH- D_3 towards 14 P450 forms were examined. Of the 14 P450s, CYP2C8, 2C9-Cys, 2C19, 2D6-Val and 2E1 were inhibited to certain extents (75–50%) at 2500 pg ml⁻¹ 1α -OH- D_3 , implying a potential drug interaction via inhibition of these P450 forms at high doses. The normal dose of 1α -OH- D_3 for clinical use is at most 100 ng kg⁻¹ body weight, but the exact concentration in the human liver is unknown. However, considering that the concentration of 1α -OH- D_3 in the

liver was determined as 600 pg ml⁻¹ in maximum when 2 µg kg⁻¹ body weight of 1α-OH-D₃ was administrated to rat (Kamachi *et al.*, unpublished data), the concentration of 1α-OH-D₃ in human liver will be <30 pg ml⁻¹. Therefore, the inhibition data suggest that 1α-OH-D₃ does not affect xenobiotic metabolism by at least the 14 tested P450s at clinical doses.

In conclusion, although vitamin D₃ 25-hydroxylase could not be identified, it has been shown that at least 14 P450s tested were not involved in the vitamin D₃ 25-hydroxylation reaction. Considering that 25-hydroxylation is an indispensable reaction for vitamin D₃ activation, further research will be needed to identify 25-hydroxylase. 1α-OH-D₃ showed low but significant inhibitory effects on the catalytic activities of 2C8, 2C9-Cys, 2C19, 2D6-Val and 2E1 for their typical substrates at high concentrations (2.5 ng ml⁻¹). However, the inhibition data at the low concentration of 1α-OH-D₃ also indicated that 1α-OH-D₃ at the clinically used concentration would not significantly influence the catalytic activity of the 14 major P450s involved in the metabolism of >90% of xenobiotics and drugs.

References

- ANDERSSON, S., DAVIS, D. L., DAHLBÄCK, H., JÖRNVALL, H. and RUSSELL, D. W., 1989, Cloning, structure, and expression of the mitochondrial cytochrome P-450 sterol 26-hydroxylase, a bile acid biosynthetic enzyme. *Journal of Biological Chemistry*, **264**, 8222–8229.
- AXÉN, E., BERGMAN, T. and WIKVALL, K., 1992, Purification and characterization of a vitamin D₃ 25-hydroxylase from pig liver microsomes. *Biochemical Journal*, **287**, 725–731.
- BUSBY, W. F., JR., ACKERMANN, J. M. and CRESPI, C. L., 1999, Effect of methanol, ethanol, dimethyl sulfoxide, and acetonitrile on *in vitro* activities of cDNA-expressed human cytochromes P-450. *Drug Metabolism and Disposition*, **27**, 246–249.
- DAHLBÄCK, H. and WIKVALL, K., 1988, 25-Hydroxylation of vitamin D₃ by a cytochrome P-450 from rabbit liver mitochondria. *Biochemical Journal*, **252**, 207–213.
- DELUCA, H. F., 1988, The vitamin D story: a collaborative effort of basic science and clinical medicine. *FASEB Journal*, **2**, 224–236.
- FU, G. K., LIN, D., ZHANG, M. Y., BIKLE, D. D., SHACKLETON, C. H., MILLER, W. L. and PORTALE, A. A., 1997b, Cloning of human 25-hydroxyvitamin D-1α-hydroxylase and mutations causing vitamin D-dependent rickets type 1. *Molecular Endocrinology*, **11**, 1961–1970.
- FU, G. K., PORTALE, A. A. and MILLER, W. L., 1997a, Complete structure of the human gene for the vitamin D 1α-hydroxylase, P450c1α. *DNA and Cell Biology*, **16**, 1499–1507.
- FUKUSHIMA, M., NIKI, R., OHKAWA, H., SHIMIZU, T., MATSUNAGA, I., NAKANO, H., TAKAGAKI, Y., NISHII, Y., OKANO, K. and SUDA, T., 1980, Comparative therapeutic effects of vitamin D₃ and its derivatives on experimental renal osteodystrophy. *Endocrinology*, **107**, 328–333.
- FUKUSHIMA, M., NISHII, Y., SUZUKI, M. and SUDA, T., 1978, Comparative studies on the 25-hydroxylations of cholecalciferol and 1α-hydroxycholecalciferol in perfused rat liver. *Biochemical Journal*, **170**, 495–502.
- GUENGERICH F. P., 1995, Human cytochrome P450 enzymes. In P. R. Ortiz de Montellano (ed.), *Cytochrome P450* (New York: Plenum Press), pp. 473–535.
- GUO, Y.-D., STRUGNELL, S., BACK, D. W. and JONES, G., 1993, Transfected human liver cytochrome P-450 hydroxylates vitamin D analogs at different side-chain positions. *Proceedings of the National Academy of Sciences, USA.*, **90**, 8668–8672.
- HAYASHI, S., NOSHIO, M. and OKUDA, K., 1986, Isolation of a cytochrome P-450 that catalyzes the 25-hydroxylation of vitamin D₃ from rat liver microsomes. *Journal of Biochemistry (Tokyo)*, **99**, 1753–1763.
- HAYASHI, S., USUI, E. and OKUDA, K., 1988, Sex-related difference in vitamin D₃ 25-hydroxylase of rat liver microsomes. *Journal of Biochemistry (Tokyo)*, **103**, 863–866.
- HEWISON, M. and O'RIORDAN, J. L. H., 1997, Immunomodulatory and cell differentiation effects of vitamin D. In D. Feldman, F. H. Glorieux and J. W. Pike (eds) *Vitamin D* (San Diego: Academic Press), pp. 447–462.
- HOLMBERG, I., 1984, Inhibition of reconstituted vitamin D₃ 25-hydroxylase by a protein fraction from rat liver microsomes. *Biochemical and Biophysical Research Communications*, **123**, 1209–1214.
- HOLMBERG, I., BERLIN, T., EWERTH, S. and BJÖRKHEM, I., 1986, 25-Hydroxylase activity in subcellular fractions from human liver. Evidence for different rates of mitochondrial hydroxylation of vitamin D₂ and D₃. *Scandinavian Journal of Clinical and Laboratory Investigation*, **46**, 785–790.

- JONES, G., STRUGNELL, S. A. and DeLUCA H. F., 1998, Current understanding of the molecular actions of vitamin D. *Physiological Reviews*, **78**, 1193–1231.
- KAWASE, A., ICHIKAWA, F., KOIKE, N., KAMACHI, S., STUMPF, W. E., NISHII, Y. and KUBODERA, N., 2000, Synthesis and pharmacokinetics of 1 α -hydroxyvitamin D₃ tritiated at 22 and 23 positions showing high specific radioactivity. *Chemical and Pharmaceutical Bulletin*, **48**, 215–219.
- KITANAKA, S., TAKEYAMA, K., MURAYAMA, A., SATO, T., OKUMURA, K., NOGAMI, M., HASEGAWA, Y., NIIMI, H., YANAGISAWA, J., TANAKA, T. and KATO, S., 1998, Inactivating mutations in the 25-hydroxyvitamin D₃ 1 α -hydroxylase gene in patients with pseudovitamin D-deficiency rickets. *New England Journal of Medicine*, **338**, 653–661.
- KOIKE, N., ICHIKAWA, F., NISHII, Y. and STUMPF, W. E., 1998, Sustained osteoblast nuclear receptor binding of converted 1 α ,25-dihydroxyvitamin D₃ after administration of ³H-1 α -hydroxyvitamin D₃: A combined receptor autoradiography and radioassay time course study with comparison to ³H-1 α ,25-dihydroxyvitamin D₃. *Calcified Tissue International*, **63**, 391–395.
- MASUMOTO, O., OHYAMA, Y. and OKUDA, K., 1988, Purification and characterization of vitamin D 25-hydroxylase from rat liver mitochondria. *Journal of Biological Chemistry*, **263**, 14256–14260.
- NELSON, D. R., 2000 [<http://drnelson.utmem.edu/nelsonhomepage.html>].
- OHYAMA, Y., MASUMOTO, O., USUI, E. and OKUDA, K., 1991, Multi-functional property of rat liver mitochondrial cytochrome P-450. *Journal of Biochemistry (Tokyo)*, **109**, 389–393.
- OHYAMA, Y., HAYASHI, S.-I., USUI, M., NOSHIO, M., OKUDA, K.-I., 1997, Assay of vitamin D derivatives and purification of vitamin D hydroxylase. In D. B. McCormick, J. W. Suttie and C. Wagner (eds), *Methods in Enzymology* (New York: Academic Press), **282**, pp. 186–199.
- OKUDA, K., USUI, E. and OHYAMA, Y., 1995, Recent progress in enzymology and molecular biology of enzymes involved in vitamin D metabolism. *Journal of Lipid Research*, **36**, 1641–1652.
- POSTLIND, H., AXÉN, E., BERGMAN, T. and WIKVALL, K., 1997, Cloning, structure, and expression of a cDNA encoding vitamin D₃ 25-hydroxylase. *Biochemical and Biophysical Research Communications*, **241**, 491–497.
- ROSEN, H., RESHEF, A., MAEDA, N., LIPPOLDT, A., SHPIZEN, S., TRIGER, L., EGGERTSEN, G., BJÖRKHEM, I. and LEITERSDORF, E., 1998, Markedly reduced bile acid synthesis but maintained levels of cholesterol and vitamin D metabolites in mice with disrupted sterol 27-hydroxylase gene. *Journal of Biological Chemistry*, **273**, 14805–14812.
- SAAREM, K., BERGSETH, S., OFTEBRO, H. and PEDERSEN, J. I., 1984, Subcellular localization of vitamin D₃ 25-hydroxylase in human liver. *Journal of Biological Chemistry*, **259**, 10936–10940.
- SHINKI, T., SHIMADA, H., WAKINO, S., ANAZAWA, H., HAYASHI, M., SARUTA, T., DeLUCA, H. F. and SUDA, T., 1997, Cloning and expression of rat 25-hydroxyvitamin D₃-1 α -hydroxylase cDNA. *Proceedings of the National Academy of Sciences, USA*, **94**, 12920–12925.
- ST-ARNAUD, R., MESSERLIAN, S., MOIR, J. M., OMDAHL, J. L. and GLORIEUX, F. H., 1997, The 25-hydroxyvitamin D 1- α -hydroxylase gene maps to the pseudovitamin D-deficiency rickets (PDDR) disease locus. *Journal of Bone and Mineral Research*, **12**, 1552–1559.
- TAKEYAMA, K., KITANAKA, S., SATO, T., KOBORI, M., YANAGISAWA, J. and KATO, S., 1997, 25-Hydroxyvitamin D₃ 1 α -hydroxylase and vitamin D synthesis. *Science*, **277**, 1827–1830.
- USUI, E., NOSHIO, M. and OKUDA, K., 1990, Molecular cloning of cDNA for vitamin D₃ 25-hydroxylase from rat liver mitochondria. *FEBS Letters*, **262**, 135–138.
- YASUKOCHI, Y. and MASTERS, B. S., 1976, Some properties of a detergent-solubilized NADPH-cytochrome c (cytochrome P-450) reductase purified by biospecific affinity chromatography. *Journal of Biological Chemistry*, **251**, 5337–5344.

Roles of cytochrome P450 3A enzymes in the 2-hydroxylation of 1,4-cineole, a monoterpene cyclic ether, by rat and human liver microsomes

M. MIYAZAWA^{†*}, M. SHINDO[†] and T. SHIMADA^{‡*}

[†] Department of Applied Chemistry, Faculty of Science and Engineering, Kinki University, Kowakae, Higashiosaka, Osaka 577-8502, Japan

[‡] Osaka Prefectural Institute of Public Health, Nakamichi 1-chome, Higashinari-ku, Osaka 537-0025, Japan

Received 22 January 2001

1. Oxidation of 1,4-cineole, a monoterpene cyclic ether, was studied in rat and human liver microsomes and recombinant cytochrome P450 (P450 or CYP) enzymes expressed in insect cells in which human P450 and NADPH-P450 reductase cDNAs have been introduced. On analysis with gas chromatography/mass spectrometry, 2-*exo*-hydroxy-1,4-cineole was identified as a principal oxidation product of 1,4-cineole catalysed by rat and human P450 enzymes.

2. CYP3A4 was a major enzyme involved in the 2-hydroxylation of 1,4-cineole by human liver microsomes, based on the following lines of evidence. First, 1,4-cineole 2-hydroxylation activities catalysed by human liver microsomes were inhibited by ketoconazole, a potent inhibitor of CYP3A activities, and an anti-CYP3A4 antibody. Second, there was a good correlation between CYP3A4 contents and 1,4-cineole 2-hydroxylation activities in liver microsomes of eighteen human samples examined. Finally, of 10 recombinant human P450 enzymes examined, CYP3A4 had the highest activity for 1,4-cineole 2-hydroxylation.

3. Liver microsomal 1,4-cineole 2-hydroxylation activities were induced in rat by pregnenolone 16 α -carbonitrile and dexamethasone and extensively inhibited by ketoconazole, indicative of the possible roles of CYP3A enzymes in this reaction.

4. Kinetic analysis showed that $V_{\max}/K_m$ for 1,4-cineole 2-hydroxylation catalysed by liver microsomes was higher in a human sample HL-104 ($4.6 \mu\text{M}^{-1} \text{min}^{-1}$) than those of rat treated with pregnenolone 16 α -carbonitrile ($0.49 \mu\text{M}^{-1} \text{min}^{-1}$) and dexamethasone ($0.36 \mu\text{M}^{-1} \text{min}^{-1}$).

5. 1,8-Cineole, a structurally related monoterpene previously shown to be catalysed by CYP3A enzymes, inhibited 1,4-cineole 2-hydroxylation catalysed by human liver microsomes, whereas 1,4-cineole did not inhibit 1,8-cineole 2-hydroxylation activities. Both compounds caused inhibition of testosterone 6 β -hydroxylation by human liver microsomes, the former compound being more inhibitory than the latter.

6. These results suggest that 1,4-cineole and 1,8-cineole, two plant essential oils present in *Citrus medica* L. var. *acida* and *Eucalyptus polybractea*, respectively, are converted to 2-hydroxylated products by CYP3A enzymes in rat and human liver microsomes. It is unknown at present whether the 2-hydroxylation products of these compounds are more active biologically than the parent compound.

Introduction

Among numerous groups of naturally occurring compounds examined so far, volatile monoterpenes have long been used as fragrances and flavouring agents.

* Author for correspondence; e-mail: miyazawa@apch.kindai.ac.jp; shimada@iph.pref.osaka.jp

1,4-Cineole, a monoterpene cyclic ether, is known to be one of the flavour constituents of lime *Citrus medica* L. var. *acida* (Kovats 1963) (figure 1).

Microorganisms are increasingly being used as biocatalysts to alter the structures of organic compounds. It was reported that *Aspergillus niger* catalyses conversion of 1,4-cineole to four hydroxylated metabolites, namely 2-*endo*-hydroxy-1,4-cineole (Miyazawa *et al.* 1991), 3-*endo*-hydroxy-1,4-cineole (Miyazawa *et al.* 1992a), 9-hydroxy-1,4-cineole (Miyazawa *et al.* 1992b) and 8-hydroxy-1,4-cineole (Miyazawa *et al.* 1992c). *Streptomyces griseus* has also been shown to oxidize 1,4-cineole to form 8-hydroxy-1,4-cineole (Rosazza *et al.* 1987, 1988). Interestingly, P450 in *Bacillus cereus* converts 1,4-cineole to 2-*exo*-hydroxy- (3.8%) and 2-*endo*-hydroxy- (21.3%) metabolites yielding a ratio of *exo*-/*endo*-products of 1:7 (Liu *et al.* 1988, 1993, Liu and Rosazza 1993).

A variety of components in numerous species of plants are oxidized by multiple forms of cytochrome P450 in mammals (Gonzalez and Nebert 1990, Nelson *et al.* 1993, Khojasteh-Bakht *et al.* 1999). P450 enzymes are shown to detoxify and/or toxify these compounds to more polar and sometimes more reactive metabolites (Thomassen *et al.* 1988, Gonzalez 1989, Guengerich and Shimada 1991, Guengerich 1992, Khojasteh-Bakht *et al.* 1999). Recently, it was reported that 1,8-cineole, a monoterpene structurally related to 1,4-cineole present as eucalyptus oil in *Eucalyptus polybractea* (figure 1), is highly catalysed by CYP3A enzymes to form 2-*exo*-hydroxy-1,8-cineole in rat and human liver microsomes (Miyazawa *et al.* 2001). Several monoterpenes such as limonene and 1,8-cineole have been shown to cause nephrotoxicity in the male rat by a mechanism involving α 2u-globulin and several investigations have suggested that hydroxylated metabolites of these monoterpenes are more active in interacting with α 2u-globulin in the rat liver

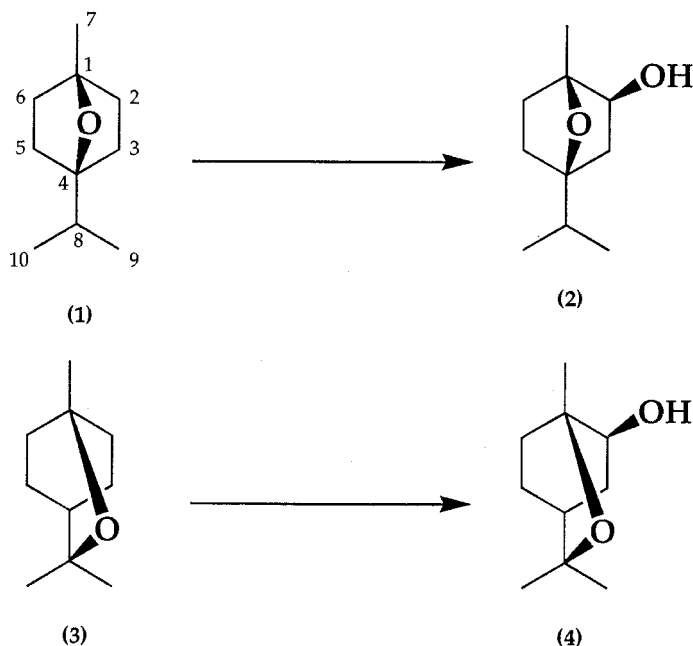


Figure 1. Structures of 1,4-cineole (1), 2-*exo*-hydroxy-1,4-cineole (2), 1,8-cineole (3) and 2-*exo*-hydroxy-1,8-cineole (4).

(Hard *et al.* 1993, Kristiansen and Madsen 1995, Hard 1998). In this regard, it is interesting to examine how 1,4-cineole is metabolized by P450 enzymes in rat and human liver microsomes.

In the present investigation, we determined the metabolism of 1,4-cineole by rat and human liver microsomes and by recombinant human P450 enzymes expressed in insect cells in which both human P450 and NADPH-P450 reductase cDNAs have been introduced (Inoue *et al.* 1999, Shimada *et al.* 1999). The effects of chemical inhibitors of P450 activities and specific antibodies to human P450 enzymes on 1,4-cineole oxidation by liver microsomes are also reported.

Materials and methods

Chemicals

1,4-Cineole (purity > 99%) was purchased from Fluka Co. (Tokyo, Japan). Ketoconazole, NADP⁺, glucose 6-phosphate and glucose 6-phosphate dehydrogenase were purchased from Sigma Chemical Co. (St Louis, MO, USA). Testosterone and its hydroxylated metabolites were obtained from sources described by Yamazaki *et al.* (1997). 2-*exo*-Hydroxy-1,4-cineole (purity > 99%) was isolated and purified from cultured medium on incubation of 1,4-cineole with *Gromellera cingulata* (M. Miyazawa and M. Shindo, unpublished data). Other reagents and chemicals used were obtained from sources as described or of highest qualities commercially available (Shimada *et al.* 1994, 1998).

Enzyme preparation

Human (male) liver samples (aged 29–67 years) were obtained from healthy organ donors or patients, mostly with liver tumours, undergoing liver resection as described (Shimada *et al.* 1994, Mimura *et al.* 1993). Male Sprague-Dawley rats (weighing ~200g) obtained from Nihon Clea Co. (Osaka, Japan) were treated with β -naphthoflavone and isosafrole (50 mg kg⁻¹, daily for 3 days), phenobarbital (80 mg kg⁻¹, daily for 3 days), and PCN and DEX (100 and 50 mg kg⁻¹, respectively, daily for 3 days). Ethanol was given to rats in drinking water for 6 days at a final concentration of 15% (v/v). Rats were starved overnight before killing. Pooled livers of three rats in these groups were used. Liver microsomes were prepared as described and suspended in 10 mM Tris-HCl buffer (pH 7.4) containing 1.0 mM EDTA and 20% glycerol (v/v) (Guengerich 1994).

Recombinant CYP1A1, 1A2, 1B1, 2A6, 2B6, 2C9, 2C19, 2D6, 2E1 and 3A4 expressed in microsomes of *Trichoplusia ni* cells infected with a baculovirus containing human P450 and NADPH-P450 reductase cDNA inserts were obtained from Gentest Co. (Woburn, MA, USA); the P450 contents in these systems were used as described in the data sheets provided by the manufacturer.

Rabbit anti-sera raised against purified human liver CYP3A4 was prepared as described (Mimura *et al.* 1993). The anti-CYP3A4 was found to cross-react with CYP3A5 as well as CYP3A4 (Yamazaki *et al.* 1994).

GC/MS analysis

A Hewlett-Packard model 5890A gas chromatograph equipped with a split injector was combined by direct coupling to a Hewlett-Packard 5972 mass spectrometer. The metabolites were separated with a TC-WAX FFS silica capillary column (0.25 mm $\times$ ~60 m) using helium (at 1 ml min⁻¹) as a carrier gas. The column temperature was programmed from 80 to 180°C linearly at 4°C min⁻¹ and then held at 180°C for 10 min. The injector temperature was at 270°C. The detector interface temperature was 280°C with the actual temperature in the MS source reaching ~180°C and the ionization voltage of 70 eV. The electron impact (EI) mode was used.

Oxidation of 1,4-cineole by P450 enzymes

1,4-Cineole oxidation activities by P450 enzymes were determined as follows. The standard reaction mixture contained rat and human liver microsomes (0.025 mg protein ml⁻¹) or recombinant P450 (50 pmol ml⁻¹) and 200 μ M 1,4-cineole in a final volume of 0.50 ml 100 mM potassium phosphate buffer (pH 7.4) containing an NADPH-generating system (0.5 mM NADP⁺, 5 mM glucose 6-phosphate, 0.5 unit of glucose 6-phosphate dehydrogenase ml⁻¹) (Shimada *et al.* 1996). Incubations were carried out at 37°C for 30 min and terminated by adding 1.0 ml ethyl acetate. The extracts (organic layer) were collected by centrifugation at 900g for 5 min and transferred to an insert for analysis by GC-MS for identification of the metabolites.

Testosterone hydroxylation assay

Testosterone hydroxylation activities were determined as described (Yamazaki *et al.* 1997). The standard incubation mixture (final volume of 0.20 ml) contained liver microsomes (0.25 μM P450), 50 mM potassium phosphate buffer (pH 7.4), an NADPH-generating system (Shimada *et al.* 1994), and testosterone (50 μM). Incubations were carried out at 37°C for 15 min and terminated by adding 1.5 ml dichloromethane and 0.3 M sodium chloride. Product formation was determined by HPLC with a C18 (5 μm) analytical column (4.6 $\times$ 150 mm). The elution was conducted with a mixture of 64% methanol (v/v) at 1.2 ml/min⁻¹ and detection was by UV absorbance at 240 nm.

Other assays

P450 contents were estimated spectrally by the original method (Omura and Sato 1964). The contents of human CYP3A proteins in liver microsomes were estimated by coupled sodium dodecyl sulphate-polyacrylamide gel electrophoresis/immunochemical development ('Western-blotting') (Guengerich *et al.* 1982). The intensities of the immunoblots were measured with an Epson GT-8000 Scanner equipped with NIH Image/Gel Analysis Program adapted for Macintosh computers. Protein concentrations were estimated by the method of Lowry *et al.* (1951).

Statistical analysis

Kinetic parameters for 1,4-cineole 2-hydroxylation by rat and human P450 enzymes were estimated using a computer program (Kareida Graph from Synergy Software, Reading, PA, USA) designed for non-linear regression analysis.

Results

Oxidation of 1,4-cineole by rat and human liver microsomes

1,4-Cineole was incubated with rat and human liver microsomes in the presence of an NADPH-generating system and detected 2-hydroxy-1,4-cineole as the only oxidation product on analysis by GC-MS equipped with EI-MS as described in the Materials and methods. Product formation was increased with substrate concentration up to 200 μM with rat and human liver microsomes. Mass fragments (m/z) of this metabolite were 170 (% relative abundance, 36; M^+), 153 (22; $\text{M}^+ - \text{OH}$), 127 (28; $\text{M}^+ - \text{C}_3\text{H}_7$), 122 (77; $\text{M}^+ - \text{C}_3\text{H}_7 - \text{CH}_3$), 109 (33; $\text{M}^+ - \text{H}_2\text{O} - \text{C}_3\text{H}_7$), and 71 (100). The metabolite formed was suggested to be 2-*exo*-hydroxy-1,4-cineole, since the molecular mass of this metabolite was increased from 153 (1,4-cineole) to 170 (a metabolite), probably reflecting an insertion of a hydroxyl group into the 1,4-cineole molecule. The position and relative stereochemistry of a hydroxyl group were determined by relative abundance of mass fragment and retention time by GC. These values were consistent with those of authentic 2-*exo*-hydroxy-1,4-cineole which was isolated from biotransformation products of 1,4-cineole with *Gromellera cingulata* (M. Miyazawa and M. Shindo, unpublished data).

1,4-Cineole 2-exo-hydroxylation activities by rat and human liver microsomes

Effects of treatment of rats with several P450 inducers on liver microsomal 1,4-cineole 2-hydroxylation activities were determined (table 1). Liver microsomes of untreated rats had very low 1,4-cineole 2-hydroxylation activity ($< 0.05 \text{ nmol min}^{-1} \text{ nmol}^{-1}$ P450). PCN and DEX increased 1,4-cineole 2-hydroxylation activities by liver microsomes whereas other chemical inducers including phenobarbital, β -naphthoflavone, isosafrole and ethanol did not.

The average rate of 1,8-cineole 2-hydroxylation by liver microsomes of 18 human samples was $0.55 \pm 0.58 \text{ nmol min}^{-1} \text{ nmol}^{-1}$ P450; the rates were similar to

Table 1. Effects of treatment with chemical inducers on liver microsomal 1,4-cineole 2-hydroxylation activities in the male rat.

Treatment of rat	P450 content (nmol mg ⁻¹ protein)	1,4-Cineole 2-hydroxylation (nmol min ⁻¹ nmol ⁻¹ P450)
None	0.67	< 0.05
β -Naphthoflavone	1.16	< 0.05
Isosafrole	1.10	< 0.05
Phenobarbital	1.48	< 0.05
Ethanol	0.96	< 0.05
PCN	1.41	0.46
DEX	1.62	0.37

1,4-Cineole 2-hydroxylation activities by liver microsomes of rat treated with various chemical inducers were determined as described in the Materials and methods. Data are averages of duplicate determinations.

those catalysed by liver microsomes of rat treated with PCN and DEX. There was good correlation between CYP3A4 contents and 1,4-cineole 2-hydroxylation activities in liver microsomes of 18 human samples ($r = 0.95$) (figure 2).

Ketoconazole significantly decreased the 1,4-cineole 2-hydroxylation activities catalysed by liver microsomes of rat treated with PCN and of human sample HL-104 (figure 3), which contained a high level of CYP3A4 in liver microsomes (Shimada *et al.* 1994). Anti-CYP3A4 IgG (5 and 10 mg IgG nmol⁻¹ P450) markedly inhibited 1,4-cineole 2-hydroxylation activities by 95 and 99%, respectively, in liver microsomes of HL-104, whereas pre-immune IgG did not cause any significant decreases in the activities.

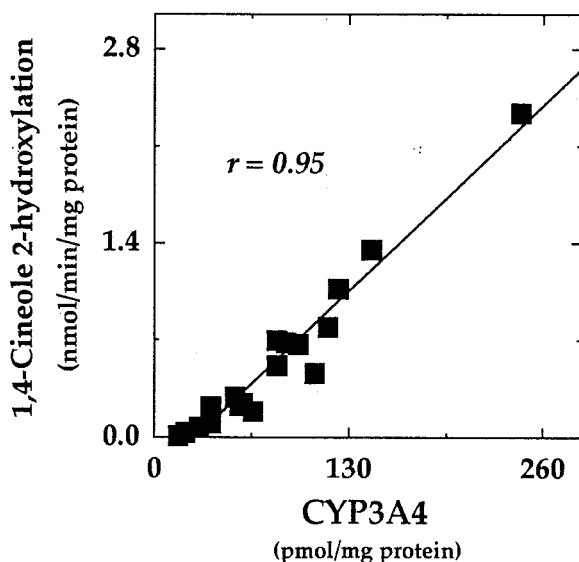


Figure 2. Correlation between the contents of CYP3A4 and activities of 1,4-cineole 2-hydroxylation in liver microsomes of 18 human samples. Data are averages of duplicate determinations.

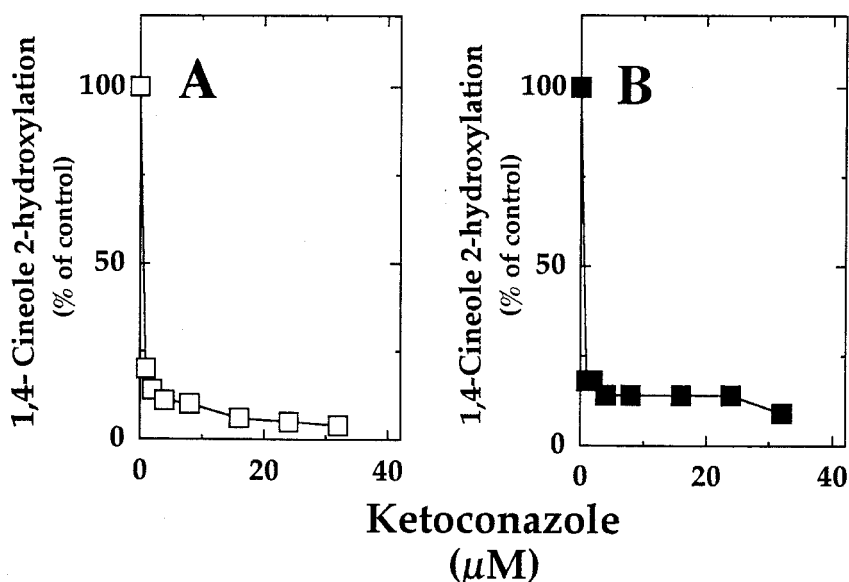


Figure 3. Effects of ketoconazole on 1,4-cineole 2-hydroxylation activities in liver microsomes of human sample HL-104 (A) and rat treated with PCN (B). Substrate concentration used was $500 \mu\text{M}$ 1,4-cineole. The corresponding control activities (in the absence of ketoconazole) were 1.64 and $0.44 \text{ nmol min}^{-1} \text{ nmol}^{-1}$ P450 for liver microsomes of human (A) and rat (B), respectively. Data are averages of duplicate determinations.

1,4-Cineole 2-hydroxylation activities by recombinant human P450 enzymes

Ten forms of recombinant human P450 enzymes expressed in insect cells were used to determine which P450 enzymes are the major catalysts for the 1,4-cineole 2-hydroxylation activities in humans (figure 4). This study did not determine whether cytochrome b_5 stimulated the activities by these P450 enzymes. CYP3A4 supported the highest rate of 1,4-cineole 2-hydroxylation, followed by 2B6 and 2A6. Other P450 enzymes including CYP1A1, 1A2, 1B1, 2C9, 2C19, 2D6 and 2E1 had very low activity or activities below the limit of detection.

Kinetic analysis of 1,4-cineole 2-hydroxylation by rat and human liver microsomes and by recombinant human P450 enzymes

Kinetic analysis of 1,4-cineole 2-hydroxylation activities by P450 enzymes was carried out in the liver microsomes of rat treated with PCN and DEX and in human sample HL-104 and in recombinant systems containing CYP2A6, 2B6 and 3A4 (table 2). Substrate concentrations used were 10, 25, 50, 100, 250, 500 and $1000 \mu\text{M}$. K_m for 1,4-cineole 2-hydroxylation with liver microsomes of rat treated with PCN and DEX was 1033 and $1123 \mu\text{M}$, respectively, and was higher than that of liver microsomes of HL-104 ($743 \mu\text{M}$). V_{max} was greater in liver microsomes of HL-104 ($3.43 \text{ nmol min}^{-1} \text{ nmol}^{-1}$ P450) than in those of the PCN-treated ($0.51 \text{ nmol min}^{-1} \text{ nmol}^{-1}$ P450) and DEX-treated rat ($0.41 \text{ nmol min}^{-1} \text{ nmol}^{-1}$ P450). K_m and V_{max} for 1,4-cineole 2-hydroxylation by recombinant human P450 enzymes were $530 \mu\text{M}$ and $3.5 \text{ nmol min}^{-1} \text{ nmol}^{-1}$ P450 for CYP2A6, $360 \mu\text{M}$ and $3.4 \text{ nmol min}^{-1} \text{ nmol}^{-1}$ P450 for CYP2B6, and $450 \mu\text{M}$ and $7.5 \text{ nmol min}^{-1} \text{ nmol}^{-1}$ P450 for CYP3A4, respectively (table 2).

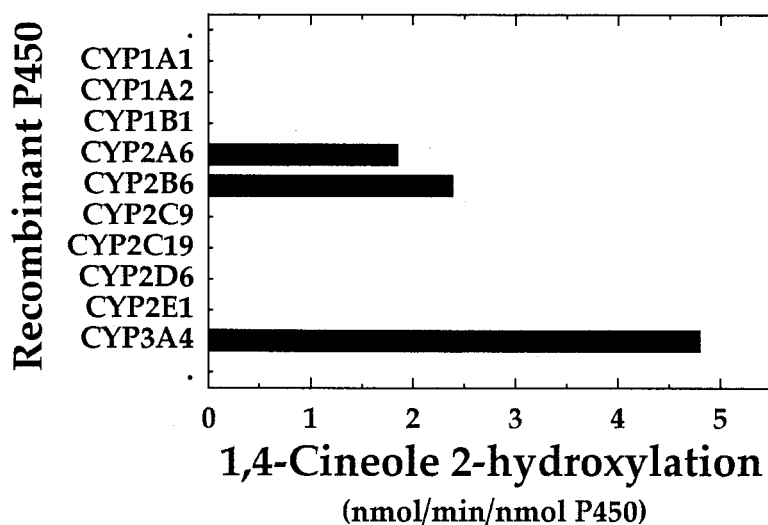


Figure 4. 1,4-Cineole 2-hydroxylation activities by recombinant human P450 enzymes expressed in insect cells. Data are averages of duplicate determinations.

Effects of 1,4-cineole and 1,8-cineole on 2-hydroxylation activities of these two compounds catalysed by human liver microsomes

1,8-Cineole inhibited non-competitively the 1,4-cineole 2-hydroxylation activities, whereas 1,4-cineole did not inhibit the 1,8-cineole 2-hydroxylation activities (figure 5A and B). Interestingly, 1,4-cineole inhibited testosterone 6 β -hydroxylation, a typical reaction catalysed by CYP3A4, more extensively than 1,8-cineole in human liver microsomes (figure 5C).

Discussion

The metabolism of 1,4-cineole by rat and human liver microsomal P450 enzymes was studied. As for the case for 1,8-cineole (Miyazawa *et al.* 2001), 1,4-cineole was also converted to the 2-*exo*-hydroxylated metabolite by rat and human liver microsomes. We could not detect any other oxidation products under the

Table 2. Kinetic analysis of 1,4-cineole 2-hydroxylation activities by liver microsomes of human sample HL-104, of rat treated with PCN and DEX, and by recombinant P450 systems containing CYP2A6, CYP2B6 and CYP3A4.

Enzymes	K_m (μM)	$V_{\max}$ (nmol min ⁻¹ nmol ⁻¹ P450)	$V_{\max}/K_m$ (μM^{-1} min ⁻¹)
Liver microsomes			
Human (HL-104)	743	3.43	4.62
PCN-treated rat	1033	0.51	0.49
DEX-treated rat	1123	0.41	0.37
Recombinant P450			
2A6	530	3.5	6.6
2B6	360	3.4	9.4
3A4	450	7.5	16.0

Kinetic parameters of 1,4-cineole 2-hydroxylation activities by liver microsomal and recombinant P450 systems were determined as described in the Materials and methods.

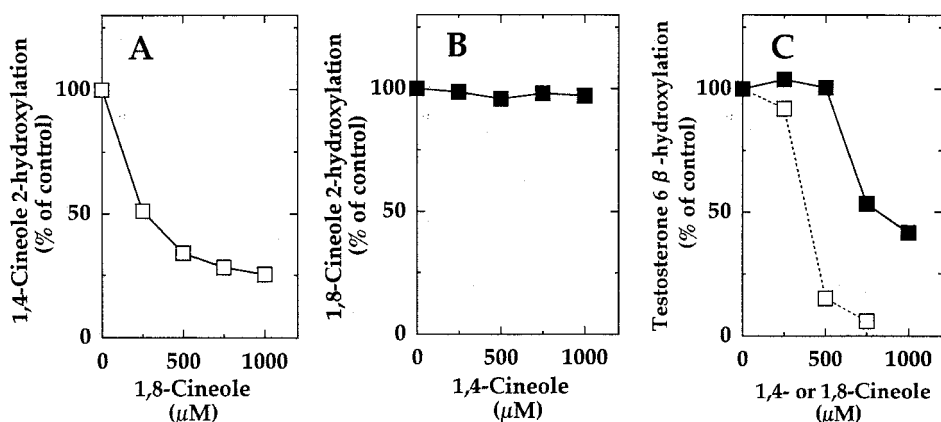


Figure 5. Effects of 1,8-cineole and 1,4-cineole on 1,4-cineole 2-hydroxylation (A) and 1,8-cineole 2-hydroxylation (B), respectively, activities in liver microsomes of human sample HL-104. Effects of 1,4- and 1,8-cineole on testosterone 6 β -hydroxylation by liver microsomes of HL-104 (C) were also determined. The 1,4-cineole 2-hydroxylation activity in the absence of 1,8-cineole (A) was $1.74 \text{ nmol min}^{-1} \text{ nmol}^{-1} \text{ P450}$ and the 1,8-cineole 2-hydroxylation activity in the absence of 1,4-cineole (B) was $63.1 \text{ nmol min}^{-1} \text{ nmol}^{-1} \text{ P450}$ in liver microsomes of human and of rat treated with PCN, respectively. The testosterone 6-hydroxylation activity in the absence of 1,4- and 1,8-cineole (C) was $10.5 \text{ nmol min}^{-1} \text{ nmol}^{-1} \text{ P450}$. Data are from a single determination.

assay conditions. The mass fragment analysis suggested the formation of 2-*exo*-hydroxy-1,4-cineole, which is identical to that produced by *Gromellera cingulata* as biocatalyst (M. Miyazawa and M. Shindo, unpublished data).

CYP3A4 was identified as a major enzyme involved in 1,4-cineole 2-hydroxylation by human liver microsomes with following lines of evidence. Ketoconazole and anti-CYP3A4 significantly inhibited the 1,4-cineole 2-hydroxylation activities and there was a good correlation between 1,4-cineole 2-hydroxylation activities and CYP3A4 in liver microsomes of 18 human samples. In recombinant human P450 systems, CYP3A4 had the highest activities of 1,4-cineole 2-hydroxylation activities, followed by CYP2A6 and 2B6; the rates catalysed by CYP2B6 and 2A6 were about half of that catalysed by CYP3A4. It is interesting to note that CYP2A6 is expressed in man in olfactory and other respiratory organs as well as in the liver (Pelkonen *et al.* 2000) with regard to the volatile nature of 1,4-cineole monoterpene. Kinetic analysis showed that the ratio of V_{max}/K_m for 1,4-cineole 2-hydroxylation activity for recombinant CYP3A4 ($16.0 \mu\text{M}^{-1} \text{ min}^{-1}$) was 2.5- and 1.7-fold higher than those in CYP2A6 ($6.6 \mu\text{M}^{-1} \text{ min}^{-1}$) and in 2B6 ($9.4 \mu\text{M}^{-1} \text{ min}^{-1}$), respectively. The levels of expression of CYP3A4 have been reported to be significantly higher than those of CYP2A6 and CYP2B6 in 30 Japanese and 30 Caucasians determined (Shimada *et al.* 1994). These results strongly suggest that CYP3A4 is a major enzyme in 1,4-cineole 2-hydroxylation in human livers.

In rat, there are at least four CYP3A enzymes and among them CYP3A1 and 3A2 are the major enzymes in liver microsomes (Maurel 1996). 1,4-Cineole 2-hydroxylation activities by liver microsomes were induced in rat by CYP3A inducers, PCN and DEX, and these activities were significantly inhibited by ketoconazole, suggesting that CYP3A1 and 3A2 enzymes contribute to the hydroxylation reaction. However, the ratio of V_{max}/K_m for 1,4-cineole 2-hydroxylation activities by liver microsomes of the PCN-treated and DEX-treated rat

was 9- and 13-fold lower, respectively, than that catalysed by human sample HL-104 which contained a high level of CYP3A4. These results suggest that there may be species-related differences in the function of CYP3A enzymes in catalysing 1,4-cineole between rat and man.

These results suggest that 1,4-cineole as well as 1,8-cineole (Miyazawa *et al.* 2001) are metabolized to their 2-*exo*-hydroxylated products by CYP3A enzymes in rat and human liver microsomes. This study found that 1,8-cineole inhibited non-competitively 1,4-cineole 2-hydroxylation activities catalysed by human liver microsomes, whereas 1,4-cineole did not affect 1,8-cineole 2-hydroxylation. Interestingly, 1,4-cineole inhibited microsomal testosterone 6 β -hydroxylation, one of the marker activities of CYP3A4 in man (Waxman *et al.* 1988, Shimada *et al.* 1994), more strongly than 1,8-cineole did. The mechanisms underlying different inhibition potencies of these two compounds on 2-hydroxylation activities of the another compound are unknown at present. The ratio of $V_{\max}/K_m$ for 1,8-cineole 2-hydroxylation activity ($182\ \mu\text{M}^{-1}\text{min}^{-1}$) (Miyazawa *et al.* 2001) was 40-fold higher than that ($4.62\ \mu\text{M}^{-1}\text{min}^{-1}$) of 1,4-cineole 2-hydroxylation in liver microsomes of sample HL-104. These results suggest that 1,8-cineole is better substrate for CYP3A4 than 1,4-cineole in human liver microsomes.

It has previously been shown that several microorganisms including *Aspergillus niger*, *Streptomyces griseus* and *Bacillus cereus* oxidize 1,4-cineole to several oxidation products including 2-*endo*-hydroxy-1,4-cineole, 3-*endo*-hydroxy-1,4-cineole, 9-hydroxy-1,4-cineole, and 8-hydroxy-1,4-cineole (Liu *et al.* 1988, 1993, Rosazza *et al.* 1987, 1988, Miyazawa *et al.* 1991, 1992a-c, Liu and Rosazza 1993). P450 in *B. cereus* reportedly converts 1,4-cineole to 2-*exo*-hydroxy-1,4-cineole as well as 2-*endo*-hydroxy-1,4-cineole in which hydroxylation is preferred at the more hindered *endo*-face; the ratio of formation of *exo*-/*endo*-products in this microorganism was shown to be 1:7. In rat and human liver microsomes, 2-*exo*-hydroxy-1,4-cineole was detected as only an oxidation product. These results suggest that metabolism of 1,4-cineole to 2-*exo*-hydroxy-1,4-cineole by rat and human liver microsomal P450 may be stereoselective as compared with microorganisms. From above findings, it is suggested that the oxygen atom is inserted from the less-hindered *exo*-face of 1,4-cineole by mammalian P450 enzymes.

In conclusion, the present study showed that 1,4-cineole, as in the case of 1,8-cineole (Miyazawa *et al.* 2001), is catalysed by CYP3A enzymes to form 2-*exo*-hydroxy-1,4-cineole in rat and human liver microsomes. CYP3A4 is a principal enzyme in this reaction catalysed by human liver microsomes. In rat, 1,4-cineole 2-hydroxylation is catalysed by CYP3A1/2 enzymes induced by PCN and DEX in liver microsomes. 1,4-Cineole did not inhibit 1,8-cineole 2-hydroxylation by human liver microsomes, whereas 1,8-cineole inhibited 1,4-cineole 2-hydroxylation activities. The mechanisms underlying the different inhibition potencies of these two compounds on the 2-hydroxylase activities of the opposite compound are unknown at present.

Acknowledgements

The work was supported in part by Grants from the Ministry of Education, Science, and Culture of Japan, the Ministry of Health and Welfare of Japan, the Developmental and Creative Studies from Osaka Prefectural Government.

References

- GONZALEZ, F. J., 1989, The molecular biology of cytochrome P450s. *Pharmacology Review*, **40**, 243–288.
- GONZALEZ, F. J. and NEBERT, D. W., 1990, Evolution of the P450 gene superfamily: animal-plant warfare, molecular drive and human genetic differences in drug oxidation. *Trends in Genetics*, **6**, 182–186.
- GUENGERICH, F. P., 1992, Human cytochrome P-450 enzymes. *Life Sciences*, **50**, 1471–1478.
- GUENGERICH, F. P., 1994, Analysis and characterization of enzymes. In A. W. Hayes (ed.), *Principles and Methods of Toxicology* (New York: Raven), pp. 1259–1313.
- GUENGERICH, F. P., WANG, P. and DAVIDSON, N. K., 1982, Estimation of isozymes of microsomal cytochrome P-450 in rats, rabbits, and humans using immunochemical staining coupled with sodium dodecyl sulfate-polyacrylamide gel electrophoresis. *Biochemistry*, **21**, 1698–1706.
- GUENGERICH, F. P. and SHIMADA, T., 1991, Oxidation of toxic and carcinogenic chemicals by human cytochrome P-450 enzymes. *Chemical Research in Toxicology*, **4**, 391–407.
- HARD, G. C., 1998, Mechanisms of chemically induced renal carcinogenesis in the laboratory rodent. *Toxicology and Pathology*, **26**, 104–112.
- HARD, G. C., RODGERS, I. S., BAETCKE, K. P., RICHARDS, W. L., MCGAUGHY, R. E. and VALCOVIC, L. R., 1993, Hazard evaluation of chemicals that cause accumulation of α 2u-globulin, hyaline droplet nephropathy, and tubule neoplasia in the kidneys of male rats. *Environmental Health Perspectives*, **99**, 313–349.
- INOUE, K., TANAKA, M., YAMAZAKI, H. and SHIMADA, T., 1999, CYP2A6 genetic polymorphisms and liver microsomal coumarin and nicotine oxidation activities in Japanese and Caucasians. *Archives in Toxicology*, **73**, 532–539.
- KHOJASTEH-BAKHT, S. C., CHEN, W., KOENIGS, L. L., PETER, R. M. and NELSON, S. D., 1999, Metabolism of (R)-(+)-pulegone and (R)-(+)-menthofuran by human liver cytochrome P-450s: evidence for formation of a furan epoxide. *Drug Metabolism and Disposition*, **27**, 574–580.
- KOVATS, E. S., 1963, Zur Kenntnis des sog. (destillierten) Limetten-Öls (*Citrus medica*, L., var. *acida*, Brandis; *Citrus aurantifolia*, Swingle). *Helvetica Chimica Acta*, **46**, 2705–2731.
- KRISTIANSEN, E. and MADSEN, C., 1995, Induction of protein droplet (α 2u-globulin) nephropathy in male rats after short-term dosage with 1,8-cineole and *l*-limonene. *Toxicology Letters*, **80**, 147–152.
- LIU, W. G., GOSWAMI, A., STEEK, R. P., CHAPMAN, R., SARIASLANI, F. S., STEENS, J. J. and ROSAZZA, J. P. N., 1988, Stereochemistry of microbiological hydroxylations of 1,4-cineole. *Journal of Organic Chemistry*, **53**, 5700–5704.
- LIU, W. G. and ROSAZZA, J. P. N., 1993, A soluble *Bacillus cereus* cytochrome P-450cin system catalyses 1,4-cineole hydroxylations. *Applied and Environmental Microbiology*, **59**, 3889–3893.
- LIU, W. G., STEENS, J. J., GOSWAMI, A. and ROSAZZA, J. P. N., 1993, Hydroxylation of deuterated 1,4-cineoles by *Bacillus cereus*. *Journal of Organic Chemistry*, **58**, 6329–6335.
- LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. and RANDALL, R. J., 1951, Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry*, **193**, 265–275.
- MAUREL, P., 1996, The CYP3 family. In C. Ioannides (ed.), *Cytochromes P450: Metabolic and Toxicological Aspects* (New York: CRC Press), pp. 241–270.
- MIMURA, M., BABA, T., YAMAZAKI, H., OHMORI, S., INUI, Y., GONZALEZ, F. J., GUENGERICH, F. P. and SHIMADA, T., 1993, Characterization of cytochrome P450 2B6 in human liver microsomes. *Drug Metabolism and Disposition*, **21**, 1048–1056.
- MIYAZAWA, M., NOMA, Y., YAMAMOTO, K. and KAMEOKA, H., 1991, Biotransformation of 1,4-cineole to 2-endo-hydroxy-1,4-cineole by *Aspergillus niger*. *Chemistry Express*, **6**, 771–774.
- MIYAZAWA, M., NOMA, Y., YAMAMOTO, K. and KAMEOKA, H., 1992a, Biotransformation of 1,4-cineole to 3-endo-hydroxy-1,4-cineole by *Aspergillus niger*. *Chemistry Express*, **7**, 125–128.
- MIYAZAWA, M., NOMA, Y., YAMAMOTO, K. and KAMEOKA, H., 1992b, Biotransformation of 1,4-cineole to 9-hydroxy-1,4-cineole by *Aspergillus niger*. *Chemistry Express*, **7**, 305–308.
- MIYAZAWA, M., NOMA, Y., YAMAMOTO, K. and KAMEOKA, H., 1992c, Biotransformation of 1,4-cineole to 8-hydroxy-1,4-cineole by *Aspergillus niger*. *Chemistry Express*, **7**, 721–724.
- MIYAZAWA, M., SHINDO, M. and SHIMADA, T., 2001, Oxidation of 1,8-cineole, the monoterpene cyclic ether originated from *Eucalyptus Polybractes*, by cytochrome P450 3A enzymes in rat and liver microsomes. *Drug Metabolism and Disposition* (in press).
- NELSON, D. R., KAMATYAKI, T., WAXMAN, D. J., GUENGERICH, F. P., ESTABROOK, R. W., FEYEREISEN, R., GONZALEZ, F. J., COON, M. J., GUNSALUS, I. C., GOTOH, O., OKUDA, K. and NEBERT, D. W., 1993, The P450 superfamily: update on new sequences, gene mapping, accession numbers, early trivial names of enzymes, and nomenclature. *DNA and Cell Biology*, **12**, 1–51.
- OMURA, T. and SATO, R., 1964, The carbon monoxide-binding pigment of liver microsomes. I. Evidence for its hemoprotein nature. *Journal of Biological Chemistry*, **239**, 2370–2378.
- PELKONEN, O., RAUTIO, A., RAUNIO, H. and PASANEN, M., 2000, CYP2A6: a human coumarin 7-hydroxylase. *Toxicology*, **144**, 139–147.

- ROSAZZA, J. P. N., GOSWAMI, A., LIU, W. G., SARIASLANI, F. S., STEENS, J. J., STEEK, R. P., BEALE, J. M., JR, CHAPMAN, R. and REEG, S., 1988, Microbial transformations of terpenes: studies with 1,4-cineole. *Journal of Industrial Microbiology*, **29**, 181–189.
- ROSAZZA, J. P. N., STEENS, J. J., SARIASLANI, F. S., GOSWAMI, A., BEALE, J. M. JR., REEG, S. and CHAPMAN, R., 1987, Microbial hydroxylation of 1,4-cineole. *Applied and Environmental Microbiology*, **53**, 2482–2486.
- SHIMADA, T., TSUMURA, F. and YAMAZAKI, H., 1999, Prediction of human liver microsomal oxidations of 7-ethoxycoumarin and chlorzoxazone using kinetic parameters of recombinant cytochrome P450 enzymes. *Drug Metabolism and Disposition*, **27**, 1274–1280.
- SHIMADA, T., YAMAZAKI, H., FOROOZESH, M., HOPKINS, N. E., ALWORTH, W. L. and GUENGERICH, F. P., 1998, Selectivity of polycyclic inhibitors for human cytochromes P450 1A1, 1A2, and 1B1. *Chemical Research in Toxicology*, **11**, 1048–1056.
- SHIMADA, T., YAMAZAKI, H., MIMURA, M., INUI, Y. and GUENGERICH, F. P., 1994, Interindividual variations in human liver cytochrome P-450 enzymes involved in the oxidation of drugs, carcinogens and toxic chemicals: studies with liver microsomes of 30 Japanese and 30 Caucasians. *Journal of Pharmacology Experimental Therapeutics*, **270**, 414–423.
- SHIMADA, T., YAMAZAKI, H., MIMURA, M., WAKAMIYA, N., GUENGERICH, F. P. and INUI, Y., 1996, Characterization of microsomal cytochrome P450 enzymes involved in the oxidation of xenobiotic chemicals in human fetal livers and adult lungs. *Drug Metabolism and Disposition*, **24**, 515–522.
- THOMASSEN, D., SLATTERY, J. T. and NELSON, S. D., 1988, Contribution of menthofuran to the hepatotoxicity of pulegone: assessment based on matched area under the curve and on matched time course. *Journal of Pharmacology Experimental Therapeutics*, **244**, 825–829.
- WAXMAN, D. J., ATTISANA, C., GUENGERICH, F. P. and LAPENSON, D. P., 1988, Human liver microsomal steroid metabolism: identification of the major microsomal steroid hormone 6 β -hydroxylase cytochrome P-450 enzyme. *Archives of Biochemistry and Biophysics*, **263**, 424–436.
- YAMAZAKI, H., GILLAM, E. M. J., DONG, M. S., JOHNSON, W. W., GUENGERICH, F. P. and SHIMADA, T., 1997, Reconstitution of recombinant cytochrome P450 2C10 (2C9) and comparison with cytochrome P450 3A4 and other forms: effects of cytochrome P450–P450 and cytochrome P450–b5 interactions. *Archives of Biochemistry and Biophysics*, **342**, 329–337.
- YAMAZAKI, H., INUI, Y., WRIGHTON, S. A., GUENGERICH, F. P. and SHIMADA, T., 1995, Procarcinogen activation by cytochrome P450 3A4 and 3A5 expressed in *Escherichia coli* and by human liver microsomes. *Carcinogenesis*, **16**, 2167–2170.

Sex-related differences in the renal disposition of the acidic metabolite of clentiazem in rat

K. HANADA[†], R. FUJISAWA[†], R. KATAOKA[†],
S. NAKAMURA[‡] and H. OGATA^{†*}

[†]Department of Biopharmaceutics, Meiji Pharmaceutical University, 2-522-1 Noshio, Kiyose, Tokyo 204-8588, Japan

[‡]Research Laboratory of Drug Metabolism, Tanabe Seiyaku Co., Ltd, 2-2-50 Kawagishi, Toda, Saitama 335, Japan

Received 11 December 2000

1. Sex-related differences in the renal excretion of the acidic compounds (+)-(2*S*,3*S*)-8-chloro-2,3,4,5-tetrahydro-3-hydroxy-2-(4-hydroxyphenyl)-4-oxo-1,5-benzothiazepin-5-acetic acid (MA4; one of the acidic metabolites of clentiazem), probenecid (PB) and methotrexate (MTX) have been investigated in the 7-week-old male and female Sprague-Dawley rat using an *in vivo* renal clearance technique.

2. The extent of plasma protein binding of MA4, PB and MTX was ~96, 95 and 65%, respectively, and it did not differ significantly between the male and female rat. On the other hand, the unbound renal clearance (CL_{rf}) of MA4 in the female was ~300 times higher than that in male, and the ratio of this clearance to the glomerular filtration rate (GFR) was ~10, suggesting that MA4 undergoes extensive active renal secretion in the female. Furthermore, the CL_{rf}/GFR ratio was significantly decreased by co-administration of PB. In contrast, no sex-related difference in the renal excretion of PB could be detected because its CL_{rf} was very low and reabsorption contributed extensively to its renal disposition. The CL_{rf}/GFR for MTX was ~2.5 but did not differ significantly between the male and female.

3. The renal organic anion transport systems in rat show sex-related differences and have different substrate-specific characteristics.

Introduction

Sex-related differences in drug disposition, such as hepatic metabolism and renal excretion, have been reported for a number of drugs (Kato *et al.* 1962, Janssen *et al.* 1976, Yamazoe *et al.* 1988, Tanaka *et al.* 1991a). Kato *et al.* (1962) were the first to report such variability, observing sex-related differences in the toxicity and metabolism of strychnine in rat. Drug-metabolizing enzymes are regulated by growth hormone, the secretion of which is in turn regulated by androgens and oestrogens (Yamazoe *et al.* 1988).

Although sex-related differences in renal excretion have not been widely studied, such differences in the nephrotoxicity and renal excretion of 6-(1-aminocyclo-hexanecarboxamido) penicillanic acid (cyclacillin) and its metabolite, 1-aminocyclo-hexanecarboxylic acid (ACHC), have been reported in the CD rat (Janssen *et al.* 1974, 1976). The renal clearance (CL_r) and clearance ratio (CL_r divided by the glomerular filtration rate [GFR]) of ACHC was higher in the female than in the male rat, suggesting that the sex-related difference in excretion may have resulted from renal reabsorption of ACHC. However, the latter authors did

* Author for correspondence: e-mail: hiroogat@my-pharm.ac.jp

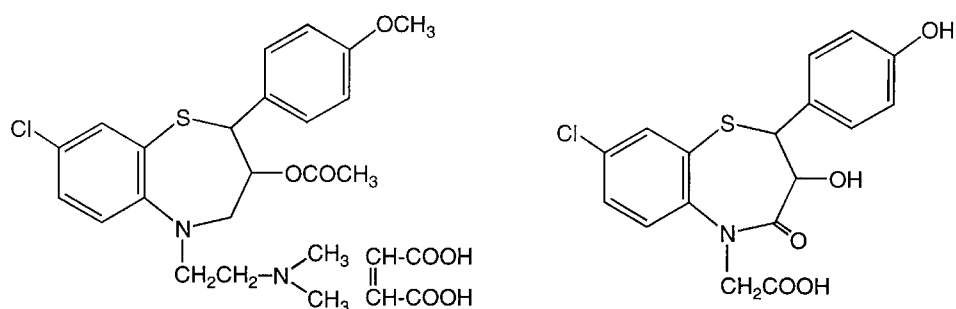


Figure 1. Chemical structures of clentiazem (left) and its metabolite, MA4 (right).

not measure the unbound fraction of ACHC in the plasma and therefore the contribution of active renal secretion could not be determined. In another study, the CL_r of perfluorooctanoate (PFO) in the female Holtzman rat was 30-fold higher than in the male (Hanhijarvi *et al.* 1982). Furthermore, the CL_r of PFO was completely inhibited by concomitant administration of probenecid, an organic anion transport inhibitor, suggesting that sex-related differences in renal excretion may result from variations in active renal secretion involving the organic anion transport system.

(+)-(2*S*,3*S*)-8-chloro-2,3,4,5-tetrahydro-3-hydroxy-2-(4-hydroxyphenyl)-4-oxo-1,5-benzothiazepin-5-acetic acid (MA4; figure 1), which is one of the metabolites of clentiazem (calcium channel antagonist; figure 1), shows sex-related differences in its hepatic metabolism, biliary excretion and renal excretion (Nakamura *et al.* 1993b). The oral clearance of clentiazem showed no significant difference between the male and female, whereas bile excretion of MA4 in the male was significantly higher than that in the female. On the other hand, the renal excretion of MA4 in the male was significantly lower than that in the female, suggesting that MA4 undergoes sex-related differences in hepatic elimination and renal excretion in rat.

The influence of sex hormones on sex-related differences in active renal secretion has been investigated in rat using zenarestat (Tanaka *et al.* 1991a,b, 1992a,b). The renal excretion of this drug was higher in the female rat than in the male. However, the relative balance of renal excretion increased significantly in the female when the rat underwent either ovariectomy or hypophysectomy, and this effect disappeared when testosterone was administered. These results suggest that sex-related differences in active renal secretion are regulated by sex hormones. However, as yet, it is unknown whether the organic anion transporters in the kidney exist in different isoforms, which may lead to such sex-related differences.

With the above in mind, the present study was conducted to clarify the substrate specificity of the organic anion transporter in the kidney *in vivo* and to obtain basic data on the renal clearances of the non-protein-bound fraction (CL_rf) of drugs in 7-week-old male and female Sprague-Dawley rats. The genders are age matched as their body weights may be significantly different and presumably also their kidney weights. During this study the metabolite of clentiazem, MA4, was used, which shows possibility of sex-related differences in its renal excretion (Nakamura *et al.* 1993b), probenecid (PB) and methotrexate (MTX).

Materials and methods

Chemicals

(+)-(2S,3S)-8-chloro-2,3,4,5-tetrahydro-3-hydroxy-2-(4-hydroxyphenyl)-4-oxo-1,5-benzothiazepin-5-acetic acid (MA4; 98% pure) was kindly donated by Tanabe Seiyaku Co., Ltd (Japan). PB, MTX and flurbiprofen were from Sigma (St Louis, MO, USA). Acetonitrile (HPLC grade) was from Wako Pure Chemical Co. (Tokyo, Japan). All other reagents were of analytical grade.

Animals

Six-week-old male and female Sprague-Dawley rats (body weight 220–270 and 130–210 g, respectively) were from Tokyo Laboratory Animals Science Co. (Tokyo, Japan). They were maintained in conditions of constant temperature and humidity and were fed a standard laboratory diet (CLEA Rodent Diet CA-1, CLEA Japan, Inc.) for 1 week. Sixteen hours prior to the experiment, they were fasted but allowed water *ad libitum*.

In vivo renal clearance study

Rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (50 mg kg^{-1}), then a polyethylene cannulae (i.d. 0.28 mm, e.d. 0.61 mm) was inserted into the femoral vein and artery and both ureters. A loading dose of MA4, PB or MTX was administered intravenously, either as a bolus injection (0.5 mg kg^{-1} for MA4) or as a 30-min infusion (at $0.4 \text{ mg min}^{-1} \text{ kg}^{-1}$ for PB and $60 \mu\text{g min}^{-1} \text{ kg}^{-1}$ for MTX: Truth Infusion Pump A-II, Truth Co., Japan). Infusion of each drug was then continued (at $13 \mu\text{g min}^{-1} \text{ kg}^{-1}$, $0.2 \text{ mg min}^{-1} \text{ kg}^{-1}$ and $30 \mu\text{g min}^{-1} \text{ kg}^{-1}$, respectively) throughout the experimental period. After 1 h at the maintenance dose, urine samples were taken for 60–90, 90–120, 120–150 and 150–180 min, and blood samples (0.3 ml) were taken at the midpoint of each urine sampling period. The blood was centrifuged at 1000g for 5 min and the plasma and urine samples were stored at about -20°C until analysis.

Protein binding

The extent of plasma protein binding of MA4, PB and MTX were investigated using the ultrafiltration technique of Hanada *et al.* (1998). Briefly, 0.5 ml serum samples from the male and female rat were incubated at 37°C for 20 min, then ultrafiltered using a VectaSpin Micro tube (molecular weight cut-off 20 000; Whatman, Maidstone, UK) at 1000g for 15 min at 37°C . No significant adsorption to the filtration device was observed. The drug concentrations in both the filtrate and an aliquot of the serum were determined by high-performance liquid chromatography (HPLC) as described below.

Analytical procedures

The HPLC system consisted of a Shimadzu HPLC apparatus (Kyoto, Japan), a LC-10A HPLC pump and a C-R6A Chromatopac integrator. The samples were introduced via a 50- μl loop (Rheodyne, Cotati, CA, USA). MA4, PB or MTX were detected using a SPD-10 AV spectrometric detector, operated at a wavelength of 245, 245 or 303 nm, with a range of 0.01, 0.005 or 0.005 AUFS, respectively. Separation of the drugs was performed at ambient temperature using a Type SG 120Å column ($5 \mu\text{m}$, $150 \times 4.6 \text{ mm}$ i.d., Shiseido, Japan) for MA4 and PB, and a Bensil C₁₈ column ($5 \mu\text{m}$, $150 \times 4.6 \text{ mm}$ i.d., Bentsch, Japan) for MTX. The mobile phases comprised acetonitrile in 15 mM phosphate buffer, pH 7.5 (11:35 v/v) for MA4 and PB, and acetonitrile in 50 mM phosphate buffer, pH 5.3 (5:95 v/v) for MTX. The mobile phase was pumped at a constant flow rate of 1.0 ml min^{-1} . Guard-PakTM Inserts Resolve C₈ (Waters Co., Milford, MA, USA) was used as guard columns.

The extraction procedures for the drugs were as follows. For MA4 and MTX, 0.2 ml acetonitrile (for MA4) or 0.1 ml perchloric acid (for MTX) was added to 0.1 ml plasma, mixed and centrifuged at 3000g for 10 min. The supernatant was injected directly into the HPLC system. Recoveries for MA4 (2, 5 and $10 \mu\text{g ml}^{-1}$) and MTX (0.5, 1.0 and $5.0 \mu\text{g ml}^{-1}$) were $100.1 \pm 3.5\%$ ($n = 5$) and $74.2 \pm 4.2\%$ ($n = 5$), respectively. The coefficients of within- and between-day variation were <5.1 and 4.7% (for MA4) and 2.7 and 3.8% (for MTX), respectively. The urine samples for MA4 and MTX determination were diluted in the relevant mobile phase. For PB, 0.1 ml 0.25 M sodium acetate buffer, pH 3.0, containing 0.1 ml flurbiprofen ($3 \mu\text{g ml}^{-1}$ in mobile phase) as an internal standard, and 5 ml ethylether were added to 50 μl plasma or 100 μl urine. The mixture was shaken vigorously for 10 min and then centrifuged at 1000g for 10 min at ambient temperature. A 4-ml aliquot of the organic layer, to which 0.1 ml 1% NH_3 in absolute ethanol had been added, was evaporated to dryness under a stream of nitrogen gas in a water-bath at 50°C . The residue was reconstituted with 0.1 ml mobile phase and a 50- μl aliquot was injected into the HPLC system. The recoveries for PB (1, 2 and $5 \mu\text{g ml}^{-1}$) was $99.7 \pm 2.7\%$ ($n = 5$) and the within- and between-day coefficients of variation were <2.1 and 4.6% ,

respectively. The urine samples were diluted by the respective mobile phases and the recoveries were almost 100%.

Inulin concentrations in both plasma and urine were determined by the anthrone method (White *et al.* 1954).

Data analysis

All data were expressed as mean $\pm$ SD. The total and renal clearances (CLt and CLr) were calculated as follows:

CLt = infusion rate of drug divided by the mean drug concentration in plasma at steady-state.

CLr = urine flow rate $\times$ drug concentration in urine, divided by the mean drug concentration in plasma at steady-state.

The statistical significance of differences in pharmacokinetic parameters were compared between the male and female rat using Student's unpaired *t*-test, and differences at *p* < 0.05 were considered significant.

Results

Sex-related differences in the renal excretion of three organic anionic compounds, MA4 (one of the acidic metabolites of clentiazem; figure 1), PB and MTX were studied during an *in vivo* renal clearance study.

Table 1 shows the pharmacokinetic parameters for MA4 in the male and female Sprague–Dawley rat. There were no significant differences in CLt and GFR (calculated with reference to the body weight of each rat) between the male and female. In contrast, the CLr of MA4 in the female was significantly greater than that in the male. The renally excreted MA4 was <1% in the male rat but was ~50% in the female. On the other hand, the extent of plasma protein binding (~96%) was similar in both sexes. In the female, the CLrf/GFR ratio for MA4 was ~10, indicating that MA4 had undergone extensive active renal secretion. On the other hand, in the male one, this ratio was <1, suggesting that active renal secretion plays little or no part in the excretion of MA4 in the male.

In a separate experiment, the effect of co-administration of MA4 with PB, a well known inhibitor of organic anion transporters, was studied. Coadministration of PB did not affect the CLt, GFR or plasma protein binding of MA4; however, CLrf/GFR was significantly decreased to about half the control level in the female

Table 1. Effects of probenecid on the pharmacokinetics of MA4 in the male and female rat.

	Control		With probenecid	
	Male	Female	Male	Female
Body weight (g)	258 $\pm$ 28	192 $\pm$ 16		
CLt (ml min ⁻¹ kg ⁻¹)	2.21 $\pm$ 0.81	2.85 $\pm$ 0.41	2.15 $\pm$ 0.94	2.59 $\pm$ 0.68
CLr (ml min ⁻¹ kg ⁻¹)	0.006 $\pm$ 0.003	1.64 $\pm$ 0.55*	0.09 $\pm$ 0.03	0.88 $\pm$ 0.48
CLr/CLt	0.003 $\pm$ 0.001	0.57 $\pm$ 0.15*	0.05 $\pm$ 0.03	0.32 $\pm$ 0.07 [#]
fup (%)	3.43 $\pm$ 0.77	3.70 $\pm$ 0.92	3.92 $\pm$ 0.82	3.75 $\pm$ 1.26
CLrf (ml min ⁻¹ kg ⁻¹)	0.17 $\pm$ 0.09	49.2 $\pm$ 26.6*	2.29 $\pm$ 0.93	24.5 $\pm$ 10.4
GFR (ml min ⁻¹ kg ⁻¹)	6.13 $\pm$ 2.14	5.02 $\pm$ 1.27	5.28 $\pm$ 1.87	4.67 $\pm$ 1.02
CLrf/GFR	0.03 $\pm$ 0.01	10.6 $\pm$ 6.54*	0.50 $\pm$ 0.33	5.76 $\pm$ 3.68 [#]

Data are mean $\pm$ SD (*n* = 5).
**p* < 0.01 (versus male), [#]*p* < 0.01 (versus MA4 alone).

CLt, total clearance; CLr, renal clearance; fup, unbound fraction in plasma; CLrf, renal clearance of unbound drug fraction; GFR, glomerular filtration rate.

Table 2. Pharmacokinetics of probenecid in the male and female rat.

	Male	Female
Body weight (g)	253 ± 32	189 ± 12*
CLt (ml min ⁻¹ kg ⁻¹)	9.88 ± 3.67	7.96 ± 2.26
CLr (ml min ⁻¹ kg ⁻¹)	0.008 ± 0.004	0.020 ± 0.013*
CLr/CLt (%)	0.089 ± 0.057	0.229 ± 0.121*
fup (%)	4.68 ± 1.35	4.78 ± 1.24
CLrf (ml min ⁻¹ kg ⁻¹)	0.029 ± 0.015	0.10 ± 0.067*
GFR (ml min ⁻¹ kg ⁻¹)	4.11 ± 1.54	5.62 ± 2.24
CLrf/GFR	0.008 ± 0.005	0.025 ± 0.025*
UFR (ml min ⁻¹ kg ⁻¹)	0.024 ± 0.003	0.026 ± 0.006
Urine pH	6.22 ± 0.16	6.76 ± 0.46*

Data are mean ± SD (*n* = 5).

**p* < 0.01 (female versus male).

UFR, urinary flow rate. For other abbreviations, see table 1.

Table 3. Pharmacokinetics of MTX in the male and female rat.

	Male	Female
Body weight (g)	200 ± 24	150 ± 20
CLt (ml min ⁻¹ kg ⁻¹)	18.2 ± 3.27	18.6 ± 4.01
CLr (ml min ⁻¹ kg ⁻¹)	8.89 ± 2.73	7.74 ± 2.59
CLr/CLt (%)	48.9 ± 11.2	41.6 ± 8.82
fup (%)	33.2 ± 5.22	35.1 ± 4.99
CLrf (ml min ⁻¹ kg ⁻¹)	13.6 ± 3.85	12.5 ± 2.79
GFR	5.31 ± 1.38	5.55 ± 2.83
CLrf/GFR	2.56 ± 0.72	2.26 ± 0.81

Data are mean ± SD (*n* = 5).

For abbreviations, see table 1.

(table 1). These results suggest that MA4 and PB may be transported by a common organic anion transport system.

To confirm the above possibility, a similar experiment was performed using PB itself. The CLt of PB was almost the same in both sexes (table 2). PB is known to show non-linear pharmacokinetics in both the saturation of hepatic metabolism and plasma protein binding (Emanuelsson and Paalzow 1988), and therefore the plasma concentration of PB was maintained at $\sim 10 \mu\text{g ml}^{-1}$ at steady-state. The CLt and plasma protein binding ($\sim 95\%$) did not differ significantly between the male and female but the fraction of PB undergoing renal excretion was very low in both sexes. The CLrf of PB in the female rat was significantly higher than that in male. However, CLrf/GFR was very low in both sexes, suggesting that sex-related differences in the renal secretion of PB itself could not be detected using this protocol.

MTX, an anionic anticancer drug, is widely used in the treatment of acute lymphocytic leukaemia and arthritis (Bleyer 1978). About 40–60% of a dose of MTX is eliminated unchanged in the urine in humans. Similarly, $\sim 50\%$ of a MTX dose is excreted in rat urine, and in the present study CLr/GFR was greater than unity (table 3), suggesting that the active renal secretion of MTX could be detected by our renal clearance method. However, the CLt of MTX showed non-linear characteristics at the dosing rate of $120 \mu\text{g min}^{-1} \text{kg}^{-1}$ (data not shown), and therefore the infusion rate was changed to 10 or $30 \mu\text{g min}^{-1} \text{kg}^{-1}$. CLt was almost

constant at both infusion rates and a rate of $30 \mu\text{g min}^{-1} \text{kg}^{-1}$ was selected. The CLt, GFR and plasma protein binding did not differ significantly between the sexes, as observed with the other compounds investigated during this study. There was also no significant difference in the CLrf/GFR of MTX between the male and female rat. These results suggest that the transporter responsible for MTX transport may not show a sex-related difference.

Discussion

The extent of plasma protein binding of MA4, PB and MTX were ~ 96 , 95 and 65%, respectively, and did not differ significantly between the male and female rat. On the other hand, the CLrf of MA4 in the female was significantly higher than that in the male, and the CLrf/GFR ratio was significantly decreased by co-administration of PB. In contrast, non-renal clearance of MA4 was also significantly different between the male and female. The biliary excretion of MA4 in the male was ~ 10 times higher than that in the female and, in an *in vitro* experiment using rat liver microsomes, the formation of the acidic metabolites was higher in the male (Nakamura *et al.* 1993b). These results suggested that hepatic elimination is predominant in the elimination of MA4 in the male, whereas both hepatic and renal excretion occurs in the elimination of MA4 in the female.

The CLrf/GFR of PB was very low in both sexes and sex-related differences in the renal secretion of PB itself could not be detected. Based on pharmacokinetic considerations, the CLr of this drug which undergoes extensive reabsorption may be dependent on the urine flow rate (UFR), because the unbound drug rapidly equilibrates between the urine and the plasma. Nevertheless, the correlation coefficient between the CLr of PB and the UFR was only 0.552, and this may reflect the lower end of the normal variation range for UFR data.

There was also no significant difference in the CLrf/GFR of MTX between the male and female rat. These results suggest that the transporter responsible for MTX transport may not show a sex-related difference. Recently, cDNA encoding two rat kidney-specific anion transporters, OAT-K1 (Saito *et al.* 1996, Masuda *et al.* 1997) and OAT-K2 (Masuda *et al.* 1999) has been isolated and cloned. These transporters show 91% amino acid identity and are located on the renal brush-border membranes. MTX is taken up by stably transfected renal cells expressing rat OAT-K1 or OAT-K2. However, the affinity of MTX transport differs between OAT-K1 and OAT-K2: the K_i for MTX against [^3H]-MTX uptake into stably transfected renal cells expressing rat OAT-K1 is $2 \mu\text{m}$ whereas that of MTX against [^3H]-taurocholate uptake into cells expressing OAT-K2 is $250 \mu\text{m}$. These differences in the affinity of MTX transport may possibly reflect as yet undetected sex-related differences in the renal excretion of MTX.

Tubular transport processes in the kidney are controlled by several hormones, such as thyroid hormones, corticosteroids and androgens. Castration of the male rat increased the half-life of *p*-aminohippurate compared with that of the female rat (Reyes *et al.* 1998). Furthermore, expression of the mRNA for renal organic anion transport peptide occurs under strong stimulatory testosterone control and weaker inhibitory oestrogen control (Lu *et al.* 1996). In the future, studies of sex-differences in active renal secretion, based on molecular analyses, will be needed to shed further light on such hormonal influences and to determine which particular transporter molecules show sex-related differences in activity.

In summary, an acidic metabolite of clemastine showed sex-related differences in renal excretion and non-renal elimination. The renal organic anion transport systems in rat exhibit sex-related differences and their substrate specificities may be different.

References

- BLEYER, W. A., 1978, The clinical pharmacology of methotrexate: new applications of an old drug. *Cancer*, **41**, 36–51.
- EMANUELSSON, B. M. and PAALZOW, L. K., 1988, Dose-dependent pharmacokinetics of probenecid in the rat. *Biopharmaceutics and Drug Disposition*, **9**, 59–70.
- HANADA, K., AKIMOTO, S., MITSUI, K., MIHARA, K. and OGATA, H., 1998, Enantioselective tissue distribution of the basic drugs disopyramide, flecainide and verapamil in rats. *Pharmaceutical Research*, **15**, 1250–1256.
- HANHIJARVI, H., OPHAUG, R. H. and SINGER, L., 1982, The sex-related difference in perfluorooctanoate excretion in the rat. *Proceedings of the Society for Experimental Biology and Medicine*, **171**, 50–55.
- INOUE, H., NAKAMURA, S., OTSUKA, H., GAINO, M., HARADA, T., MATSUKI, K. and TAKEDA, M., 1994, Synthesis of the metabolites of clemastine. *Chemical and Pharmaceutical Bulletin*, **42**, 167–172.
- JANSSEN, F. W., YOUNG, E. M. and RUELIUS, H. W., 1976, Effect of sex hormones on the disposition in rats of 1-aminocyclohexane-carboxylic acid, a metabolite of a semisynthetic penicillin. *Drug Metabolism and Disposition*, **6**, 540–546.
- JANSSEN, F. W., YOUNG, E. M., KIRKMAN, S. K., AGERSBORG, H. P. K., JR, TUCKER W. E., JR and RUELIUS, H. W., 1974, Metabolic and pathologic investigation of 1-aminocyclohexane-carboxylic acid (ACHC), a metabolite of 6-(1-aminocyclohexane-carboxamido) penicillanic acid (cyclacillin). *Toxicology and Applied Pharmacology*, **29**, 19–34.
- KATO, R., CHIESARA, E. and VASSANELLI, P., 1962, Increased activity of microsomal strychnine-metabolizing enzyme induced by phenobarbital and other drugs. *Biochemical Pharmacology*, **11**, 913–922.
- LU, R., KANAI, N., BAO, Y., WOLKO, A. W. and SCHUSTER, V. L., 1996, Regulation of renal oatp mRNA expression by testosterone. *American Journal of Physiology*, **39**, F332–F337.
- MASUDA, S., IBARAMOTO, K., TAKEUCHI, A., SAITO, H., HASHIMOTO, Y. and INUI, K., 1999, Cloning and functional characterization of a new multispecific organic anion transporter, OAT-K2, in rat kidney. *Molecular Pharmacology*, **55**, 743–752.
- MASUDA, S., SAITO, H., NONOGUCHI, H., TOMITA, K. and INUI, K., 1997, mRNA distribution and membrane localization of the OAT-K1 organic anion transporter in rat renal tubules. *FEBS Letters*, **407**, 127–131.
- NAKAMURA, S., SUGAWARA, Y., FUKUSHIMA, T., ITO, Y., OHASHI, M. and TAKAITI, O., 1993a, Metabolism of clemastine in rats. *Biological and Pharmaceutical Bulletin*, **16**, 635–646.
- NAKAMURA, S., SUGAWARA, Y., FUKUSHIMA, T., ITO, Y., OHASHI, M. and TAKAITI, O., 1993b, Disposition and metabolic rate of clemastine in rats and dogs. *Biological and Pharmaceutical Bulletin*, **16**, 647–655.
- REYES, J. L., MELENDEZ, E., ALEGRIA, A. and JARAMILLO-JUAREZ, F., 1998, Influence of sex differences on the renal secretion of organic anions. *Endocrinology*, **139**, 1581–1587.
- SAITO, H., MASUDA, S. and INUI, K., 1996, Cloning and functional characterization of a novel rat organic anion transporter mediating basolateral uptake of methotrexate in the kidney. *Journal of Biology and Chemistry*, **271**, 20719–20725.
- TANAKA, Y., DEGUCHI, Y., ISHII, I. and TERAI, T., 1991a, Sex differences in excretion of zenarestat in rat. *Xenobiotica*, **21**, 1119–1125.
- TANAKA, Y., FUJIWARA, T. and ESUMI, Y., 1992b, Sex difference in the excretion of zenarestat in mice, rats, dogs and humans. *Xenobiotica*, **22**, 941–947.
- TANAKA, Y., KADOH, Y. and ISHIKAWA, H., 1992a, Effects of hypophysectomy and testosterone treatment on the urinary excretion of zenarestat in rats. *Xenobiotica*, **22**, 51–55.
- TANAKA, Y., KADOH, Y., MUKUMOTO, S. and ISHIKAWA, H., 1991b, The role of age and sex hormones on the urinary excretion of zenarestat in rats. *Xenobiotica*, **21**, 1273–1279.
- WHITE, R. P. and SAMSON, F. E., JR, 1954, Determination of inulin in plasma and urine by use of anthrone. *Journal of Laboratory and Clinical Medicine*, **43**, 475–478.
- YAMAZOE, Y., MURAYAMA, N., SHIMADA, M., YAMAUCHI, K., NAGATA, K., IMAOKA, S., FUNAE, Y. and KATO, R., 1988, A sex-specific form of cytochrome P-450 catalyzing propoxycoumarin O-depropylation and its identity with testosterone 6 beta-hydroxylase in untreated rat livers: reconstitution of the activity with microsomal lipids. *Journal of Biochemistry*, **104**, 785–790.

Metabolism of 2-(4-methylsulphonyl-2-nitrobenzoyl)-1,3-cyclohexanedione (mesotrione) in rat and mouse

A. J. GLEDHILL*, B. K. JONES and W. J. D. LAIRD

Zeneca Central Toxicology Laboratory, Alderley Park, Macclesfield, Cheshire SK10 4TJ, UK

Received 11 December 2000

1. The metabolic fate of [^{14}C]-2-(4-methylsulphonyl-2-nitrobenzoyl)-1,3-cyclohexanedione (mesotrione) has been determined in the male and female rat and mouse following a single oral dose of either 1 or 100 mg kg $^{-1}$, in rat given 14 consecutive oral doses of 1 mg kg $^{-1}$, and in the surgically prepared, bile duct-cannulated rat following a single oral dose of 50 mg kg $^{-1}$. The excretion of a single i.v. dose of 1 mg kg $^{-1}$ in the male and female rat was also investigated.

2. Mesotrione was extensively absorbed and rapidly excreted via urine in both rat and mouse. The absorbed dose was not well metabolized in either species. Unabsorbed material was subject to metabolic action by the gut microflora.

3. The major metabolic pathway was hydroxylation of the aromatic ring. There was evidence for cleavage of the dione and aromatic rings followed by reduction of the nitro group in the gastrointestinal tract.

4. There were no species differences in the metabolism and excretion of mesotrione, which could explain the species differences in toxicity reported for this class of compounds.

Introduction

2-(4-Methylsulphonyl-2-nitrobenzoyl)-1,3-cyclohexanedione (mesotrione) is a triketone herbicide originally discovered at the Western Research Centre, Richmond, CA, USA (Lee *et al.* 1997). The metabolism of this compound in rat and mouse has not previously been published. The mode of action of triketone herbicides, in both plants and mammals, is the selective inhibition of 4-hydroxyphenylpyruvate dioxygenase (HPPD), the second enzyme in the catabolic cascade of tyrosine (Ellis *et al.* 1995). The toxicological consequence of this inhibition is a rise in plasma tyrosine. Although triketones inhibit HPPD in all species studied, the degree of the triketone-induced tyrosinaemia has been previously reported to be different in different species, with rat showing much higher levels of plasma tyrosine than mouse (Lock *et al.* 2000).

In order to determine if the species difference in toxicity could be due to species differences in the metabolism and/or disposition of triketones, studies were performed in rat and mouse using a single oral dose of [^{14}C]-labelled mesotrione at a low and high dose level (selected to correspond with dose levels used in toxicology studies with this compound). Studies were also performed in rat only, using multiple oral doses or a single intravenous (i.v.) dose of mesotrione

* Author for correspondence.

for comparison with a single oral dose. The biliary excretion of mesotrione in rat was also investigated. This paper reports the results of these studies, in which the major metabolic pathways and the rates and routes of elimination of mesotrione in rat and mouse were elucidated, and it discusses the findings with respect to the basis of species difference in triketone-induced tyrosinaemia.

Materials and methods

[¹⁴C]-aromatic ring-labelled mesotrione (specific activity 1.12 or 1.42 GBq mmol⁻¹ and radiochemical purity >97%), [¹⁴C]-dione ring-labelled mesotrione (specific activity 1.35 GBq mmol⁻¹ and radiochemical purity >99%) and unlabelled mesotrione (purity >99%) were supplied by Zeneca Agrochemicals (Fernhurst, UK).

Reference standards of 4-methylsulphonyl-2-aminobenzoic acid (AMBA), 2-nitro-4-methylsulphonyl-benzoic acid (MNBA), 4- and 5-hydroxy-2-(4-methylsulphonyl-2-nitrobenzoyl)-1,3-cyclohexanedione were supplied by Zeneca Agrochemicals. Optiphase MP, Hi-Safe 3, Carbo-sorb E[®] and Permaflour E⁺[®] were obtained from Canberra Packard (Pangbourne, UK). Liquid chromatography (LC) columns were obtained from Hichrom Ltd (Theale, UK). LC solvents were obtained from Fischer Scientific (Loughborough, UK). Pelleted PCD rat diet was obtained from Special Diets Services Ltd (Stepfield, UK).

Animals

The studies were conducted in accordance with the UK Home Office Animals (Scientific Procedures) Act 1986, and local ethical guidelines. Wistar-derived male and female rats (Alpk:AP;SD strain, body weight range 175–300g at the time of dosing) were obtained from the Barriered Animal Breeding Unit (Zeneca Pharmaceuticals). Male and female mouse (CD-1:Cr1(ICR) BR strain, body weight range 18–25g at the time of dosing) were obtained from Charles River (Margate, UK). The animals were maintained under controlled environmental conditions (19–25°C, 30–70% relative humidity, with an artificial light cycle giving 12 h light, 12 h dark). Urinary and faecal metabolite profiles were obtained from two groups of five male and five female rats given a single oral dose of either 1 or 100 mg [¹⁴C]-aromatic ring-labelled mesotrione kg⁻¹, or 1 mg [¹⁴C]-aromatic ring-labelled mesotrione kg⁻¹ following 14 consecutive daily doses of unlabelled mesotrione. Urinary and faecal metabolite profiles were obtained from two groups of four male and four female mice given a single oral dose of either 1 or 100 mg [¹⁴C]-aromatic mesotrione. Animals were fasted for 12 h prior to dosing and for 2 h after dosing. All doses were administered by gavage using aqueous sodium bicarbonate (pH 7.0–7.5) as the dose vehicle. The dose was administered at 4 ml kg⁻¹ bodyweight and the amount of radioactivity administered was between 4 and 16 MBq kg⁻¹. Urinary and faecal elimination was determined from a group of five male and five female rats administered a single i.v. dose of [¹⁴C]-aromatic ring-labelled mesotrione kg⁻¹ in pyrogen free water at a dose volume of 4 ml kg⁻¹.

In addition, in order to obtain enough mass of material for metabolite identification, two groups of two male and two female rats were bile duct cannulated under Fluothane (AstraZeneca Pharmaceuticals, Macclesfield, UK) anaesthesia. The cannulae were exteriorized and the animals allowed to recover. Analgesia was administered for the first 24 h after surgery and as required thereafter. On the morning following surgery each group was given a single oral dose of 50 mg of one of the two radiolabelled forms of mesotrione kg⁻¹ as described above. These animals were allowed unlimited access to food (pelleted PCD rat diet) and a glucose/salt solution (5% glucose, 0.9% NaCl, 0.05% KCl) throughout the duration of the experiment.

Urine and faeces were collected into containers cooled by solid carbon dioxide. Urine was collected during 0–6 and 6–12 h, faeces were collected during 0–12 h, and urine and faeces were collected during 12–24, 24–36 and 36–48 and 48–72 h after dosing. For the bile duct-cannulated rat, bile was collected at room temperature during 0–6, 6–12, 12–24, 24–36 and 36–48 h after dosing and urine and faeces were collected as described above until 48 h after dosing. All samples were stored at approximately –20°C prior to analysis.

Determination of radioactivity

A Packard Tricarb scintillation counter was used for all radioactivity determinations. Duplicate, weighed aliquots of urine and bile were mixed with scintillant (either Optiphase MP or Hi-Safe 3) and analysed for radioactivity to a 1% standard deviation of the count or for a maximum of 10 min each, whichever occurred first. The results obtained were corrected for background activity and counting efficiency using [¹³³Ba] as the external standard. Faecal samples were extracted with acetonitrile and the organic layer counted as described above. The residue was air dried and ground, and then replicate weighed aliquots were oxidized in a Packard Tricarb sample oxidizer. The [¹⁴C]-carbon dioxide

generated was absorbed into Carbo-sorb E[®] and mixed with Permafluor E+[®] scintillant prior to analysis by liquid scintillation counting as described above.

Liquid chromatography (LC)

The analysis of excreta, bile and reference standards, LC-mass spectroscopy and the quantitation of metabolites was performed using the following conditions: column: Hichrom HIPRB (15 cm × 4.6 mm); mobile phase A: water modified with 1% formic acid; mobile phase B: acetonitrile modified with 1% formic acid; gradient series: start at 90%A and 10%B, then change to 50%A and 50%B over 25 min then to 0%A and 100%B over 5 min; flow rate: 1 ml min⁻¹; UV detection: 270 nm; and radiochemical detection: Berthold LB-506 solid cell detector.

To obtain a sufficient mass of metabolites for mass spectroscopy and NMR spectroscopy, samples of urine were fractionated by preparative LC using the method described above with a 20 cm × 10 mm semipreparative column with a flow rate of 4 ml min⁻¹. Individual fractions were collected manually into round-bottom flasks. Following evaporation under reduced pressure, the fractions were reconstituted into a small volume of mobile phase and analysed by analytical LC, to assess purity.

Mass spectroscopy (MS)

- Direct insertion probe MS: a VG70-70E mass spectrometer (VG Analytical, Wythenshaw, UK) with a model 11-250 data system was used to obtain mass spectra of mesotrione. Samples were introduced directly into the source via the probe. Both electron impact (EI⁺) and chemical ionization (CI⁺, with methane as the reagent gas) spectra were recorded.
- LC-electrospray-MS (LC-MS): analysis was carried out using a Finnigan TSQ-7000 triple quadrupole instrument fitted with an electrospray (ESP) source (Finnigan MAT, San José, CA, USA). The LC conditions described for spectroscopy were used. Spectra were recorded in positive and negative-modes.

Proton nuclear magnetic resonance spectroscopy (¹H NMR)

Purified metabolites or reference standards were dissolved in an appropriate deuterated solvent and spectra were recorded using a Bruker WM 400 MHz instrument (Bruker, Coventry, UK).

Results

Excretion of radioactivity by rat

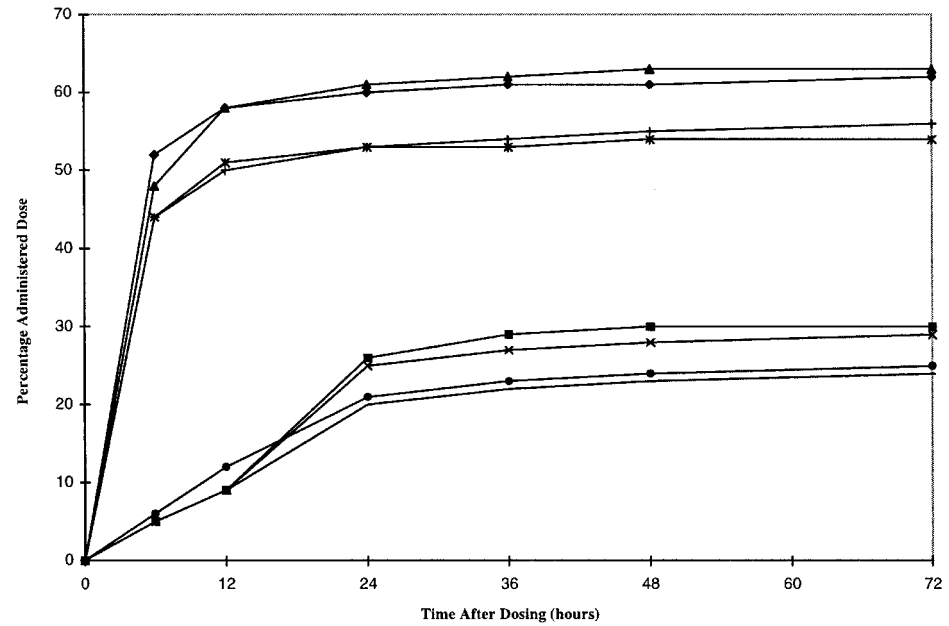
The cumulative excretion data are presented in figure 1.

The excretion of mesotrione was very similar for all dose regimens. Mesotrione was rapidly absorbed and quickly eliminated, and the majority of radioactivity was eliminated via urine within the first 6 h after dosing. The radioactivity in faeces from the i.v. dose was 7 and 2% in males and females respectively, suggesting biliary secretion (or other secretory route) of the compound was of the order of 7 and 2% of the dose in males and females respectively.

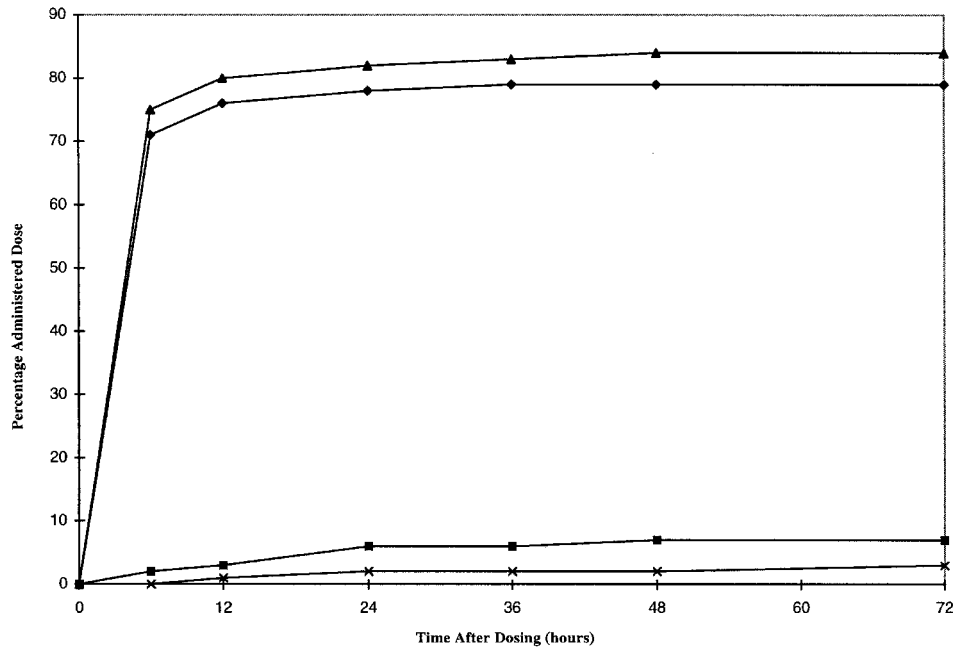
Excretion of radioactivity by the bile duct-cannulated rat

The cumulative excretion data for rat with bile duct cannulae are shown in figure 2.

For both radiolabelled forms the majority of the dose was eliminated via urine with the rate being slightly slower than that seen in intact animals. However, urinary elimination was still essentially complete 12 h after dosing. The amount of radioactivity eliminated in bile (2–14%) was similar to faecal elimination following an i.v. dose to intact animals even though there was a 50-fold difference in dose level. Approximately 25% of the dose was eliminated in faeces, and this was considered to represent unabsorbed material.



(a)



(b)

Figure 1. Cumulative excretion of radioactivity by rats administered mesotrione. Each value is the mean of five animals. For clarity, error bars not shown. (a) Administration of a single oral dose of 1 or 100 mg kg⁻¹. —◆—, Male high-dose urine; —■—, male high-dose faeces; —▲—, female high-dose urine; —×—, female high-dose faeces; —*—, male low-dose urine; —●—, male low-dose faeces; —|—, female low-dose urine; —|—, female low-dose faeces. (b) Administration of a single i.v. dose of 1 mg kg⁻¹. —◆—, Male i.v. dose urine; —■—, male i.v. dose faeces; —▲—, female i.v. dose urine; —×—, female i.v. dose faeces.

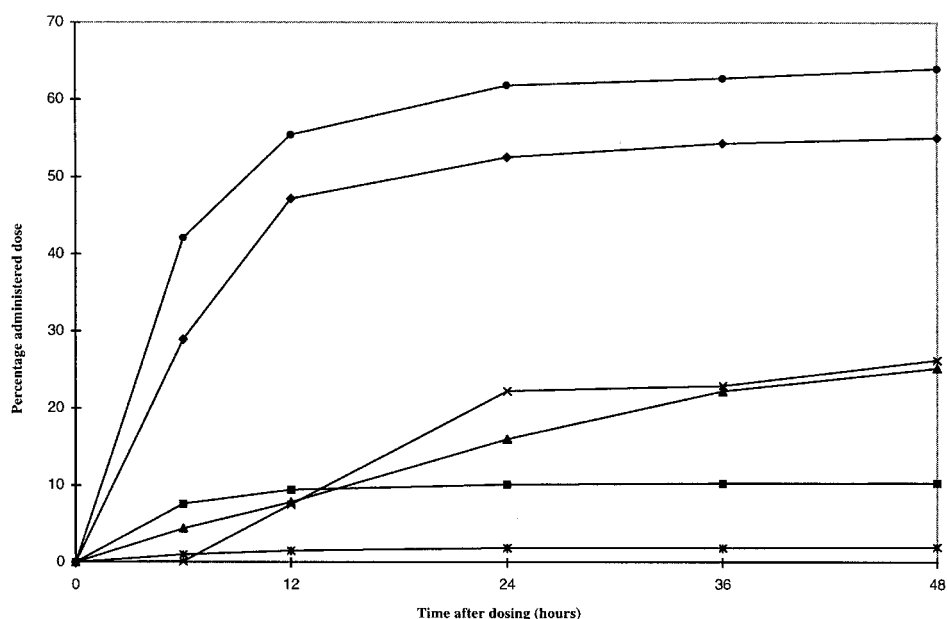


Figure 2. Cumulative excretion of radioactivity by the bile duct-cannulated rat administered a single oral dose of 50 mg mesotrione kg^{-1} . Each value is a mean of two animals. —◆—, Male urine; —■—, male bile; —▲—, male faeces; —×—, female faeces; —*—, female bile; —●—, female urine.

Excretion of radioactivity by mouse

The cumulative excretion of a single oral dose of either 1 or 100 mg [^{14}C]-aromatic ring-labelled mesotrione kg^{-1} is shown in figure 3.

The excretion of mesotrione by mouse was similar to that seen in rat. For both dose levels much of the dose was eliminated via urine in the first 6 h after dosing. For males administered a low dose, urinary elimination appeared to be lower than that measured for the other groups, but this was considered to be an anomalous result due to urinary contamination of faecal pellets and cage debris for one animal.

Identification of metabolites in rat

All the metabolites observed in urine and/or bile were identified:

- Peak 1: this was the major component in urine and bile. The structure and mass spectrum for this compound is shown in figure 4. LC-MS analysis of this peak gave spectra almost identical to those obtained for the reference standard mesotrione and the peak co-eluted with it. There was no molecular ion in the positive-mode LC-MS spectrum but the ion cluster $(2M + 1)^+$ at m/z 679 and the acetonitrile adduct $(M + 1 + 41)$ at m/z 381 confirmed a molecular weight of 339. The negative-mode LC-MS spectrum showed a molecular ion $(M - 1)^-$ at m/z 338 (base peak), which had a formic acid adduct at m/z 384, and formed a sodium ion cluster $(2M - 1 + 23)^-$ at m/z 699. The NMR spectrum obtained was identical to that of a reference standard of mesotrione (NMR data are tabulated in table 1). These data showed that peak 1 was mesotrione.
- Peak 2: this metabolite accounted for 5% of the radioactivity in male urine but

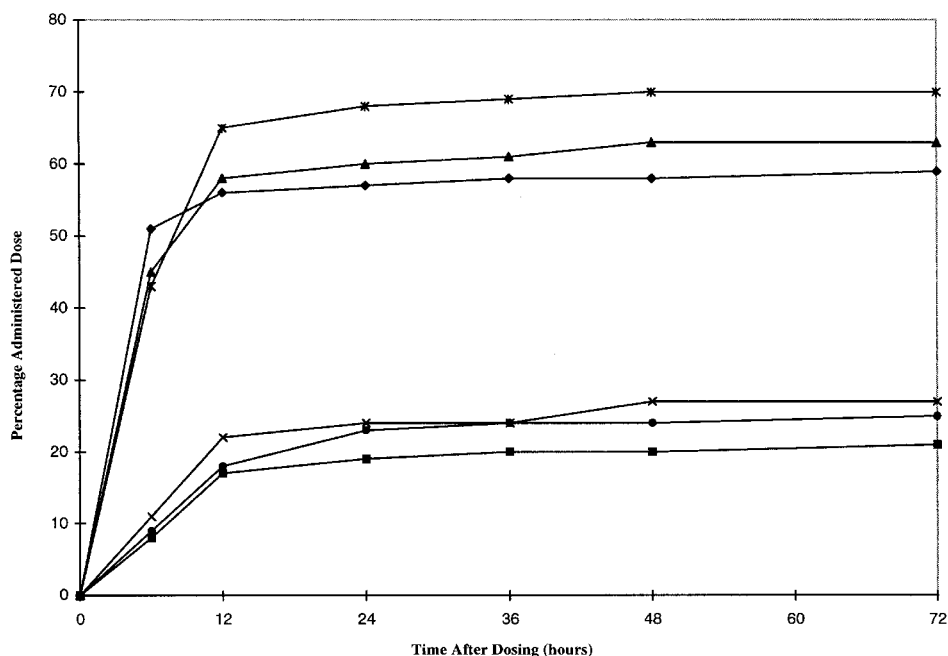


Figure 3. Cumulative excretion of radioactivity by mouse administered a single oral dose of 1 mg or 100 mg mesotrione kg⁻¹. Each value is the mean of four animals. Data for low-dose males are not included because of possible contamination of faeces by urine. —◆—, Female low-dose urine; —■—, female low-dose faeces; —▲—, male high-dose urine; —×—, male high-dose faeces; —*—, female high-dose urine; —●—, female high-dose faeces.

was present in female urine only in trace amounts. It was isolated and identified by LC-MS and by co-chromatography with a reference standard of 4-hydroxy-2-(4-methylsulphonyl-2-nitrobenzoyl)-1,3-cyclohexanedione (trivial name: 4-hydroxy mesotrione). The structure (4-hydroxy mesotrione) and mass spectrum of this metabolite are shown in figure 5. The negative-mode LC-MS spectrum gave a characteristic molecular ion ($M - 1$)⁻ at m/z 354.

- Peak 3: this metabolite was present in trace amounts in urine and larger amounts in the solvent extract of faeces, though it was not present in bile. It was isolated from urine and identified by LC-MS and NMR. The structure and mass spectrum of this compound are shown in figure 6. The negative-mode LC-MS spectrum showed a molecular ion ($M - 1$)⁻ at m/z 244. A fragment ion seen at m/z 200 was formed by the loss of CO₂H from the molecule. A formic acid adduct ion ($M + 45$)⁻ was seen at m/z 290 and a sodium cluster ($2M - 1 + 23$)⁻ seen at m/z 511. The NMR spectrum gave a signal pattern that corresponded to a 1,3,4 tri-substituted aromatic ring and the signal from a methylsulphonyl group. Therefore, peak 3 was identified as 2-nitro-4-methylsulphonyl-benzoic acid (trivial name: MNBA).
- Peak 4: this metabolite was present in trace amounts in urine and larger amounts in the solvent extract of faeces, although it was not present in bile. It was isolated from urine and identified by LC-MS, NMR and by co-chromatography with a reference standard of 4-methylsulphonyl-2-aminobenzoic acid (trivial name: AMBA). The structure and mass spectrum of this compound are shown in figure 7. The positive-mode LC-MS spectrum (not shown) gave a peak at m/z

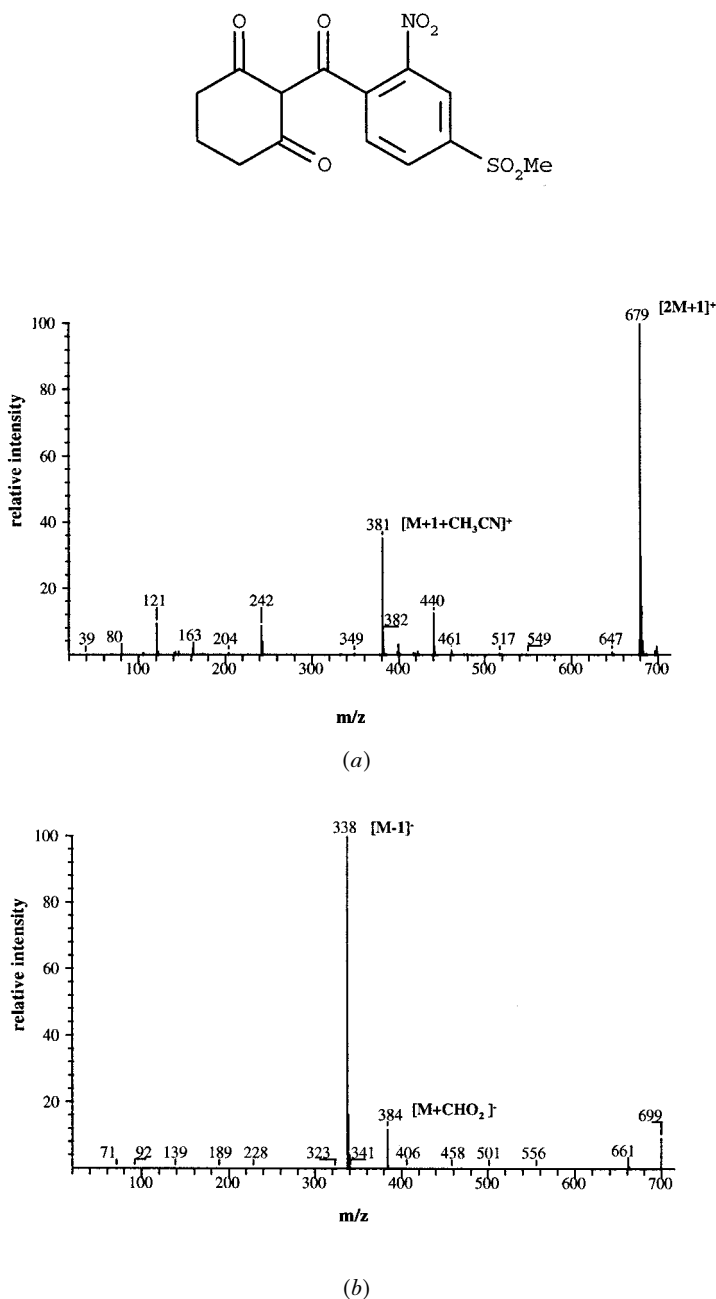


Figure 4. Structure and electrospray mass spectrum of mesotrione isolated from rat urine. (a) Positive mode, (b) negative-mode.

198, which was due to $(M-OH)^+$. The negative-mode spectrum gave an adduct with formic acid at m/z 260. The NMR spectrum showed a signal pattern that corresponded to a 1,3,4 tri-substituted aromatic ring and the signal from a methylsulfonyl group and confirmed the identity of this peak to be AMBA.

- Peak 5: this metabolite was identified in male urine by co-chromatography with

Table 1. ¹H-NMR data for metabolites isolated from urine.

Position	Mesotrione	MNBA	AMBA
1	δ1.98 ppm, m, 2H		
2	δ2.55 ppm, bs, 4H		
Me	δ3.19 ppm, s, 3H	δ3.41 ppm, s, 3H	δ3.2 ppm, s, 3H
a	δ7.54 ppm, d, 1H	δ8.55 ppm, d, 1H	δ7.88 ppm, d, 1H
b	δ8.25 ppm, d, 1H	δ8.04 ppm, d, 1H	δ7.16 ppm, d, 1H
c	δ8.59 ppm, s, 1H	δ8.22 ppm, s, 1H	δ7.28 ppm, s, 1H

δ, chemical shift; s, singlet; m, multiplet; bs, broad singlet; d, doublet.

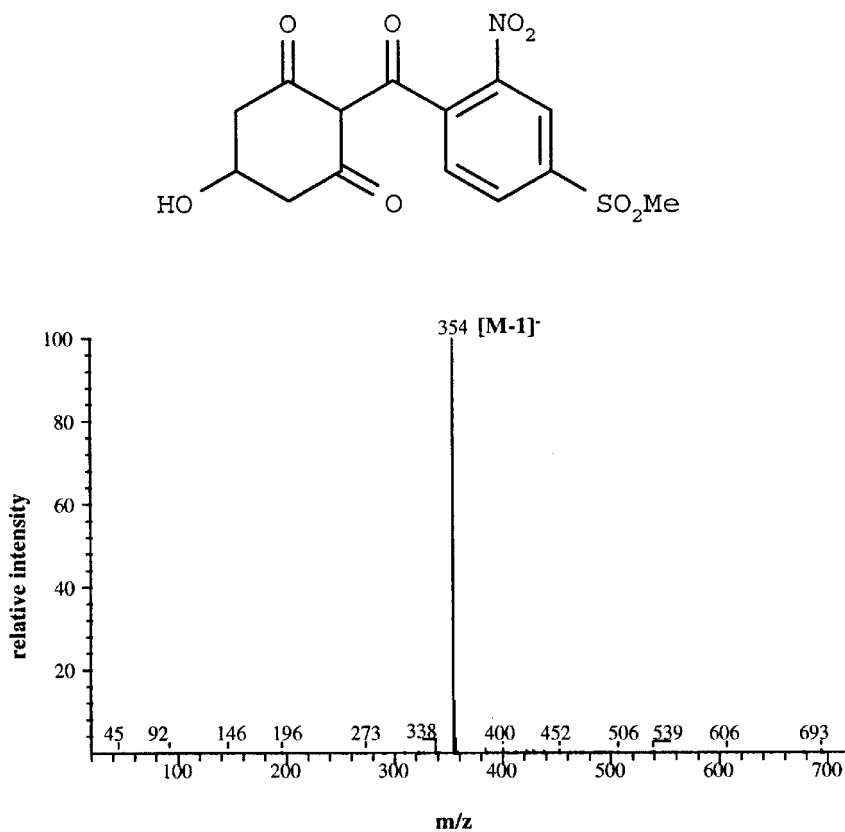


Figure 5. Structure and negative-mode electrospray mass spectrum of 4-hydroxy mesotrione isolated from urine.

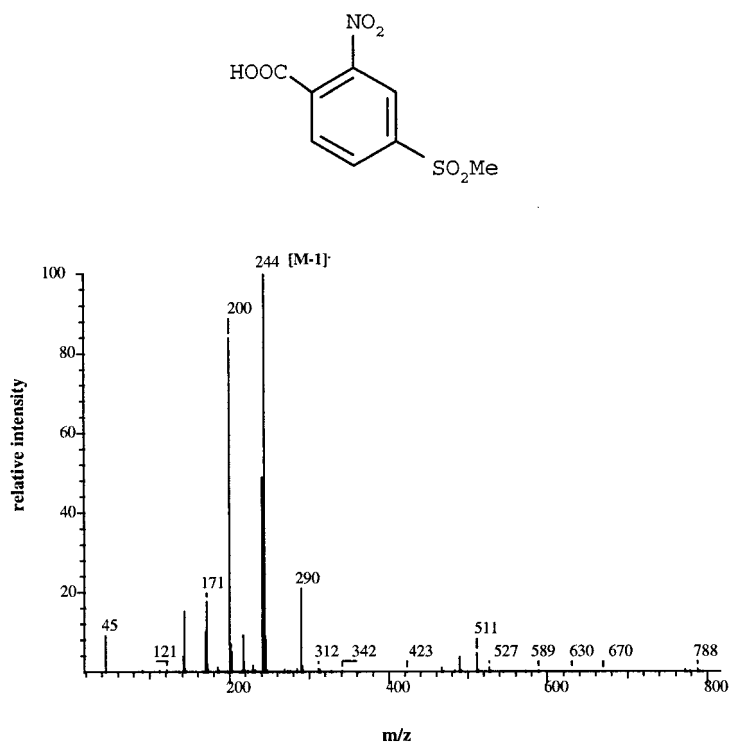


Figure 6. Structure and negative-mode electrospray mass spectrum of MNBA isolated from urine.

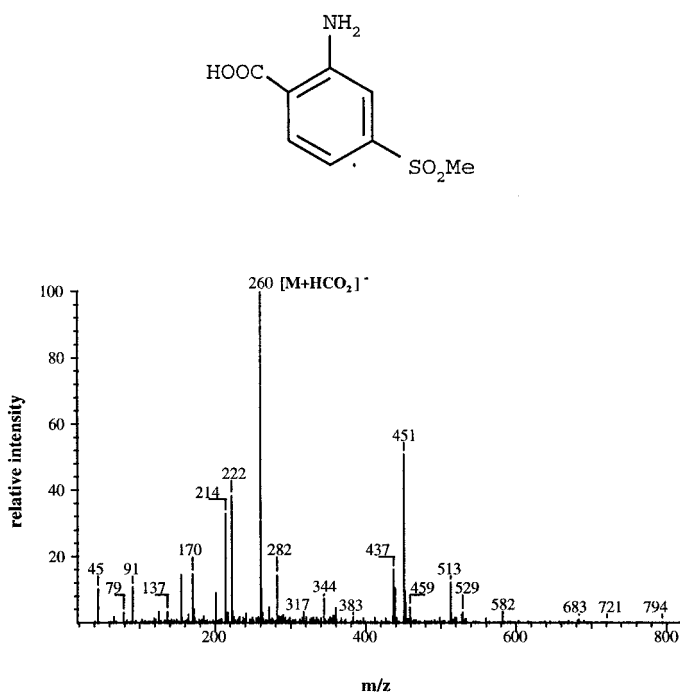


Figure 7. Structure and negative-mode electrospray mass spectrum of AMBA isolated from urine.

Table 3. Quantitation of metabolites in the bile duct-Cannulated rat administered 50 mg mesotrione kg^{-1} .

	Dione ring [^{14}C]-labelled mesotrione									
	Aromatic ring [^{14}C]-labelled mesotrione				Urine				Bile	
	Urine		Bile		Faeces		Urine		Bile	
	Male	Female	Male	Female	Male	Female	Male	female	Male	Female
Peak 1	48	60	8	2	2	0	43	51	11	3
Peak 2	0	0	2	0	2	2	3	0	1	0
Peak 3	0	1	0	0	0	1	—	—	—	—
Peak 4	0	1	0	0	8	10	—	—	—	—
Peak 5	5	0	0	0	1	0	0	0	0	0
Unknowns	9	1	0	0	8	5	1	0	2	1

Values are expressed as percentage of administered dose.

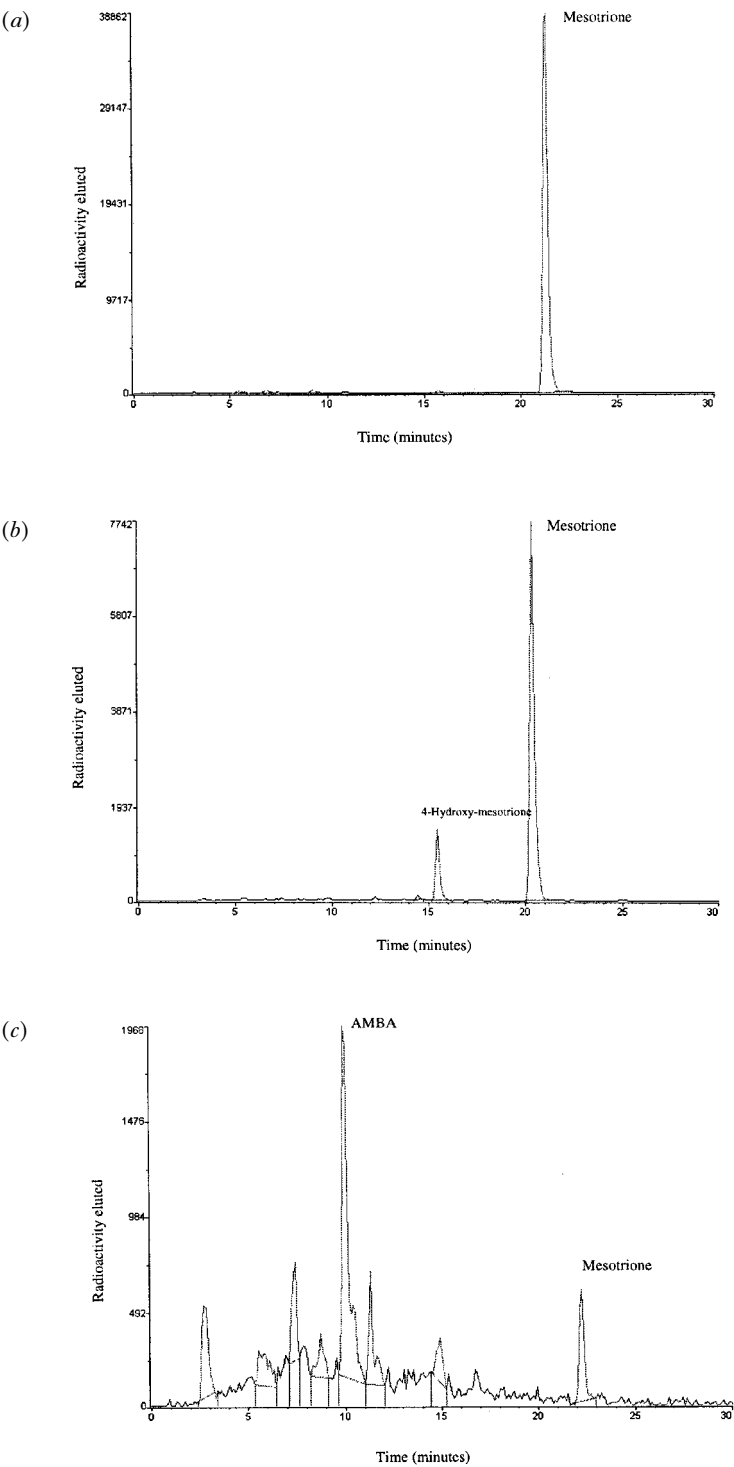


Figure 8. Representative LC chromatograms of rat urine, bile and faeces. (a) 0-6 h urine from the bile duct-cannulated rat; (b) 0-6 h bile from the bile duct-cannulated rat; (c) 0-12 h faeces from the bile duct-cannulated rat.

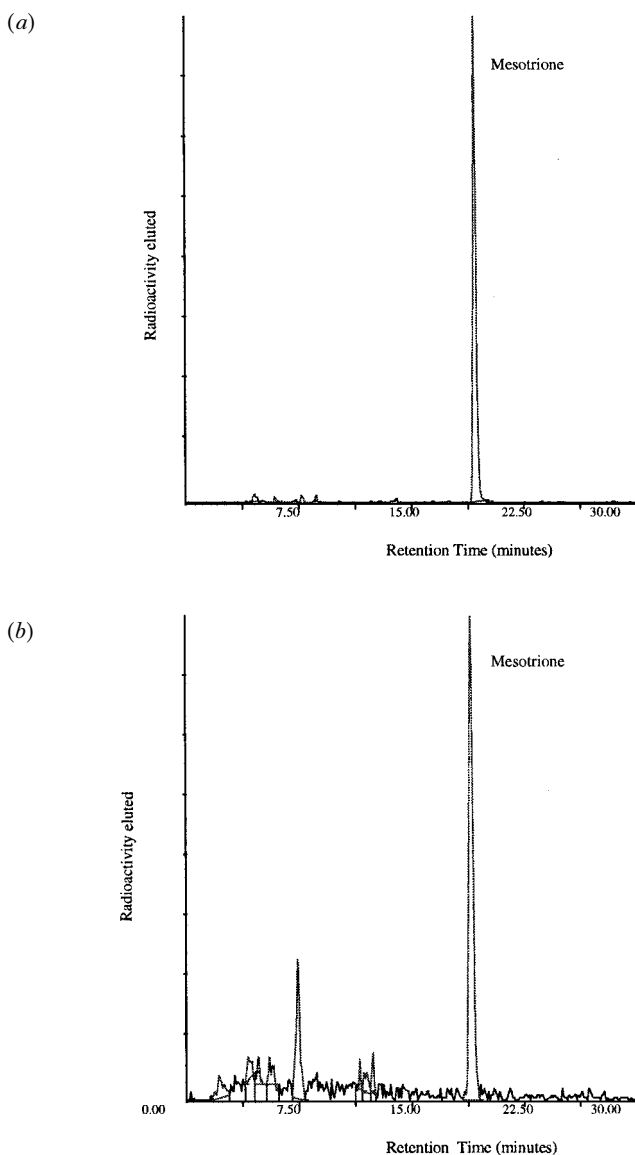


Figure 9. Representative LC chromatograms of mouse urine and faeces extract. (a) 0-12 h urine; (b) 0-12 h faeces extract.

a reference standard of 5-hydroxy-2-(4-methylsulphonyl-2-nitrobenzoyl)-1,3-cyclohexanedione (trivial name: 5-hydroxy mesotrione). LC-MS spectra obtained for this peak were too weak to give any molecular weight information.

Identification of metabolites in mouse

Metabolites were identified directly from urine or solvent extract of faeces by LC-MS. The metabolites identified in the mouse were the same as those identified in the rat metabolism studies.

Quantification of metabolites in rat and mouse

Metabolites were quantified by measuring the peaks present in the [^{14}C]-chromatograms of urine, bile and solvent extract of faeces. Since column recoveries were $>95\%$, the percentage of each peak was then multiplied by the proportion of dose represented by each sample to calculate the percentage of dose represented by each metabolite. The results for intact animals are presented in table 2 and for bile duct-cannulated animals in table 3. Representative LC traces for urine, bile and faeces extract in the rat are presented in figure 8 and urine and faeces extract for the mouse in figure 9.

Mesotrione was the most abundant radioactive component present in rat and mouse urine and rat bile for all dose regimes, accounting for $>90\%$ of radioactivity in urine and bile. Other metabolites accounted for $<5\%$ of the administered dose in urine or bile, and 4-hydroxy mesotrione was the most abundant.

Mesotrione, MNBA and AMBA and a number of unidentified metabolites were present in various proportions in faeces extracts from all studies, including the bile duct cannulation experiments, even though MNBA and AMBA were not present in bile.

Discussion

Following the administration of a single oral dose of either 1 or 100 mg [^{14}C]-mesotrione kg^{-1} to rat or mouse, or 14 consecutive doses of 1 mg kg^{-1} followed by 1 mg [^{14}C]-mesotrione kg^{-1} to rat, mesotrione was well absorbed and rapidly excreted, with the majority of the dose eliminated within the first 6 h after dosing. The excretion of an i.v. dose of 1 mg [^{14}C]-mesotrione kg^{-1} to rat showed a similar pattern, with most of the dose eliminated in urine in the 6 h from dosing.

Rat and mouse excreted absorbed mesotrione predominantly unchanged in the urine, where it represented at least 90% of urinary radioactivity. Mesotrione was also the major radioactive component in rat bile. There were no differences in the urinary metabolite profile between different dose levels for both rat and mouse, or following multiple dosing in the rat. Therefore, the metabolism of mesotrione was not saturable or auto-inducible at the dose levels investigated. The other metabolites present in urine represented only a small proportion of the dose. They were identified as hydroxylated mesotrione and the cleavage products, MNBA and AMBA. Again, these metabolites were common to rat and mouse and were not dose-dependent. Hydroxylated mesotrione was the only other metabolite detected in bile. The urinary metabolite profile following an i.v. dose was not determined but since an oral dose of mesotrione showed little first-pass metabolism, one would expect that the metabolite profile of an i.v. dose to be similar to that following an oral dose. The proposed metabolic pathway for mesotrione in both the rat and mouse is presented in figure 10.

The proportion of dose elimination in faeces following an i.v. dose of mesotrione to rat corresponded well with the proportion of the dose eliminated in bile. There was a slight sex difference, with females excreting slightly less of the dose via bile than males but this was considered not significant. From the metabolite profiles of solvent extract of faeces from bile duct-cannulated animals and the reductive nature of the biotransformation, it was clear that the array of metabolites present in faeces resulted from the action of gut microflora. MNBA, AMBA and mesotrione were identified in faeces. Other faecal metabolites were not identified. The presence of MNBA and

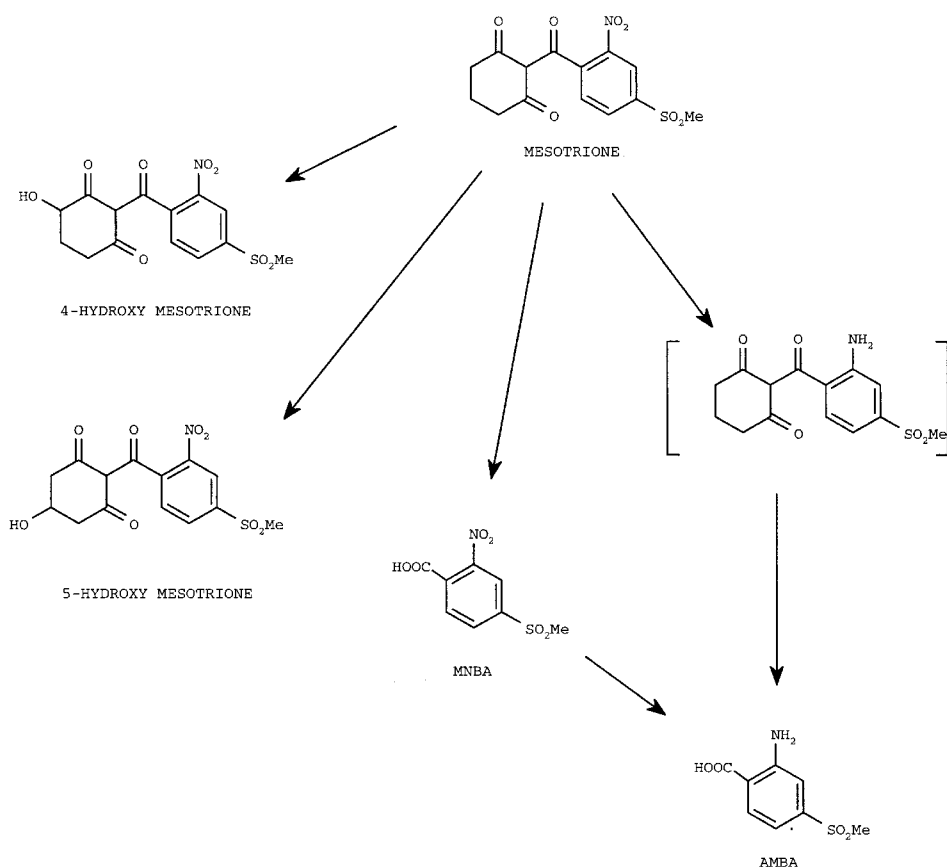


Figure 10. Proposed metabolic pathway of mesotrione in rat and mouse.

AMBA in urine may be attributable to their absorption from the gastrointestinal tract, especially since experiments undertaken with [^{14}C]-dione ring-labelled mesotrione showed no evidence of cleaved dione ring products in urine or bile, which would be expected if the cleavage occurred systemically.

In conclusion, the results from this series of experiments show that there were no differences in the absorption, metabolism and excretion of mesotrione between rat and mouse which could explain the reported species difference in the toxicity of triketones.

References

- ELLIS, M. K., WHITFIELD, A. C., GOWAN, L. A., AUTON, T., PROVAN, W. M., LOCK, E. A. and SMITH, L. L., 1995, Inhibition of 4-hydroxyphenyl pyruvate dioxygenase by 2-(2-nitro-4-trifluoromethylbenzoyl)cyclohexane-1,3-dione and 2-(2-chloro-4-methanesulphonylbenzoyl)cyclohexane-1,3-dione. *Toxicology and Applied Pharmacology*, **133**, 12–19.
- LEE, D. L., PRISBYLLA, M. P., CROMARTIE, T. H., DAGARIN, D. P., HOWARD, W. H., PROVAN, W. M., ELLIS, M. K., FRASER, T. and MUTTER, L. C., 1997, The discovery and structural requirements of inhibitors of *p*-hydroxyphenylpyruvate dioxygenase. *Weed Science*, **45**, 601–609.
- LOCK, E. A., GASKIN, P., ELLIS, M. K., PROVAN, W. M., ROBINSON, M. and SMITH, L. L., 2000, Tissue distribution of 2-(2-nitro-4-trifluoromethylbenzoyl)cyclohexane-1,3-dione (NTBC) and its effects on enzymes involved in tyrosine catabolism in the mouse. *Toxicology* (in press).

Pharmacokinetics of trimethylamine in rats, including the effects of a synthetic diet

I. P. NNANE* and L. A. DAMANI‡

† Temple University School of Pharmacy, 3307 N. Broad Street, Philadelphia, PA 19140, USA

‡ Department of Pharmacy, Faculty of Medicine, Chinese University of Hong Kong, Shatin, N.T., Hong Kong

Received 9 February 2001

1. The pharmacokinetic profile of trimethylamine (TMA) was examined in the male Wistar rat and the effects of a synthetic diet on TMA pharmacokinetics were also evaluated.

2. The concentrations of TMA and its *N*-oxide in blood were analysed by a sensitive headspace gas chromatographic assay.

3. The pharmacokinetics of TMA were essentially linear for intravenous (i.v.) bolus doses of 10–40 mg kg⁻¹. Over the range of administered i.v. doses, the concentrations of TMA in blood declined approximately monoexponentially with half-lives of 2.03–2.48 h. The *V*_d of TMA ranged from 3.2 to 4.39 l kg⁻¹ and clearance ranged from 18.78 to 23.92 ml min⁻¹ kg⁻¹. The peak concentration of TMA in blood occurred at 1 h after oral administration of a 20 mg kg⁻¹ dose and the bioavailability for the oral dose averaged 81%.

4. Peak concentrations of trimethylamine *N*-oxide (TMAO) in blood were attained at 0.75 and 1 h after i.v and oral administration of TMA (20 mg kg⁻¹), respectively.

5. Feeding the male Wistar rat with a synthetic diet resulted in a twofold decrease in the clearance of TMA. Furthermore, the concentration of TMAO in blood after i.v. administration of TMA peaked at 1.25 h in rat placed on the synthetic diet as opposed to 0.75 h in rat placed on normal laboratory rat chow. The altered pharmacokinetic profile of TMA and its *N*-oxide suggest a diminished drug-elimination capacity in rat placed on the synthetic diet.

6. Dietary modulation of flavin-containing monooxygenase (FMO) activity may explain the effects of diet on the pharmacokinetics of TMA and its *N*-oxide.

Introduction

Trimethylamine (TMA) is a simple tertiary aliphatic amine and its natural occurrence has been recognized for a long time (Al-Waiz *et al.* 1992). It undergoes extensive *N*-oxidation *in vivo* to form the polar metabolite trimethylamine *N*-oxide (TMAO). The *N*-oxidation reaction requires a direct two-electron oxidation of the heteroatom via an ionic mechanism and is mediated almost exclusively by the flavin-containing monooxygenase (FMO) (Ziegler 1990).

FMO was first purified and characterized to homogeneity by Ziegler and Mitchell (1972) and catalyses a broad range of xenobiotics bearing nucleophilic nitrogen and sulphur atoms. The mechanism of FMO-catalysed oxidations is distinctly different from that of cytochrome P450 and is well suited for the detoxification of soft nucleophiles present in most plant-derived foods (Damani

* Author for correspondence; e-mail: innane@unix.temple.edu

1989). FMO is not inducible and there are no selective mechanism-based inhibitors for this enzyme at present. However, FMO is regulated developmentally in many species and selective nutritional control of liver FMO activity has been reported (Kaderlik *et al.* 1991, Damani and Nnane 1996). Interestingly, dietary modulation of the liver FMO activity *in vivo* may have profound effects on the pharmacokinetic profile of TMA and its *N*-oxide.

The objective of this paper was to evaluate the pharmacokinetic profile of TMA in the male Wistar rat placed on normal laboratory rat chow or on a chemically defined diet since little is known to date about the pharmacokinetics of TMA and the effects of a synthetic diet on TMA disposition in rats.

Materials and methods

Chemicals

TMA, TMAO and isopropylamine were purchased from Aldrich Chemical Company (Gillingham, UK). The chemical purity of all these compounds was >98%. Heparin (sodium salt) was purchased from Sigma Chemical Company (Poole, UK). Polyvinyl tubing (0.63 mm i.d., 1.4 mm o.d.), polythene tubing (0.58 mm i.d., 0.96 mm o.d.) and silastic medical grade tubing (1.19 mm i.d., 1.70 mm o.d., vinyl) were obtained from Portex (Hythe, UK). Monojet[®] 23-gauge needles and 1 ml syringes were purchased from Macarthy's Medical Limited (Romford, UK). The anaesthetic agents Hypnorm[®] and Hypnovel[®] were purchased from Janssen Pharmaceuticals Limited (Grove, Oxford, UK) and Roche Products Limited (Welwyn Garden City, UK), respectively.

Animal studies

Male Wistar rats (230–280 g) were obtained from Bantin and Kingman Limited (Aldbrough, Hull, UK). The animals were maintained in a controlled environment of constant temperature (20°C), 50% relative humidity and 12-h light/dark cycles for at least 4 days before use. The health of all animals was monitored throughout the study by determining changes in body weights. Male Wistar rats (230–280 g) were divided into two groups of six. The control group was maintained on normal laboratory rat chow (22.8% protein, 52.9% carbohydrate, 4.5% fat, 14% amino acids and supplemented with vitamins and minerals) while the other group was placed on a synthetic diet (47% dextrin (type 2), 23% sucrose, 10% corn oil, 14% amino acids and supplemented with vitamins and minerals) for 7 days. The diets were purchased from Special Diet Services (SDS) Limited (Essex, UK). The rats were surgically prepared, under hypnorm/hypnovel anaesthesia, by implanting indwelling cannulae into the jugular vein and the carotid artery 24 h before drug administration and blood sampling. TMA was dissolved in saline to produce a concentration of 10 mg ml⁻¹. TMA was administered as a single bolus dose through the jugular vein or given orally by gavage (20 mg kg⁻¹). Arterial blood samples (250 µl) were collected in heparinized tubes pre-dose, and post-dose at 5, 15, 30, 45 min, 1, 2, 3, 4, 5, 6, 7, 8 and 24 h. Heparinized saline (250 µl) was injected into the animals immediately after sample collection. The pharmacokinetics of i.v. doses of TMA were investigated at 10, 20 and 40 mg kg⁻¹ (*n* = 6) and at an oral dose of 20 mg kg⁻¹ in rats placed on normal laboratory rat chow and also at 20 mg kg⁻¹ dose in rats placed on the synthetic diet.

Sample preparation

The concentrations of TMA and TMAO in blood were analysed using a modification of the headspace gas chromatographic method of Al-Waiz (1987). For TMA measurement, the collected blood (250 µl) was placed in 5 ml serum bottles. The internal standard, aqueous isopropylamine (10 µl 0.2% v/v), was added and this was followed by addition of potassium carbonate (10 mg) and potassium hydroxide (10 mg) to bring the pH to ~10. The vials were capped, vortex-mixed and heated at 80°C for 20–30 min in a water bath. An aliquot (5 ml) of the headspace generated was injected directly onto the GLC column (2 m × 0.4 cm, 4% Carbowax 20 M/0.8% w/w KOH on Carbowax B).

The concentrations of TMAO in blood were analysed as follows: blood samples containing TMA and TMAO were analysed for TMA as described above. The same samples, after the TMA assay, were heated for 3–5 min at 50°C in uncovered serum bottles and a gentle stream of nitrogen was passed through the samples to remove any remaining volatile TMA. The samples were then acidified to pH 1–2 with five drops of concentrated hydrochloric acid (HCl) and 50 µl TiCl₃ (15% w/v) solution was added to reduce the non-volatile TMAO to volatile TMA. The serum bottles were resealed and vortex-mixed and the mixture kept in a water bath (50°C) for 30 min. The samples were then cooled (4°C) and analysed for TMA as described above. The amount of initial TMAO present was calculated by taking

into account the conversion ratio of obtaining TMAO from TMA and the proportion of spontaneous breakdown of TMAO to TMA in rat whole blood.

Chromatographic conditions

Gas chromatography was performed on an HP5840A gas chromatograph (Hewlett-Packard Company, Avondale, USA). The GLC apparatus was equipped with a flame ionization detector, a computing integrator and a glass column (2 m × 0.4 cm). GLC separation of TMA and the internal standard isopropylamine was achieved on a glass column packed with 4% Carbowax 20 M/0.8% KOH on Carbopack B graphitized carbon support (60–80 mesh) obtained from Supelco (Bellefonte, PA, USA). The optimum operating conditions for analysis of TMA were; nitrogen flow rate, 32 ml min⁻¹; injection port temperature, 170 °C; column temperature, 70 °C (isothermal); and detector temperature, 200 °C.

Analysis of data

Non-compartmental pharmacokinetic parameters were calculated with the computer program Topfit v.1.0 using the standard non-compartmental equations. The terminal elimination rate constants (k_{el}) were calculated by log-linear regression of the concentration versus time data and the elimination half-lives ($t_{1/2}$) were calculated from the following equation; $t_{1/2} = 0.693/k_{el}$. The extrapolated areas under the plasma concentration versus time curves (AUC) were calculated from the linear trapezoid rule. Systemic clearance (Cl) and volume of distribution (V_d) were calculated from the following equations; $Cl = \text{dose}/AUC$ and $V_d = Cl/k_{el}$, respectively. Cl and V_d , after oral administration of TMA, were not corrected for bioavailability (F). F was calculated from the ratio of the extrapolated areas under the plasma concentration versus time curves following oral administration of TMA ($AUC_{p.o.}$) to the areas under the plasma concentration versus time curves after i.v. dosing ($AUC_{i.v.}$) of the same dose (20 mg kg⁻¹); $F = AUC_{p.o.}/AUC_{i.v.}$. The maximum concentrations of TMA or TMAO in blood (C_{max}) and the times to reach peak concentrations (T_{max}) were determined from the observed concentrations of TMA or TMAO in blood versus time data. Statistical analyses were performed by the Students' *t*-test or one-way ANOVA as appropriate. $p < 0.05$ was considered statistically significant.

Results

Chromatography

TMA and the internal standard isopropylamine were separated satisfactorily by the 4% Carbowax 20 M/0.8% KOH on Carbopack B column. The peaks were sharp and symmetrical and were well resolved from other low aliphatic amines such as mono- and di-methylamines and ammonia. The headspace of pre-dose blood was free from interfering substances at the retention time of interest (data not shown).

The calibration curves were linear ($r = 0.998$) and reproducible in the following ranges 0.1–1.0 and 1.0–10.0 µg ml⁻¹. The precision and accuracy of the assay were determined by calculating coefficient of variation (COV) and the mean percentage difference, MD(%), respectively. COV and MD(%) at the lower concentrations were < 20 and < 10% at the higher concentrations.

Pharmacokinetics

The concentrations of TMA in blood declined approximately monoexponentially with half-lives of 2.03–2.48 h after i.v. administration of TMA (figure 1). The pharmacokinetics of TMA were essentially linear for i.v. doses of 10–40 mg kg⁻¹ (table 1). At the 20 mg kg⁻¹ i.v. dose level, V_d of TMA was 3.2 l kg⁻¹ and the systemic clearance was 18.78 ml min⁻¹ kg⁻¹. The pharmacokinetic parameters of TMA were not significantly different after administration of a 10 or 40 mg kg⁻¹ dose. TMAO was also detected in blood and the peak concentration of TMAO in blood was attained at 0.75 or 1 h after i.v. administration of TMA doses of

Table 1. Pharmacokinetic parameters of TMA and TMAO at different I.V. bolus doses of TMA in the male Wistar rat fed on normal rat chow.

Parameter	Dose (mg kg ⁻¹)		
	10	20	40
TMA			
k_{el} (h ⁻¹)	0.36 ± 0.08	0.35 ± 0.09	0.28 ± 0.04
$t_{1/2}$ (h)	2.03 ± 0.50	2.12 ± 0.60	2.48 ± 0.40
AUC (µg ml ⁻¹ h)	8.92 ± 0.74	17.43 ± 4.98	29.55 ± 3.45
Cl (ml min ⁻¹ kg ⁻¹)	18.78 ± 1.49	20.97 ± 5.92	23.92 ± 3.43
V_d (l kg ⁻¹)	3.20 ± 0.22	3.64 ± 0.95	4.39 ± 0.66
TMAO			
C_{max} (µg ml ⁻¹)	3.19 ± 0.17	6.38 ± 0.86	11.72 ± 0.74
T_{max} (h)	0.75 ± 0.0	0.75 ± 0.25	1.0 ± 0.15
AUC (µg ml ⁻¹ h)	12.8 ± 3.02	23.43 ± 2.98	44.88 ± 2.42
k_{el} (h ⁻¹)	0.29 ± 0.04	0.27 ± 0.03	0.24 ± 0.02

Values are means ± SD (*n* = 6).

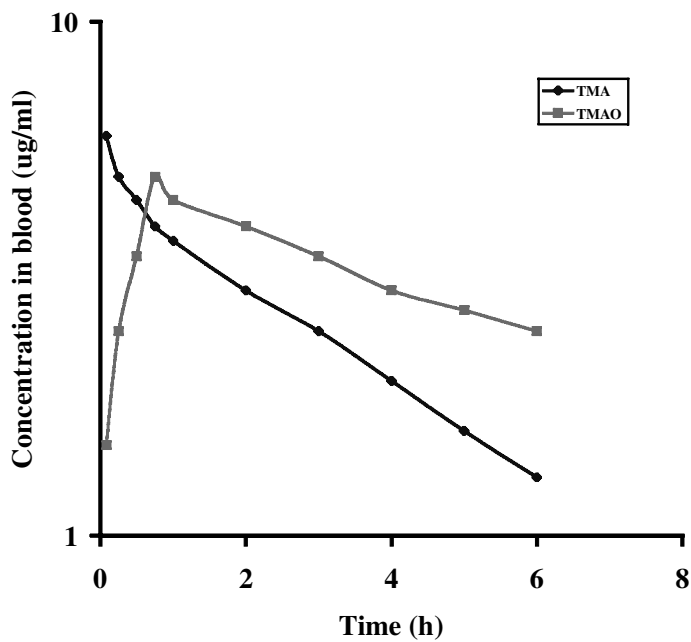


Figure 1. Mean blood concentration of TMA and TMAO after i.v. administration of TMA (20mg kg⁻¹) to the male Wistar rat placed on normal rat chow. Values are the mean from six rats. SD = ±3–6% of the means.

10–40 mg kg⁻¹ (figure 1). The concentrations of TMAO in blood after administration of the parent amine declined approximately monoexponentially with a slope shallower than that for the parent amine. Thus, the elimination of TMAO was elimination rate-limited. Furthermore, C_{max} and AUC for TMAO essentially increased proportionately to the dose after administration of the parent amine (table 1).

Table 2. Pharmacokinetic parameters of TMA and TMAO after oral administration of TMA (20 mg kg^{-1}) in the male Wistar rat fed on normal rat chow.

Parameter	TMA	TMAO
$k_{el} \text{ (h}^{-1}\text{)}$	0.42 ± 0.11	0.34 ± 0.06
$t_{1/2} \text{ (h)}$	1.65 ± 0.42	1.80 ± 0.05
AUC ($\mu\text{g ml}^{-1} \text{ h}$)	14.17 ± 1.05	17.4 ± 2.83
F	81.21 ± 2.01	—
*Cl ($\text{ml min}^{-1} \text{ kg}^{-1}$)	19.63 ± 1.58	—
* $V_d \text{ (l kg}^{-1}\text{)}$	3.15 ± 0.75	—
$C_{\max} \text{ (}\mu\text{g ml}^{-1}\text{)}$	5.02 ± 1.14	5.92 ± 1.15
$T_{\max} \text{ (h)}$	1.0 ± 0.15	1.0 ± 0.25

Values are means $\pm$ SD ($n = 6$). *Not corrected for bioavailability (F).

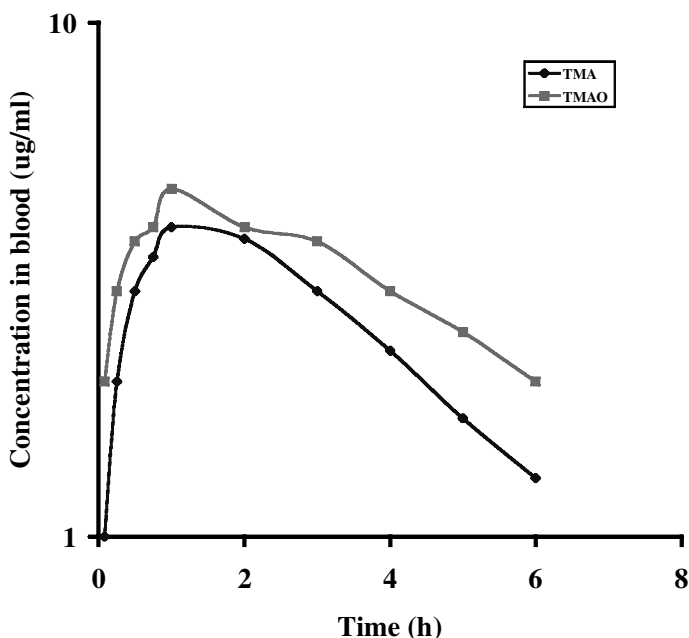


Figure 2. Mean blood concentration of TMA and TMAO after oral administration of TMA (20 mg kg^{-1}) to the male Wistar rat placed on normal rat chow. Values are the mean from six rats. SD = ± 3 –6% of the means.

The concentrations of TMA in blood after oral administration of a 20 mg kg^{-1} dose are shown in figure 2. The peak concentrations of TMA in blood occurred at 1 h and then declined monoexponentially (figure 2 and table 2). About 81% of TMA (20 mg kg^{-1}) was absorbed into the systemic circulation unchanged. TMAO was formed fairly rapidly after administration of TMA (20 mg kg^{-1}) orally. Peak concentrations of TMAO in blood were detected at 1 h after administration of TMA (figure 2).

The mean concentrations of TMA and TMAO in blood after i.v. administration of TMA (20 mg kg^{-1}) to rat placed on normal laboratory rat chow and on the synthetic diet are shown in figure 3. Feeding the male Wistar rat with the synthetic diet resulted in a twofold increase in the area under the curve (AUC). The

systemic clearance (Cl) of TMA was also halved in rat placed on the synthetic diet. The terminal half-life ($t_{1/2}$) of TMA was prolonged by placing rats on the synthetic diet (figure 3 and table 3). Important differences in the pharmacokinetic parameters of TMAO after administration of the parent amine were also observed between rats placed on a normal laboratory rat chow and those on the synthetic diet. The concentrations of TMAO in blood were higher and peaked at 0.75 h in rats placed on normal laboratory rat chow as opposed to 1.25 h in rats placed on the synthetic amino acid diet. However, the AUC of TMAO was not significantly altered after feeding rats with the synthetic diet (figure 3 and table 3).

Table 3. Pharmacokinetic parameters of TMA and TMAO after i.v. administration of TMA (20 mg kg⁻¹) in the male Wistar rat fed on normal rat chow or on a synthetic diet.

Parameter	Normal rat chow	Synthetic diet	Statistical significance (<i>p</i>)
TMA			
k_{el} (h ⁻¹)	0.35 ± 0.09	0.22 ± 0.06	< 0.05
t_2 (h)	2.12 ± 0.60	3.20 ± 0.37	< 0.05
AUC (µg ml ⁻¹ h)	17.43 ± 4.98	34.65 ± 5.64	< 0.01
Cl (ml min ⁻¹ kg ⁻¹)	20.97 ± 5.92	9.83 ± 1.56	< 0.01
V_d (l kg ⁻¹)	3.64 ± 0.95	2.83 ± 0.54	> 0.05
TMAO			
C_{max} (µg ml ⁻¹)	6.38 ± 1.86	4.33 ± 0.86	< 0.05
T_{max} (h)	0.75 ± 0.52	1.25 ± 0.23	< 0.05
AUC (µg ml ⁻¹ h)	27 ± 10.0	33.2 ± 5.01	> 0.05
k_{el} (h ⁻¹)	0.27 ± 0.03	0.20 ± 0.01	< 0.05

Values are means ± SD (*n* = 6).

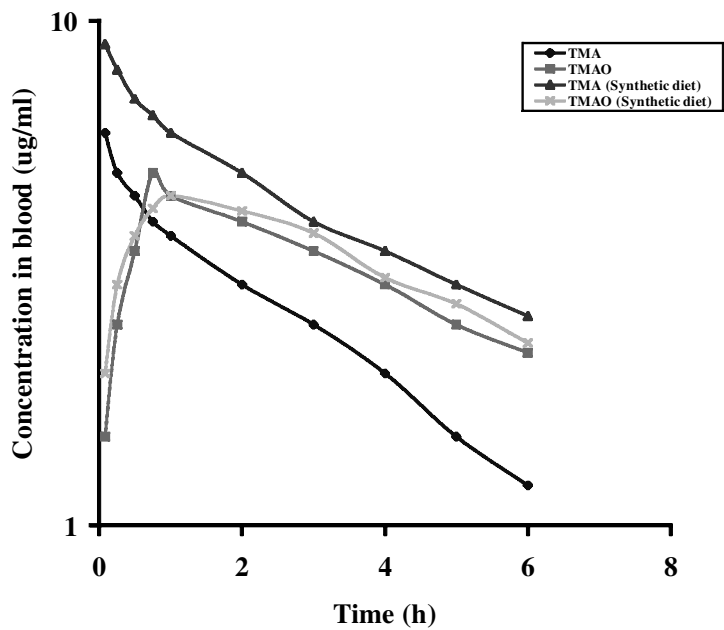


Figure 3. Comparative mean blood concentration of TMA and TMAO after i.v. administration of TMA (20 mg kg⁻¹) to the male Wistar rat placed on normal rat chow or synthetic diet. Values are the mean from six rats. SD = ±3–6% of the means.

Discussion

TMA is a simple tertiary amine subject to metabolism by FMO (Al-Waiz *et al.* 1987). However, the pharmacokinetics and the effects of a synthetic diet on the concentrations of TMA in blood versus time profile of this FMO substrate in animals have not been investigated until now. The present investigation shows that the concentrations of TMA in blood following administration of a dose of 20 mg kg^{-1} declined with a half-life of $\sim 2 \text{ h}$ and that TMA is extensively distributed into rat tissues with a V_d of $\sim 3.2 \text{ l kg}^{-1}$. The study also shows that the AUC for TMA increased proportionately to the given dose up to 40 mg kg^{-1} , indicating that the processes of elimination of TMA were not saturated in this dose range and that the pharmacokinetics of TMA are essentially linear. *N*-oxidation of TMA to the corresponding *N*-oxide is the main metabolic pathways observed for this compound (Al-Waiz *et al.* 1987). The *N*-oxide was present in rat blood after administration of TMA and the terminal elimination half-lives of TMAO were longer than that of the parent amine. Furthermore, the AUC for TMAO was greater than that of TMA. These observations suggest that TMA was cleared much more rapidly than the *N*-oxide.

The differences in pharmacokinetic parameters for TMA and its *N*-oxide observed between the two groups may reflect diminished metabolic activity or elimination capacity in rats placed on the synthetic diet. TMA is a substrate for FMO (Al-Waiz *et al.* 1987) and previous studies have shown that the synthetic diet used in this study selectively modulates the activity of FMO in rat (Kaderlik *et al.* 1991, Damani and Nnane 1996). TMA could therefore be a useful *in vivo* marker of hepatic microsomal FMO activity in intact animals with the pharmacokinetic parameters serving as indices of enzyme activity.

In conclusion, TMA, a simple tertiary amine and substrate for FMO, exhibited essentially linear pharmacokinetics in the dose range examined and the observed disposition profile of TMA was altered by a synthetic diet.

Acknowledgements

The authors thank Professor D. M. Zielger (University of Austin, TX) for providing the protocol for the synthetic diet.

References

- AL-WAIZ, M., MIKOV, M., MITCHELL, S. C. and SMITH, R. L., 1992, The exogenous origin of trimethylamine in the mouse. *Metabolism*, **41**, 135–136.
- AL-WAIZ, M., MITCHELL, S. C., IDLE, J. R. and SMITH, R. L., 1987, The metabolism of ^{14}C -labelled trimethylamine and its *N*-oxide in man. *Xenobiotica*, **17**, 551–558.
- DAMANI, L. A. (ed.), 1989, *Sulphur-Containing Drugs and Related Organic Compounds*, vol. 1, Part A (Chichester: Ellis Horwood), pp. 135–150.
- DAMANI, L. A. and NNANE, I. P., 1996, The assessment of the flavin-containing monooxygenase activity in intact animals. *Drug Metabolism and Drug Interactions*, **13**, 1–28.
- KADERLIK, R. K., WESER, E. and ZIEGLER, D. M., 1991, Selective loss of liver flavin-containing monooxygenase in rats on chemically defined diets. *Progress in Pharmacology and Clinical Pharmacology*, **8**, 95–103.
- ZIEGLER, D. M., 1990, Flavin-containing monooxygenases: enzymes adapted for multi-substrate Specificity. *TIPS*, **19**, 321–324.
- ZIEGLER, D. M. and MITCHELL, C. H., 1972, Microsomal oxidase. IV. Properties of a mixed-function amine oxidase isolated from pig liver microsomes. *Archives in Biochemistry and Biophysics*, **150**, 116–125.

Differential inhibition of human CYP1A1 and CYP1A2 by quinidine and quinine

M. S. CHING*, C. L. BLAKE, N. A. MALEK, P. W. ANGUS and H. GHABRIAL

Department of Medicine, University of Melbourne, Austin & Repatriation Medical Centre, Victoria 3081, Australia

Received 19 March, 2001

1. The inhibition of recombinant CYP1A1 and CYP1A2 activity by quinidine and quinine was evaluated using ethoxyresorufin *O*-deethylation, phenacetin *O*-deethylation and propranolol desisopropylation as probe catalytic pathways.

2. With substrate concentrations near the K_m of catalysis, both quinidine and quinine potently inhibited CYP1A1 activity with $[I]_{0.5} \sim 1\text{--}3\ \mu\text{M}$, whereas in contrast, there was little inhibition of CYP1A2 activity. The Lineweaver–Burk plots with varying inhibitor concentrations suggested that inhibition by quinidine and quinine was competitive.

3. There was only trace metabolism of quinidine by recombinant CYP1A1, whereas rat liver microsomes as a control showed extensive consumption of quinidine and metabolite production.

4. This work suggests that quinidine is a non-classical inhibitor of CYP1A1 and that it is not as highly specific at inhibiting CYP2D6 as previously thought.

Introduction

There is considerable interest in identifying substrates and inhibitors that are handled differently by the CYP1A subfamily members 1A1 and 1A2, either for their ability to distinguish between these enzymes or to provide insight into how these enzymes function. Whereas CYP1A substrates are often metabolized by both enzymes with similar activity, and CYP1A inhibitors often inhibit both enzymes with similar potencies, a few compounds can differentiate between CYP1A1 and CYP1A2 to various extents. For example, 7-ethoxyresorufin is the preferred substrate for CYP1A1, whilst 7-methoxyresorufin is the preferred substrate for CYP1A2 (Lewis and Lake 1996). Furafylline potently and selectively inhibits CYP1A2-mediated phenacetin *O*-deethylation in human liver microsomes, provided appropriately low concentrations ($< 20\ \mu\text{M}$) of inhibitor are used so that there is little inhibition of CYP1A1 (Tassaneeyakul *et al.* 1994, Newton *et al.* 1995). Furafylline is therefore used as an inhibitor to study the role of CYP1A2 in the metabolism of xenobiotics in human liver microsomes (Kunze and Trager 1993, Clarke *et al.* 1994). Subsequently, Pastrakuljic *et al.* (1997) postulated that the compounds α -naphthoflavone, apigenin, isosafrole and fluvoxamine might differentially inhibit CYP1A2 versus CYP1A1 because of their structural similarity to furafylline (i.e. the numbers of atoms in planar structure) and previous reports of CYP1A2 inhibition by these compounds. Pastrakuljic *et al.* demonstrated that the

* Author for correspondence; e-mail: ching@austin.unimelb.edu.au

two flavones α -naphthoflavone and apigenin inhibit both isoenzymes with similar potency, whereas isosafrole was 14-fold more potent and fluvoxamine was 800-fold more potent in inhibiting CYP1A2 than in inhibiting CYP1A1.

Recently Sai *et al.* (2000), in examining the specificities of eight chemical inhibitors with recombinant cytochrome P450 enzymes, reported that the specific CYP2D6 inhibitor quinidine inhibited CYP1A1 to some degree; the $[I]_{0.5}$ of quinidine was 36 μM with 7-ethoxycoumarin as the substrate. The present study describes the observation that quinidine, as well as its isomer quinine, can both selectively inhibit CYP1A1 over CYP1A2, and with seemingly greater potency than previously reported by Sai *et al.*

Materials and methods

Chemicals

7-Ethoxyresorufin, resorufin, phenacetin, paracetamol, propranolol and quinine were obtained from Sigma Chemical Co. (St Louis, MO, USA) and quinidine (97% pure, remainder quinine) was obtained from Aldrich Chemical Co. (Milwaukee, WI, USA). 3-Hydroxyquinidine was a gift from Dr Kim Brosen (Odense University, Denmark) and quinidine *N*-oxide was synthesised (purity 95%) by the *m*-chloroperbenzoic acid method of Guentert *et al.* (1982). Glucose 6-phosphate dehydrogenase and the disodium salts of glucose 6-phosphate and NADP were purchased from Boehringer Mannheim (Mannheim, Germany). All other chemicals were of analytical grade and all chromatography solvents were of HPLC grade (Mallinckrodt Baker, KT, USA).

Expression of CYP1A1 and CYP1A2 in yeast

CYP1A1 and CYP1A2 were separately expressed in *Saccharomyces cerevisiae* strain AH22, as described by Ching *et al.* (1991, 1996). CYP1A1 microsomes were prepared from AH22 yeast cells transformed with the CYP1A1 expression plasmid AH22pCK1-1, CYP1A2 microsomes were prepared from AH22 yeast cells transformed with the CYP1A2 expression plasmid AH22pCB-1. The P450 content of CYP1A was measured by the reduced carbon monoxide difference spectroscopy method (Ching *et al.* 1991), and control yeast microsomes were also prepared from AH22 yeast cells transformed with plasmid pMA91.

Kinetics of 7-ethoxyresorufin and phenacetin O-deethylation

The kinetics of 7-ethoxyresorufin O-deethylation by CYP1A1 and CYP1A2 were determined as follows. For CYP1A1, a 0.5 mM 7-ethoxyresorufin solution was made in methanol:DMSO (1:1), and this was diluted with 10 mM Tris HCl (pH 7.4) to produce a range of 10-fold concentrated stock solutions such that 50 μl stock added to the microsomal incubation reaction (final volume 0.5 ml) gave final 7-ethoxyresorufin concentrations of 0.02, 0.04, 0.07, 0.10, 0.20, 0.40 and 1.00 μM . The highest content of DMSO in the CYP1A1 7-ethoxyresorufin incubations was therefore 0.1%. For CYP1A2 where a higher substrate concentration range was required, a 2.5 mM 7-ethoxyresorufin solution was made in DMSO, and this was diluted with DMSO to produce a range of 250-fold concentrated stock solutions such that 2 μl stock added to the incubation reaction (final volume 0.5 ml) gave final 7-ethoxyresorufin concentrations of 0.83, 0.99, 1.21, 1.54, 2.21, 3.32, 5.53 and 11.06 μM . The content of DMSO in all of the CYP1A2 7-ethoxyresorufin incubations was therefore 0.4%. The final incubation mixtures comprised 1–10 μl of CYP1A yeast microsomal suspension, 0.1 ml 0.2 M potassium phosphate buffer, pH 7.4, 0.1 ml potassium phosphate buffer containing the NADPH-generating system, which consisted of 2 μmol glucose 6-phosphate, 0.2 μmol NADP, 0.2 units glucose 6-phosphate dehydrogenase and 1 μmol MgCl_2 , and the remaining volume was 1.15% (w/v) KCl. After pre-incubating the mixture at 37°C for 3 min, the reaction was started by the addition of 7-ethoxyresorufin and proceeded for 2 min for CYP1A1 or 6 min for CYP1A2 in a shaking water bath. The reaction was terminated by the addition of 0.5 ml methanol and utilization of the original substrate was < 5% (Segel 1975). The sample was microcentrifuged and the supernatant directly analysed for resorufin. Preliminary experiments demonstrated that resorufin formation was linear with respect to time (1–10 min for CYP1A1, 2–10 min for CYP1A2) and amount of enzyme added in the incubation (0.5–8 pmole for CYP1A1, 4–40 pmole for CYP1A2).

The kinetics of phenacetin O-deethylation by CYP1A1 and CYP1A2 were determined as follows. For both CYP1A1 and CYP1A2, phenacetin (30 mM) was dissolved in and diluted with methanol, so that 5 μl 100-fold concentrated stock solutions added to the incubation reaction (final volume 0.5 ml)

gave final phenacetin concentrations of 10, 20, 40, 60, 80, 100, 200 and 300 μM . The incubation mixtures were the same as that described above. After pre-incubating the mixture at 37°C for 3 min, the reaction was started by the addition of phenacetin and proceeded for 10 min in a shaking water bath. Reactions were terminated by the addition of 50 μl 6% perchloric acid and utilization of the original substrate was < 5% (Segel 1975). The sample was microcentrifuged and the supernatant was directly analysed for paracetamol. Preliminary experiments demonstrated that paracetamol formation was linear with respect to time (5–15 min for CYP1A1 and 1A2) and amount of enzyme added in the incubation (15–60 pmole for CYP1A1 and 1A2).

Inhibition by quinidine and quinine

$[I]_{0.5}$ of quinidine and quinine inhibition of 7-ethoxyresorufin and *O*-deethylation phenacetin *O*-deethylation by CYP1A1 and CYP1A2 were determined. Quinidine and quinine concentrations were varied from 1 to 200 μM using a constant 7-ethoxyresorufin concentration of 0.04 and 1.4 μM for CYP1A1 and CYP1A2 respectively, or a constant phenacetin concentration of 60 μM for both CYP1A1 and CYP1A2.

$[I]_{0.5}$ of quinidine and quinine inhibition on propranolol *N*-desisopropylation by CYP1A2 was determined, in which case quinidine and quinine concentrations were varied from 1 to 200 μM and constant propranolol concentrations of 150 and 90 μM for (+) and (–) propranolol were used respectively for CYP1A2. Incubations involving propranolol *N*-desisopropylation were carried out as described (Ching *et al.* 1996).

In further experiments to examine the type of inhibition demonstrated by quinidine and quinine with CYP1A1, 7-ethoxyresorufin as the substrate was varied from 0.02 to 0.40 μM , propranolol as the substrate was varied from 2.5 to 200 μM , and inhibitor concentrations of quinidine and quinine from 0.25 to 6.0 μM .

HPLC analysis of resorufin and paracetamol

The analysis of resorufin was based on the method of Murray *et al.* (1993). A 30 μl aliquot of the incubation supernatant was injected into the chromatograph (Waters Associates, Milford, MA, USA), which comprised a 6000A pump, a 717plus auto-injector, a Novapak C18 4 μm , 3.9 $\times$ 150 mm stainless steel column, a 470 fluorescence detector (excitation 565 nm, emission 590 nm) and a Maxima data acquisition system. The mobile phase was acetonitrile:water (13:87) with 1% (v/v) triethylamine adjusted to pH 7.8 with orthophosphoric acid, and the flow rate was 1 ml min⁻¹. Resorufin had a retention time of 5 min and calibration curves were plotted as resorufin peak height versus standard concentration of 2.5–80 nm for CYP1A1 and 6.25 to 200 nm for CYP1A2. The limit of sensitivity was $\sim$ 0.1 pmole resorufin on column, and the assay coefficient of variation was < 4% at both 2.5 and 80 nm.

The analysis of paracetamol was based on the method of Solomon (1993). A 30 μl aliquot of the incubation supernatant was injected into the chromatograph as above, except that detection of paracetamol was performed using ECD with a BAS LC-4A amperometric detector and a BAS glassy carbon electrode (0.8V versus Ag/AgCl). The mobile phase was methanol:water (5:95) with 0.005 M trisodium citrate and 0.2 M sodium perchlorate adjusted to pH 5.0 with perchloric acid. The flow rate was 1 ml min⁻¹, the retention time of paracetamol was 7 min and calibration curves were plotted as paracetamol peak height versus standard concentration. The limit of sensitivity was $\sim$ 1.5 pmole of paracetamol on column, and the assay coefficient of variation was 7% at 0.125 μM and 2% at 2.0 μM .

Metabolism of quinidine by CYP1A1

The potential metabolism of quinidine by CYP1A1 was examined using the standard microsomal incubation methods as described above with 0.10 ml of either CYP1A1 yeast microsomal suspension or rat liver microsomes in a final volume of 0.5 ml. Incubations using rat liver microsomes acted as a positive control to prove that the present microsomal system could support the metabolism of quinidine. Reactions proceeded for 1 hr and were terminated by the addition of 20 μl perchloric acid. The HPLC method used to determine 3-hydroxyquinidine and quinidine *N*-oxide was based on the method of Ching *et al.* (1991). After centrifugation to pellet the precipitated microsomal protein, 30 μl of the supernatant was directly injected into the chromatograph as described above. Detection was by fluorescence (model 470; Waters) with an excitation/emission of 246/412 nm. The analytical column was a reverse-phase Luna phenyl-hexyl 5 μm , 250 $\times$ 3 mm stainless steel column (Phenomenex, CA, USA) protected by an in-line SecurityGuard precolumn module with 4 $\times$ 2 mm phenylpropyl precolumn inserts (Phenomenex). The mobile phase was acetonitrile (6% v/v), methanol (6% v/v) and 1% triethylamine in deionized water and adjusted to pH 2.5 with concentrated orthophosphoric acid. Chromatography was carried out at a constant flow rate of 0.8 ml min⁻¹ giving a back pressure of $\sim$ 3200 psi.

Data analysis

For both 7-ethoxyresorufin and phenacetin *O*-deethylation, velocity (v) was determined by the rate of appearance of resorufin or paracetamol measured as $\text{nmol min}^{-1} \text{nmol}^{-1}$ CYP1A1 or CYP1A2, and the Henri-Michaelis-Menten equation was fitted to the (v) versus substrate $[S]$ data using the iterative non-linear least-squares regression program Minim (v. 3.0.8; R.D. Purves, University of Otago, New Zealand). Results are reported as mean $\pm$ standard deviation (SD) unless otherwise stated.

Results

In control AH22::pMA91 microsomes, there was no evidence of 7-ethoxyresorufin *O*-deethylation (data not shown). CYP1A1 and CYP1A2 catalysed formation of resorufin, although both the affinity of resorufin ($K_m = 0.050 \pm 0.003 \mu\text{M}$, $n = 4$) and V_{max} ($4.85 \pm 1.29 \text{ nmol min}^{-1} \text{nmol}^{-1}$) for CYP1A1 were greater than the corresponding values for CYP1A2 ($K_m = 1.38 \pm 0.18 \mu\text{M}$, $n = 3$, $V_{\text{max}} = 2.01 \pm 0.20 \text{ nmol min}^{-1} \text{nmol}^{-1}$) (figure 1). In control AH22::pMA91 microsomes, there was no evidence of phenacetin *O*-deethylation (data not shown). CYP1A1 and CYP1A2 catalysed formation of paracetamol, and the affinity of phenacetin appeared to be slightly greater for CYP1A1 ($K_m = 38 \pm 15 \mu\text{M}$, $n = 4$) than for CYP1A2 ($K_m = 116 \pm 16 \mu\text{M}$, $n = 4$) whilst V_{max} was somewhat greater for CYP1A2 ($2.08 \pm 0.15 \text{ nmol min}^{-1} \text{nmol}^{-1}$, $n = 4$ versus $0.73 \pm 0.16 \text{ nmol min}^{-1} \text{nmol}^{-1}$ for CYP1A1, $n = 4$) (figure 2).

Quinidine and quinine both potently inhibited 7-ethoxyresorufin *O*-deethylation by CYP1A1 (figure 3) with $[I]_{0.5}$ 1.1 and $1.0 \mu\text{M}$ respectively. By contrast, there was negligible inhibition of 7-ethoxyresorufin *O*-deethylation by CYP1A2. Quinidine also inhibited phenacetin *O*-deethylation by CYP1A1 ($[I]_{0.5} = 3.1 \mu\text{M}$) but had no effect upon phenacetin metabolism by CYP1A2 (figure 3). An identical picture emerged with propranolol; neither quinidine nor quinine showed inhibition of (+) or (−) propranolol *N*-desisopropylation by CYP1A2, but both compounds potently inhibited *N*-desisopropylation of (−) propranolol by CYP1A1 (data not shown). The $1/v$ versus $1/[S]$ Lineweaver-Burk plots of quinidine and quinine

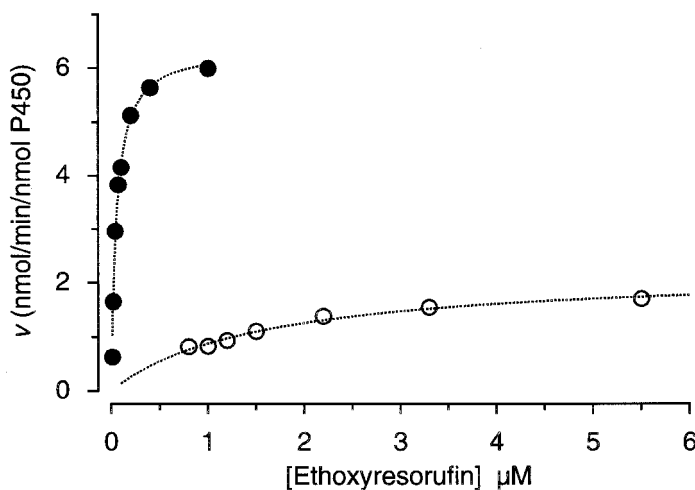


Figure 1. Representative v versus $[S]$ curve of resorufin formation following incubation of 7-ethoxyresorufin with CYP1A1 (●) and CYP1A2 (○).

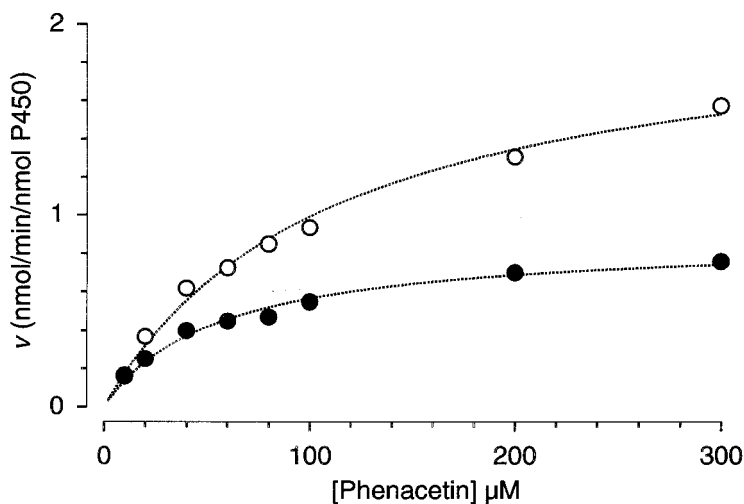


Figure 2. Representative v versus $[S]$ curve of paracetamol formation following incubation of phenacetin with CYP1A1 (●) and CYP1A2 (○).

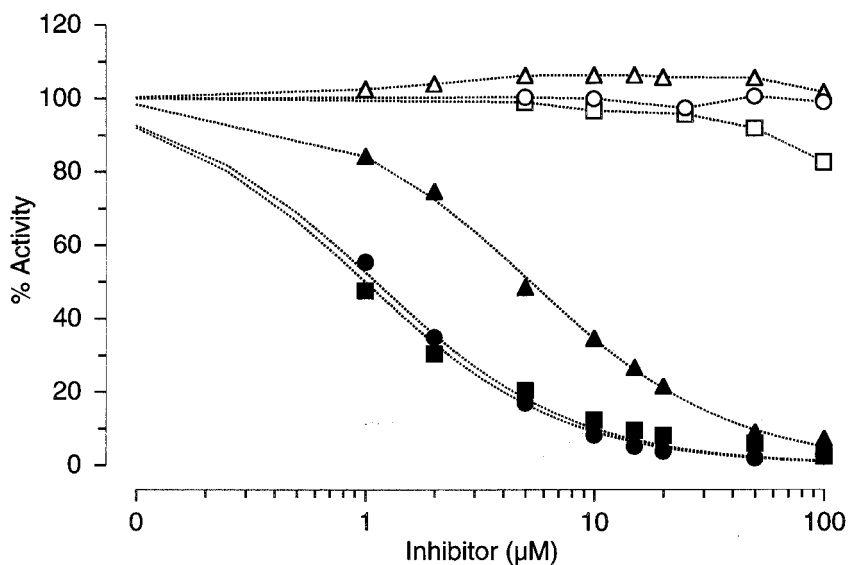


Figure 3. Representative plot showing a decrease in % activity of 7-ethoxyresorufin *O*-deethylation by CYP1A1 in the presence of quinidine (●) and quinine (■). Neither quinidine (○) nor quinine (□) inhibited 7-ethoxyresorufin *O*-deethylation by CYP1A2. Also shown is the plot of decrease in % activity of phenacetin *O*-deethylation by CYP1A1 in the presence of quinidine (▲). Quinidine (△) had no effect on phenacetin *O*-deethylation by CYP1A2.

inhibition of 7-ethoxyresorufin *O*-deethylation and propranolol *N*-desisopropylation by CYP1A1 are shown in figure 4.

In rat liver microsomes, after 1 h of incubation with quinidine, $\sim 90\%$ of the original quinidine was consumed, production of 3-hydroxyquinidine was evident in the chromatogram but production quinidine *N*-oxide was not evident (figure 5).

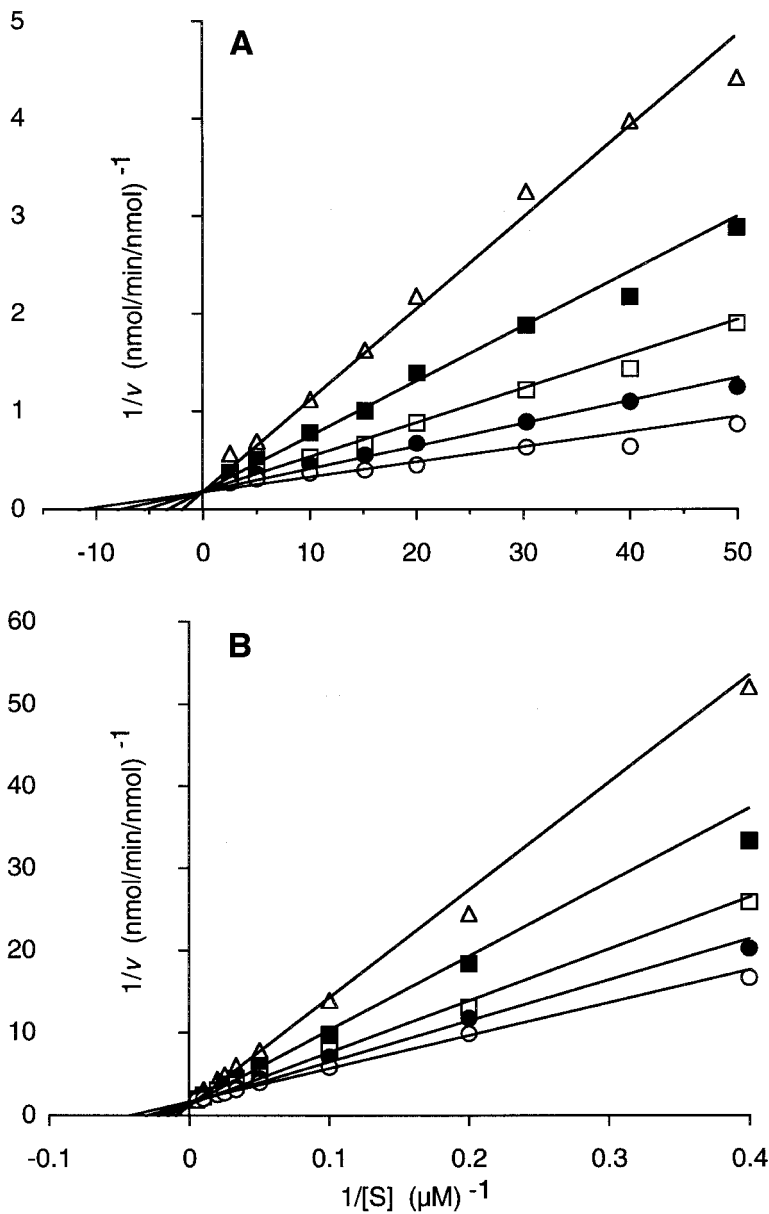


Figure 4. Representative Lineweaver–Burk plots of quinidine (A) inhibition of propranolol *N*-desisopropylation and quinine (B) inhibition of 7-ethoxyresorufin *O*-deethylation by CYP1A1. Inhibitor concentrations (μM) are: no inhibitor (○), 0.5 (●), 1.0 (□), 2.0 (■) and 4.0 (△).

In recombinant CYP1A1 microsomes, after 1 h of incubation, there was negligible loss quinidine, no production of quinidine *N*-oxide, but there was some evidence of production of 3-hydroxyquinidine as shown by the small peak at 4.5 min (figure 5); this peak that was not present in blank drug-free microsomes or in microsomes spiked with quinidine but not incubated.

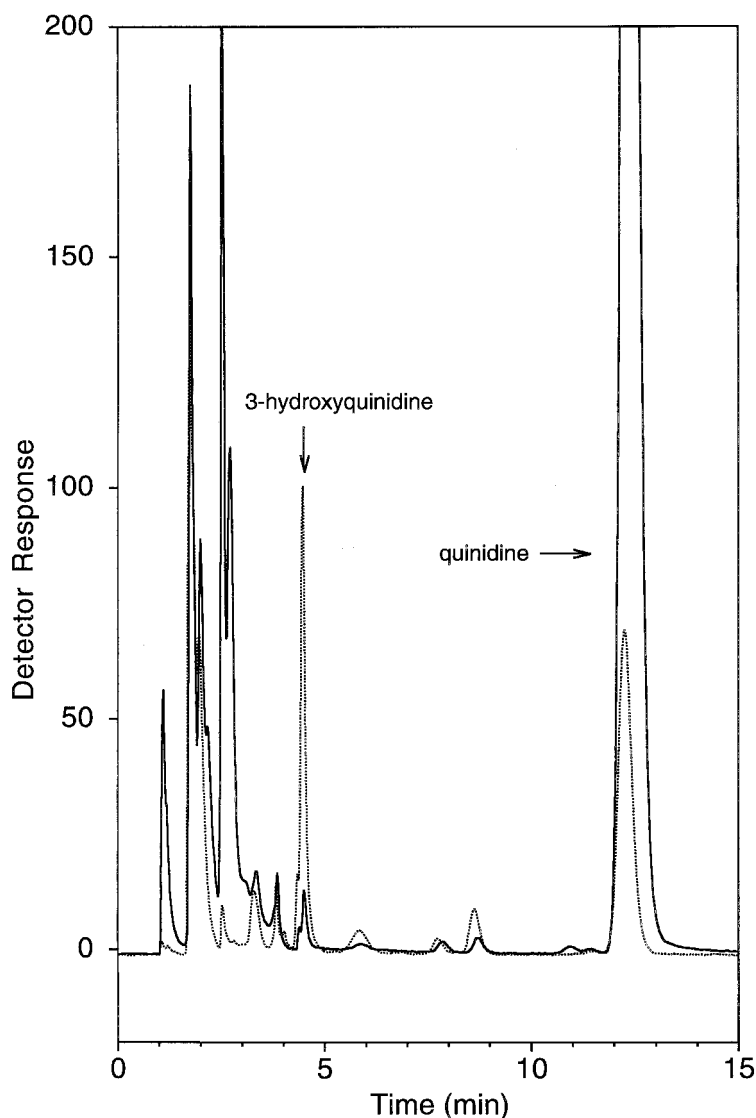


Figure 5. HPLC chromatogram showing production of 3-hydroxyquinidine after 1 h incubation with rat liver microsomes (dashed line) and recombinant CYP1A1 (solid line). There was no evidence of quinidine *N*-oxide production (retention time 9.2 min) in the chromatograms. There was negligible consumption of quinidine with recombinant CYP1A1 but $\sim 90\%$ consumption of quinidine with rat liver microsomes.

Discussion

Both yeast expressed CYP1A1 and CYP1A2 catalysed the metabolism of 7-ethoxyresorufin, K_m and V_{max} were much greater for CYP1A1 than that for CYP1A2 (figure 1). An estimate of intrinsic clearance (V_{max}/K_m) of 7-ethoxyresorufin is ~ 60 -fold higher for recombinant CYP1A1 than CYP1A2, which agrees with other reports that the affinity and intrinsic clearance of 7-ethoxyresorufin are both considerably greater for CYP1A1 than CYP1A2 (Penman *et al.* 1994, Pastrakuljic *et al.* 1997). Both CYP1A enzymes catalysed the metabolism of

phenacetin (figure 2) and the affinity was slightly greater for CYP1A1 ($K_m = 38 \mu\text{M}$) than for CYP1A2 ($K_m = 116 \mu\text{M}$); these K_m are in the same order of magnitude as those reported by Tassaneeyakul *et al.* (1993), although in the latter study the K_m of phenacetin *O*-deethylation by CYP1A1 ($108 \mu\text{M}$) was greater than that for CYP1A2 ($25 \mu\text{M}$) when expressed in COS cells.

We have observed that both quinidine and quinine are able to differentially inhibit CYP1A1 and CYP1A2 when using 7-ethoxyresorufin *O*-deethylation, phenacetin *O*-deethylation and propranolol *N*-desisopropylation as probes of CYP1A activity (figure 3). This inhibition is relatively potent with an $[I]_{0.5} \sim 1 \mu\text{M}$ for CYP1A1 when using substrate concentrations approximately equal to the K_m . The Lineweaver–Burk plots (figure 4) also suggest that the inhibition is competitive in nature. Although the specificity of several substrates and inhibitors of CYP1A1 and CYP1A2 has been examined by Tassaneeyakul *et al.* (1993), and Pastrakuljic *et al.* (1997), and the specificity of several human cytochrome P450 inhibitors (including quinidine) has been examined by Newton *et al.* (1985), Bourri  *et al.* (1996) and Ono *et al.* (1996), the potential of quinidine to inhibit human CYP1A1 was not examined in any of those studies. More recently, however, Sai *et al.* (2000) appeared to be the first to report on human recombinant CYP1A1 inhibition by quinidine, and in this latter study, the $[I]_{0.5}$ of quinidine was $36 \mu\text{M}$ (with 7-ethoxycoumarin as the substrate), which is considerably less potent compared with what we observed. In that study, however, since the substrate concentration of 7-ethoxycoumarin used was very high ($100 \mu\text{M}$) and the K_m was not characterized, it is probable that the $[I]_{0.5}$ of quinidine reported ($36 \mu\text{M}$) does not reflect the K_i and the true potency of inhibition by quinidine. Consequently, Sai *et al.* (2000) made no specific comment regarding the quinidine inhibition of CYP1A1. The present work suggests that if micromolar quinidine concentrations are used to abolish hepatic CYP2D6 microsomal activity in order to probe hepatic CYP1A microsomal activity (e.g. as recently used by Johnson *et al.* 2000 in a study of propranolol 4-hydroxylation), then potential hepatic CYP1A1 activity may also be inhibited and its contribution masked.

Given that quinidine and quinine can interact with CYP1A1, we therefore examined the possibility of whether CYP1A1 has a role in the metabolism of quinidine. Metabolism of quinidine to 3-hydroxyquinidine by rat liver microsomes (as a positive control) was evident by appearance of the metabolite peak at 4.5 min and diminution of the parent quinidine peak by 90% (figure 5), consistent with a previous report that quinidine can undergo 3-hydroxylation by rat liver microsomes (Rakhit and Mico 1985). In CYP1A1 microsomes, however, although there was evidence of 3-hydroxyquinidine appearance, the small magnitude of the peak at 4.5 min and the fact that there was negligible consumption of quinidine suggests that CYP1A1 is involved in quinidine 3-hydroxylation to only a minor degree. These findings imply that the interaction of quinidine with CYP1A1 is similar to the interaction with CYP2D6 (Guengerich *et al.* 1986, Otton *et al.* 1988) in that the drug can occupy the active site of CYP1A1 but at the same time it is not a major substrate for this isoenzyme. This is consistent with what has been previously shown about the roles of human P450s in quinidine and quinine metabolism. CYP3A4 is the major P450 enzyme involved in quinidine 3-hydroxylation and *N*-oxidation (Guengerich *et al.* 1986) in man. Furthermore, using nine

human recombinant P450 enzymes cloned in lymphoblastoid cells, Zhao *et al.* (1996) reported that CYP3A4 and to a lesser degree CYP2C19 are the major human enzymes involved in 3-hydroxylation of *quinine* whilst several other enzymes including CYP1A1 appeared to show only trace activity for quinine 3-hydroxylation.

The observations of the present work contradict the conventional thinking of the specific molecular structural requirements of the substrates that interact with CYP1A1. It is thought that CYP1A essentially has an exclusive preference to metabolise flat planar molecules with high molecular area/depth² ratios = 4–5, and medium molecular volume of 175–220 Å³ (Ioannides and Parke 1987, Lewis 2000). In contrast, the area/depth² ratios of typical substrates for CYP2C9, CYP2C19, CYP2D6 and CYP3A4 have area/depth² ratios in the range 1–3. An exception is CYP2E1 whose substrates have area/depth² ratios in the range 1–6 but they are also of small molecular volume (60–150 Å³) compared with CYP1A substrates (Lewis 2000). According to the recent model of CYP1A1 by Lewis and Lake (1996), the CYP1A active site is an approximately rectangular slot with the coplanar rings of 181Phe and 437Tyr facing parallel to each other ~6.2 Å apart, thereby restricting access to the active site only to planar poly(hetero)aromatic substrates with molecular depth no larger than about ~6 Å. Using the molecular modelling program Sybyl v.6.4 (Tripos Associates) and assuming a molecular sphere radius of 1.4 Å as Lewis (2000) did, the molecular volume of quinidine is 297.5 Å³ and its surface area is 295.3 Å² in the low energy state. From this, one can calculate a molecular area/depth² ratio of quinidine of 2.5 if molecular depth (10.9 Å) is determined using a plane normal to the aromatic rings, or a molecular area/depth² ratio = 4.8 if molecular depth (7.8 Å) is determined using a plane of best fit through all atoms. In either case, the molecular area/depth² ratio and/or molecular depth of quinidine are outside the range of classical CYP1A substrates, meaning that quinidine appears to be an atypical inhibitor of CYP1A1.

Lewis and Lake (1996) also suggested that there were few significant differences between the putative active sites of CYP1A1 and CYP1A2, with the coplanar rings of 181Phe and 437Tyr involved in π - π stacking with aromatic rings on substrate molecules and 266Phe and 330Phe assisting in access/orientation of planar substrates; these residues are present in both CYP1A1 and CYP1A2 in many species. The present work, however, suggests that the active sites of CYP1A1 and CYP1A2 must be dissimilar enough so that CYP1A1 can accommodate quinidine and quinine binding and CYP1A2 cannot. It would be most interesting to explore if any of the current models of CYP1A postulated by Zvelebil *et al.* (1991), Lewis and Lake (1996) and Lozano *et al.* (1997), and consideration of the conformation of quinidine itself can rationalize the findings in the present study.

Acknowledgements

The study was supported by the NH&MRC (Australia) and the Sir Edward Dunlop Foundation. M.S.C. is a Senior Research Officer of the NH&MRC. The authors thank Dr Tony Hughes of the Department of Pharmacology, University of Melbourne, for assistance with calculations using the program Sybyl.

References

- BOURRIÉ, M., MEUNIER, A., BERGER, Y. and FABRE, G., 1996, Cytochrome P450 isoform inhibitors as a tool for the investigation of metabolic reactions catalyzed by human liver microsomes. *Journal of Pharmacology and Experimental Therapeutics*, **277**, 321–332.
- CHING, M. S., BICHARA, N., BLAKE, C. L., GHABRIAL, H., TUKEY, R. H. and SMALLWOOD, R. A., 1996, Propranolol 4- and 5-hydroxylation and *N*-desisopropylation by cloned human cytochrome P4501A1 and 1A2. *Drug Metabolism and Disposition*, **24**, 692–694.
- CHING, M. S., BLAKE, C. L., GHABRIAL, H., ELLIS, S. W., LENNARD, M. S., TUCKER, G. T. and SMALLWOOD, R. A., 1995, Potent inhibition of yeast expressed CYP2D6 by dihydroquinidine, quinidine, and its metabolites. *Biochemical Pharmacology*, **50**, 833–837.
- CHING, M. S., LENNARD, M. S., TUCKER, G. T., WOODS, H. F., KELLY, D. E. and KELLY, S. L., 1991, The expression of human cytochrome P4501A1 in the yeast *Saccharomyces cerevisiae*. *Biochemical Pharmacology*, **42**, 753–758.
- CLARKE, S. E., AYRTON, A. D. and CHENERY, R. J., 1994, Characterization of the inhibition of P4501A2 by furafylline. *Xenobiotica*, **24**, 517–526.
- GUENGERICH, F. P., MÜLLER-ENOCH, D. and BLAIR, I. A., 1986, Oxidation of quinidine by human liver cytochrome P-450. *Molecular Pharmacology*, **30**, 287–295.
- GUENTERT, T. W., DALY, J. J. and RIEGELMAN, S., 1982, Isolation, characterisation and synthesis of a new quinidine metabolite. *European Journal of Drug Metabolism and Pharmacokinetics*, **7**, 31–38.
- IOANNIDES, C. and PARKE, D. V., 1987, The cytochromes P-448—a unique family of enzymes involved in chemical toxicity and carcinogenesis. *Biochemical Pharmacology*, **36**, 4197–4207.
- JOHNSON, J. A., HERRING, V. L., WOLFE, M. S. and RELLING, M. V., 2000, CYP1A2 and CYP2D6 4-hydroxylate propranolol and both reactions exhibit racial differences. *Journal of Pharmacology and Experimental Therapeutics*, **294**, 1099–1105.
- Kunze, K. L. and TRAGER, W. F., 1993, Isoform-selective mechanism based inhibition of human cytochrome P450 by furafylline. *Chemical Research in Toxicology*, **6**, 649–656.
- LEWIS, D. F. V., 2000, On the recognition of mammalian microsomal cytochrome P450 substrates and their characteristics. *Biochemical Pharmacology*, **60**, 293–306.
- Lewis, D. F. V. and LAKE, B. G., 1996, Molecular modelling of CYP1A subfamily members based on an alignment with CYP102: rationalization of CYP1A substrate specificity in terms of active site amino acid residues. *Xenobiotica*, **26**, 723–753.
- LOZANO, J. J., LÓPEZ-DE-BRÍÑAS, E., CENTENO, N. B., GUIGÓ, R. and SANZ, F., 1997, Three-dimensional modelling of human cytochrome P450 1A2 and its interaction with caffeine and MeIQ. *Journal of Computer Aided Molecular Design*, **11**, 395–408.
- MURRAY, B. P., EDWARDS, R. J., MURRAY, S., SINGLETON, A. M., DAVIES, D. S. and BOOBIS A. R., 1993, Human hepatic CYP1A1 and CYP1A2 content, determined with specific anti-peptide antibodies, correlates with the mutagenic activation of PhIP. *Carcinogenesis*, **14**, 585–592.
- Newton, D. J., Wang, R. W. and LU, A. Y. H., 1985, Cytochrome P450 inhibitors. Evaluation of specificities in the *in vitro* metabolism of therapeutic agents by human liver microsomes. *Drug Metabolism and Disposition*, **23**, 154–158.
- ONO, S., HATANAKA, T., HOTTA, H., SATOH, T., GONZALEZ, F. J. and TSUTSUI, M., 1996, Specificity of substrate and inhibitor probes for cytochrome P450s: evaluation of *in vitro* metabolism using cDNA-expressed human P450s and human liver microsomes. *Xenobiotica*, **26**, 681–693.
- OTTON, S. V., BRINN, R. U. and GRAM, L. F., 1988, *In vitro* evidence against the oxidation of quinidine by the sparteine/debrisoquine monoxygenase of human liver. *Drug Metabolism Disposition*, **16**, 15–17.
- PASTRAKULJIC, A., TANG, B. K., ROBERTS, E. A. and KALOW, W., 1997, Distinction of CYP1A1 and CYP1A2 activity by selective inhibition using fluvoxamine and isosafrole. *Biochemical Pharmacology*, **53**, 531–538.
- PENMAN, B. W., LIPING, C., GELBOIN, H. V., GONZALEZ, F. J. and CRESPI, C. L., 1994, Development of a human lymphoblastoid cell line constitutively expressing human liver CYP1A1 cDNA: substrate specificity with model substrates and promutagens. *Carcinogenesis*, **15**, 1931–1937.
- RAKHIT, A. and MICO, B., 1985, Kinetics of microsomal metabolism of quinidine in rats. *Research and Communications and Chemical Pathology and Pharmacology*, **49**, 109–124.
- SAI, Y., DAI, R., YANG, T. J., KRAUSZ, K. W., GONZALEZ, F. J., GELBOIN, H. V. and SHOU, M., 2000, Assessment of specificity of eight chemical inhibitors using cDNA-expressed cytochrome P450. *Xenobiotica*, **30**, 327–343.
- SEGEL, H., 1975, Enzyme Kinetics—Behaviour and Analysis of Rapid Equilibrium and Steady-State Enzyme Systems (New York: Wiley), pp. 18–99.
- SOLOMON, B. P., 1987, Determination of acetaminophen in plasma. *Current Separations*, **8**, 58–59.
- TASSANEYAKUL, W., BIRKETT, D. J., VERONESE, M. E., McMANUS, M. E., TUKEY, R. H. and MINERS, J. O., 1994, Direct characterization of the selectivity of furafylline as an inhibitor of human cytochromes P450 1A1 and 1A2. *Pharmacogenetics*, **4**, 281–284.

- ZHAO, X.J., YOKOYAMA, H., CHIBA, K., WANWIMOLRUK, S. and ISHIZAKI, T., 1996, Identification of human cytochrome P450 isoforms involved in the 3-hydroxylation of quinine by human liver microsomes and nine recombinant human cytochromes P450. *Journal of Pharmacology and Experimental Therapeutics*, **279**, 1327–1334.
- ZVELEBIL, M. J. J., WOLF, C. R. and STERNBERG, M. J. E., 1991, A predicted three-dimensional structure of human cytochrome P450: implications for substrate specificity. *Protein Engineer*, **4**, 271–282.

Enzymatic and non-enzymatic reduction of brucine *N*-oxide by aldehyde oxidase and catalase

K. TAKEKAWA^{†‡}, K. SUGIHARA[†], S. KITAMURA^{†*} and S. OHTA[†]

[†] Institute of Pharmaceutical Science, Hiroshima University School of Medicine, Kasumi 1-2-3, Minami-ku, Hiroshima 734-8551, Japan

[‡] Safety Research Laboratory, Tanabe Seiyaku Co., Ltd., Yodogawa-ku, Osaka 532-8505, Japan

Received 28 February 2001

1. Brucine *N*-oxide was reduced by aldehyde oxidase in rabbit liver cytosol in the presence of an electron donor, 2-hydroxypyrimidine, under anaerobic conditions. The flavoprotein purified from rabbit liver exhibited significant reductase activity in the presence of electron donors.

2. Brucine *N*-oxide was also reduced by rabbit liver cytosol and blood in the presence of both a reduced pyridine nucleotide and FAD under anaerobic conditions. The *N*-oxide reductase activities were inhibited by carbon monoxide and air. However, these activities were not abolished when liver cytosol and blood were boiled. Rabbit erythrocytes exhibited the reductase activity, but not plasma.

3. When liver cytosol or blood was separated by DEAE-cellulose column chromatography, the fractions with the reducing activity in the presence of both NADH and FAD also showed catalase activity.

4. Catalase catalysed the brucine *N*-oxide reduction in the presence of both NAD(P)H and FAD. Hematin also exhibited the reductase activity in the presence of both NAD(P)H and FAD. Photochemically reduced FAD was effective in the reduction instead of NAD(P)H and FAD.

5. Brucine *N*-oxide reduction proceeds via two routes in liver cytosol and blood. One is enzymatic reduction by aldehyde oxidase; the other is non-enzymatic reduction catalysed by the haem group of catalase in the presence of reduced flavin.

Introduction

Several aliphatic tertiary amines are metabolically oxidized to the corresponding *N*-oxides (Damani 1982) and is thought to be an important detoxification pathway of tertiary amines. Alkaloids are also oxidized to the alkaloid *N*-oxides (Phillipson and Handa 1978) and alkaloid *N*-oxides such as indicine *N*-oxide, which have pharmacological activity, also occur naturally in plants (Phillipson 1971). The enzyme systems involved in the oxidative metabolism of tertiary amines have been well investigated, and the roles of cytochrome P450 and flavin-containing monooxygenase have been established (Ziegler 1980). The *N*-oxides, including alkaloid *N*-oxides, are usually reduced to the parent tertiary amines and this interconversion between tertiary amines and *N*-oxides is a well-

* Author for correspondence; kitamura@pharm.hiroshima-u.ac.jp

known metabolic pathway (Bickel 1969). Such interconversion in the body might be effective in maintaining the pharmacological and toxicological effect of tertiary amines, including alkaloids.

Aliphatic tertiary amine *N*-oxides are known to be reduced enzymatically to the corresponding amines by liver microsomal cytochrome P450 supplemented with NADPH or NADH and this has been demonstrated for indicine *N*-oxide, imipramine *N*-oxide, tiaramide *N*-oxide and *N,N*-dimethylaniline *N*-oxide (Sugiyama *et al.* 1976, Iwasaki *et al.* 1977, Powis and Wincentsen 1980). In contrast, we investigated the reduction of *N*-oxide compounds by mammalian liver cytosol using imipramine *N*-oxide, cyclobenzaprine *N*-oxide and *S*-(-)-nicotine-1'-*N*-oxide as aliphatic tertiary amine *N*-oxides, and also nicotinamide *N*-oxide as an aromatic type *N*-oxide (Sugihara *et al.* 1996, Kitamura and Tatsumi 1984a, b). Mammalian liver cytosol exhibits significant *N*-oxide reductase activity toward these *N*-oxides when supplemented with an electron donor for aldehyde oxidase (EC 1231), such as 2-hydroxypyrimidine, *N*¹-methylnicotinamide or benzaldehyde, and the flavoenzyme purified from rabbit liver functioned as an *N*-oxide reductase. Furthermore, we demonstrated that aliphatic tertiary amine *N*-oxides are non-enzymatically reduced by cytochrome P450 in rat liver microsomes (Takekawa *et al.* 2001).

Brucine *N*-oxide is a major metabolite of brucine, found in seeds of *Strychnos nux-vomica* together with strychnine (Phillipson and Handa 1978). In the present study, the reduction of brucine *N*-oxide, as a model alkaloid *N*-oxide, by rabbit liver cytosol and blood was investigated. This paper provides evidence that this heterocyclic *N*-oxide is reduced enzymatically by aldehyde oxidase in liver cytosol and also reduced non-enzymatically by catalase in liver cytosol and blood in the presence of both a reduced pyridine nucleotide and a flavin. This is a novel reaction of catalase.

Materials and methods

Materials

Brucine *N*-oxide (99%), brucine (98%) and imipramine hydrochloride (>99%) were purchased from Aldrich Chemical Co. Inc. (Milwaukee, WI, USA). Nicotinamide (>99%), nicotinamide *N*-oxide (99%), menadione (98%), 2-hydroxypyrimidine hydrochloride (>98%) and *N*¹-methylnicotinamide chloride (98%) were from Tokyo Chemical Co. Ltd. (Tokyo, Japan). Cyclobenzaprine hydrochloride (>99%), bovine liver catalase, bovine blood haemoglobin and bovine blood hematin were from Sigma Chemical Co. (St Louis, MO, USA). Imipramine *N*-oxide (>99%) and cyclobenzaprine *N*-oxide (>99%) were prepared by the method of Kitamura and Tatsumi (1984b). Reduced FAD was prepared by the method of Yubisui *et al.* (1980).

Liver homogenate preparation

Japanese White male rabbits (2.6–3.2 kg) were exsanguinated. Livers were immediately perfused with 1.15% KCl and homogenized in 4 vols of the KCl solution using a Potter-Elvehjem homogenizer. The homogenate was centrifuged for 20 min at 9000g, and the supernatant fraction was centrifuged for 60 min at 105 000g. The cytosol fraction was obtained from the supernatant.

Preparation of blood and erythrocytes

Blood samples were collected from the jugular vein. Blood was placed in polystyrene tubes containing heparin as an anticoagulant and was centrifuged for 5 min at 4°C at 1000g. The plasma was removed, and the pellet (erythrocyte fraction) was washed with 2 vols cold isotonic saline. Erythrocytes were suspended and haemolyzed in 1 vol. of 0.01 M K₂Na-phosphate buffer (pH 7.4).

Purification of aldehyde oxidase

Aldehyde oxidase was purified from rabbit liver according to the method of Felsted *et al.* (1973), except that gel filtration was adopted instead of preparative acrylamide electrophoresis, as described by Kitamura *et al.* (1984). The purified enzyme was homogeneous by the criterion of sodium dodecyl sulphate-polyacrylamide gel electrophoresis with Rapid CBB (Kanto Co. Ltd, Tokyo, Japan) staining.

Assay method for N-oxide reductase

The incubation mixture consisted of 0.5 μmol brucine *N*-oxide or other *N*-oxides, 2 μmol NADPH or NADH, 0.1 μmol FAD and liver cytosol, blood or a haemoprotein (0.5 nmol) in a final volume of 1 ml 0.1 M K,Na-phosphate buffer (pH 7.4). The incubation was performed using a Thunberg tube under anaerobic conditions. The side arm contained NADPH or NADH, and the main compartment contained all the other components. The tube was gassed for 3 min with deoxygenated nitrogen, evacuated with an aspirator for 5 min and again gassed with the nitrogen. The reaction was started by mixing the components of the side arm and the main compartment together, continued for 10 min at 37°C and stopped by adding 0.1 ml 1 N NaOH. The mixture, after addition of 12.5 μg of acetanilide or 25 μg of phenacetin as an internal standard in the case of brucine *N*-oxide, or imipramine *N*-oxide and cyclobenzaprine *N*-oxide, respectively, was extracted once with 5 ml ethyl acetate and the extract was evaporated to dryness *in vacuo*. The residue was dissolved in 0.1 ml methanol and then subjected to high-performance liquid chromatography (HPLC). In other cases, the incubation mixture, which consisted of brucine *N*-oxide, an electron donor of aldehyde oxidase and an enzyme source in the phosphate buffer, was incubated for 10 min. After incubation, the mixture was extracted and assayed by HPLC as above. The time-course of the reaction was examined by using rabbit liver cytosol in the presence of NADPH and FAD, or 2-hydroxypyrimidine. When brucine *N*-oxide was incubated for 0, 3, 6, 10 and 15 min, the reaction proceeded linearly for at least the initial 15 min.

When nicotinamide *N*-oxide was used as a substrate in this experiment, the incubation was performed using 0.2 μmol of the *N*-oxide compound with catalase, in the same manner as described above. The assay for nicotinamide was conducted by the method of Kitamura and Tatsumi (1984a).

HPLC

HPLC was performed in a Hitachi L-6000 high-performance liquid chromatograph equipped with a UV absorption detector. The instrument was fitted with a 150 $\times$ 4.6 mm Inertsil ODS-3 (GL Sciences, Inc., Tokyo, Japan) column. The mobile phase was acetonitrile-0.1 M KH_2PO_4 (1:4) for the separation of brucine and brucine *N*-oxide. The chromatograph was operated at a flow rate of 0.5 ml min⁻¹ at ambient temperature and at a wavelength of 254 nm. Elution times of brucine *N*-oxide and brucine were 4.6 and 10.0 min, respectively. For the separation of imipramine *N*-oxide, cyclobenzaprine *N*-oxide and their reduction metabolites, the mobile phase was acetonitrile-0.1 M KH_2PO_4 (3:7). The chromatograph was operated at a flow rate of 1.0 ml min⁻¹ and at a wavelength of 254 nm. Elution times of imipramine *N*-oxide, imipramine, cyclobenzaprine *N*-oxide and cyclobenzaprine were 18.2, 16.0, 19.0 and 16.3 min, respectively. Aliphatic tertiary amines formed were determined from the relative peak areas.

Assay of catalase activity

The rate of disappearance of H_2O_2 was followed by observing the rate of decrease in absorbance at 240 nm. One unit of activity corresponds to the decomposition of 1.0 μmol H_2O_2 per min in 0.1 M phosphate buffer (pH 7.0) at 25°C.

UV spectral analysis

UV spectra were measured in a Hitachi 320 apparatus using a Thunberg-type cuvette under anaerobic conditions at 25°C. The incubation mixture consisted of 0.5 μmol of *N*-oxide, 0.1 μmol reduced FAD and 0.5 nmol catalase in a final volume of 2 ml 0.1 M K,Na-phosphate buffer (pH 7.4).

Determination of protein

Protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as a standard protein.

Results

Reduction of brucine N-oxide by rabbit liver cytosol

Rabbit liver cytosol exhibited *N*-oxide-reducing activity toward brucine *N*-oxide in the presence of an electron donor of aldehyde oxidase, 2-hydroxypyrimidine.

imine or N^1 -methylnicotinamide, under anaerobic conditions. However, the reductase activity was not observed with xanthine, an electron donor of xanthine oxidase (table 1). 2-Hydroxypyrimidine or N^1 -methylnicotinamide-dependent activity was abolished when the cytosol was boiled (table 1). The activity was inhibited by *p*-benzoquinone, chlorpromazine and amytal (inhibitors of aldehyde oxidase) at a concentration of 10^{-4} M (data not shown). The flavoprotein purified from rabbit liver exhibited the reductase activity in the presence of its electron donors, 2-hydroxypyrimidine, N^1 -methylnicotinamide and benzaldehyde. The 2-hydroxypyrimidine-dependent activity was inhibited by *p*-benzoquinone (figure 1). In contrast, rabbit liver cytosol also exhibited *N*-oxide reductase activity toward brucine *N*-oxide in the presence of NADH or NADPH under anaerobic conditions. The reductase activity was markedly enhanced in the presence of both NADPH and FAD, or NADH and FAD. These activities were not abolished by boiling the liver cytosol (table 1). The *N*-oxide reductase activities were not affected by the addition of potassium cyanide (1 mM) or EDTA (1 mM). However, about 95% of the initial activity was inhibited by oxygen and carbon monoxide (data not shown).

Reduction of brucine N-oxide by rabbit blood

Brucine *N*-oxide was also reduced to brucine by rabbit blood in the presence of both NAD(P)H and FAD under anaerobic conditions. The *N*-oxide reductase activity was inhibited by carbon monoxide and sodium azide, both of which are haem ligands. However, the activity was not abolished by boiling the blood. The activity of boiled blood was also markedly inhibited by carbon monoxide and sodium azide (table 2). Rabbit erythrocytes exhibited activity with NAD(P)H and FAD, but plasma did not (data not shown).

These facts suggest that brucine *N*-oxide reductase activity is due to hemo-proteins in rabbit liver cytosol or blood, in addition to aldehyde oxidase in the liver cytosol.

Table 1. Reduction of brucine *N*-oxide by rabbit liver cytosol.

Addition	Brucine formed ^a (nmol min ⁻¹ mg protein ⁻¹)	
	Cytosol	Boiled cytosol ^b
None	n.d.	n.d.
NADH	0.3 ± 0.1	0.2 ± 0.1
NADPH	0.4 ± 0.1	0.3 ± 0.1
NADH, FAD	2.7 ± 0.4	3.0 ± 0.5
NADPH, FAD	2.6 ± 0.4	2.9 ± 0.5
2-Hydroxypyrimidine	1.0 ± 0.2	n.d.
N^1 -methylnicotinamide	0.8 ± 0.1	n.d.
Xanthine	n.d.	n.d.

Each value represents the mean ± SD of four experiments. n.d., Not detected.

^a Brucine *N*-oxide was incubated with liver cytosol in the presence of cofactors for 10 min under anaerobic conditions. Brucine formed was determined by HPLC. Other details are as described in the Materials and methods.

^b Liver cytosol was heated for 10 min in a boiling water bath.

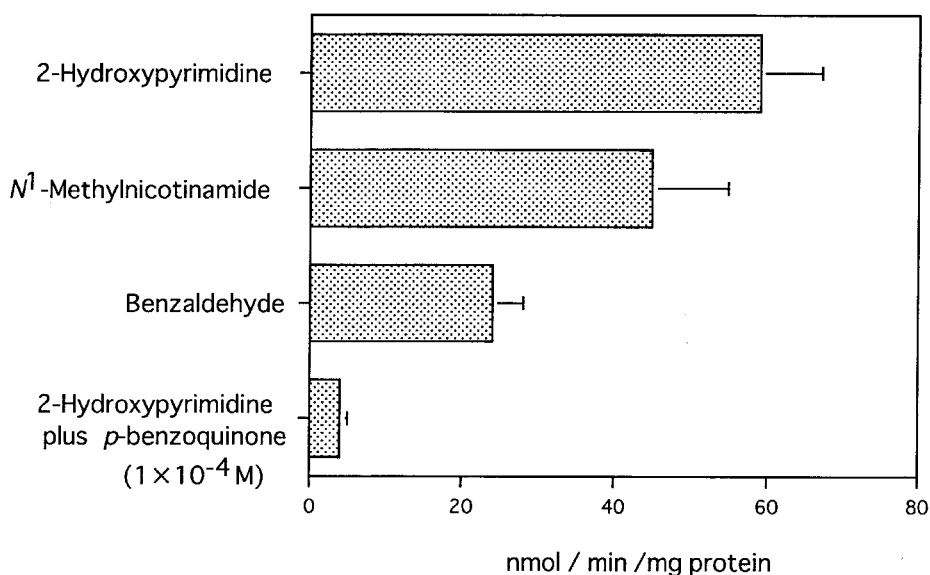


Figure 1. Reduction of brucine *N*-oxide by aldehyde oxidase from rabbit liver cytosol. Each value represents the mean $\pm$ SD of four experiments. Reactions were conducted with liver aldehyde oxidase (0.1 mg protein) in the presence of an electron donor at 37°C for 10 min under anaerobic conditions. Brucine formed was determined by HPLC. Other details are as described in the Materials and methods.

DEAE-cellulose column chromatography of rabbit liver cytosol and blood

Next, we examined the components in liver cytosol and blood that mediate the *N*-oxide reduction in the presence of NAD(P)H and FAD. The 30–45% ammonium sulphate fraction of rabbit liver cytosol was subjected to DEAE-cellulose column chromatography, and the fractions were assayed for brucine *N*-oxide reductase activity. The fractions that exhibited *N*-oxide reducing activity in the presence of both NADH and FAD also showed catalase activity (figure 2A). When

Table 2. Reduction of brucine *N*-oxide by haemolyzed rabbit blood.

Addition	Brucine formed ^a (nmol min ⁻¹ mg protein ⁻¹)	
	Blood	Boiled blood ^b
None	n.d.	n.d.
NADH	0.8 $\pm$ 0.2	0.8 $\pm$ 0.3
NADPH	0.7 $\pm$ 0.2	0.5 $\pm$ 0.2
NADH, FAD	36.7 $\pm$ 4.4	32.4 $\pm$ 5.5
NADPH, FAD	35.7 $\pm$ 3.8	32.0 $\pm$ 6.2
NADH, FAD, sodium azide ^c	5.4 $\pm$ 1.2	4.7 $\pm$ 1.8
NADH, FAD, CO ^d	n.d.	n.d.

Each value represents the mean $\pm$ SD of four experiments. n.d., Not detected.

^a Brucine *N*-oxide was incubated with rabbit blood in the presence of cofactors for 10 min under anaerobic conditions. Brucine formed was determined by HPLC. Other details are as described in the Materials and methods.

^b Blood was heated for 10 min in a boiling water bath.

^c Sodium azide was added to the incubation mixture at the concentration of 1×10^{-4} M.

^d Incubation was performed in an atmosphere of carbon monoxide.

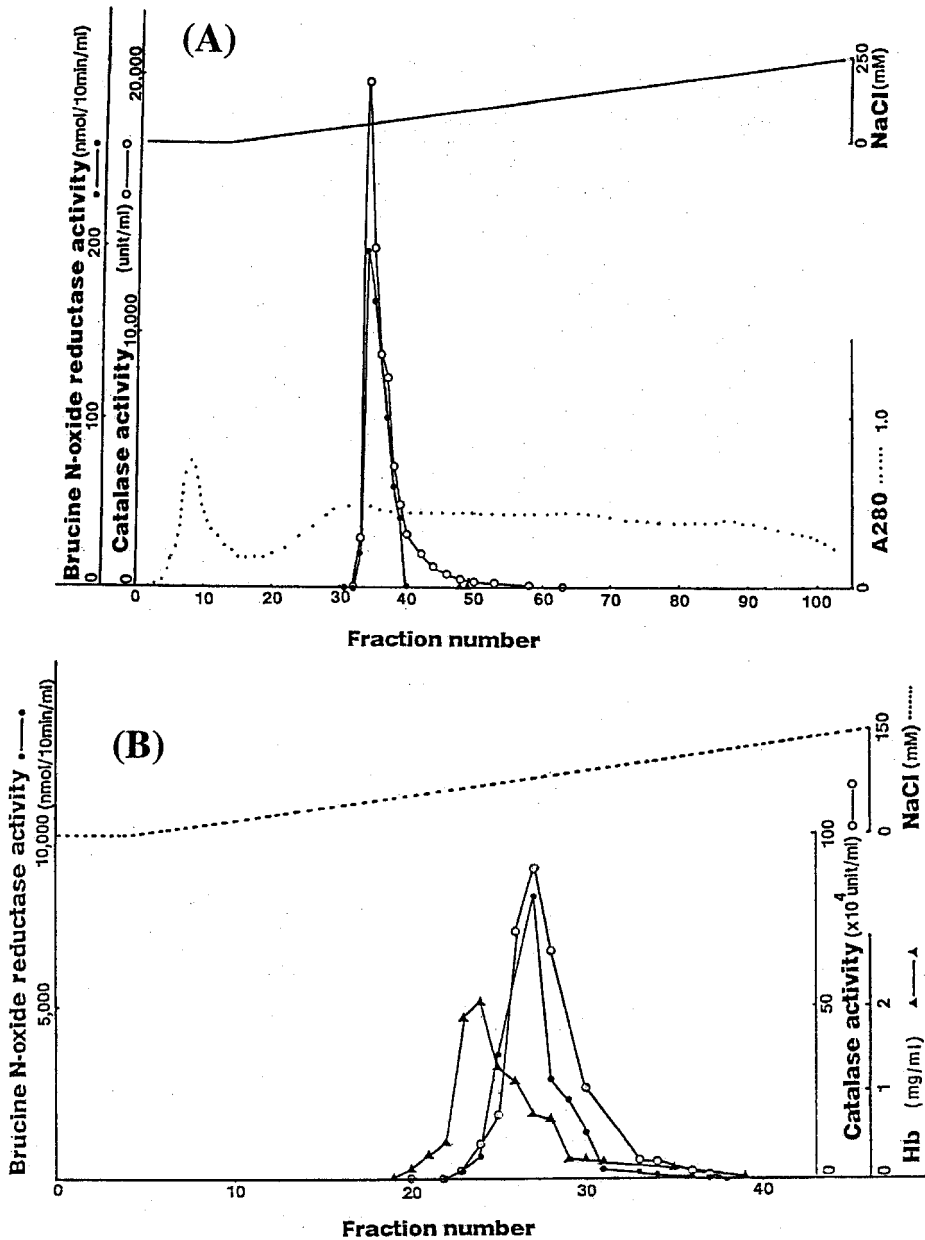


Figure 2. Elution pattern of brucine *N*-oxide reductase activity in DEAE-cellulose column chromatography of the 30–45% ammonium sulphate fraction of rabbit liver cytosol (A) and the 0–50% ammonium sulphate fraction of haemolyzed erythrocytes (B). Brucine *N*-oxide reductase activity was assayed with 50 μ l of each fraction in the presence of both NADH and FAD at 37°C for 10 min under anaerobic conditions. Brucine formed was determined by HPLC. Other details are as described in the Materials and methods. Hb: haemoglobin.

0–50% haemolyzed erythrocytes were subjected to DEAE cellulose column chromatography, the *N*-oxide reductase activity eluted with catalase activity, but not with haemoglobin (figure 2B). These facts suggest that catalase in rabbit liver cytosol or blood was involved in the *N*-oxide reduction.

Table 3. Reduction of brucine *N*-oxide by bovine liver catalase.

Addition	Brucine formed ^a (nmol min ⁻¹ mg protein ⁻¹)	
	Catalase	Boiled catalase ^b
None	n.d.	n.d.
NADH	4 ± 2	1 ± 1
NADPH	6 ± 2	2 ± 2
NADH, FAD	291 ± 40	125 ± 20
NADPH, FAD	302 ± 44	128 ± 26
NADH, FMN	310 ± 52	—
NADH, riboflavin	491 ± 37	—
NADH, FAD, sodium azide ^c	50 ± 13	34 ± 8
NADH, FAD, CO ^d	n.d.	n.d.

Each value represents the mean ± SD of four experiments. nd, Not detected; —, not determined.

^a Brucine *N*-oxide was incubated with catalase in the presence of cofactors for 10 min under anaerobic conditions. Brucine formed was determined by HPLC. Other details are as described in the Materials and methods.

^b Catalase was heated for 10 min in a boiling water bath.

^c Sodium azide was added to the incubation mixture at the concentration of 1×10^{-4} M.

^d Incubation was performed in an atmosphere of carbon monoxide.

Reduction of brucine *N*-oxide by catalase

Catalase from bovine blood exhibited an *N*-oxide-reducing activity in the presence of both NAD(P)H and FAD. The NADH-linked activity was also markedly enhanced by the addition of FMN and riboflavin. The NADH- and FAD-dependent activity was inhibited by carbon monoxide and sodium azide. When catalase was boiled, the activity was not abolished. The activity was also inhibited by carbon monoxide and sodium azide (table 3). When catalase was replaced by haemoglobin, peroxidase, myoglobin or cytochrome *c*, reducing activity was still observed, but these haemoproteins were not as effective as catalase for *N*-oxide reduction. The boiled haemoproteins also exhibited reductase activity (figure 3). Hematin also exhibited significant reductase activity in the presence of both NADPH and FAD under anaerobic conditions at a rate of $566 \text{ nmol min}^{-1} \text{ mg}^{-1}$. The reducing ability of catalase towards different types of *N*-oxide compounds was examined. Catalase exhibited the highest activity toward brucine *N*-oxide, a heterocyclic tertiary amine *N*-oxide, followed by cyclobenzaprine *N*-oxide and imipramine *N*-oxide, noncyclic tertiary amine *N*-oxides. However, nicotinamide *N*-oxide, an aromatic heterocyclic *N*-oxide, was not reduced by this system (figure 4).

Reduced flavin-dependent *N*-oxide reduction by catalase

Brucine *N*-oxide was not reduced by photochemically reduced FAD (table 4). However, catalase exhibited reducing activity in the presence of reduced FAD in place of NAD(P)H and FAD. The reduced FAD-dependent activity was inhibited by carbon monoxide. Haemoglobin and myoglobin also exhibited *N*-oxide reductase activity in the presence of reduced FAD. However, these activities were much less than that of catalase as observed in the case of NAD(P)H- and FAD-dependent reduction (table 4). However, ferric chloride, ferrous chloride or protoporphyrin did not exhibit reductase activity (data not shown).

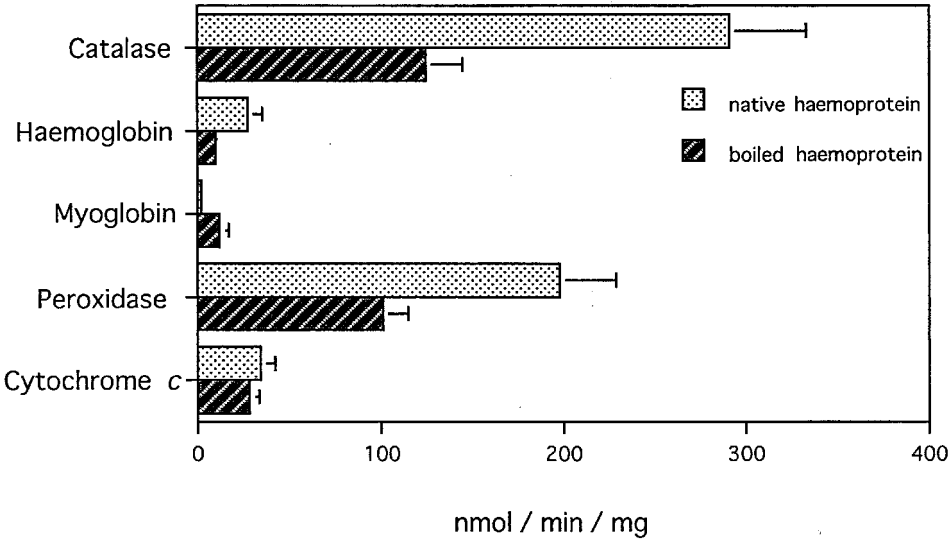


Figure 3. Reduction of brucine *N*-oxide by some haemoproteins. Each value represents the mean $\pm$ SD of four experiments. Reactions were conducted with 0.5 nmol native or boiled haemoprotein in the presence of 2 μ mol NADPH and 0.1 μ mol FAD at 37°C under anaerobic conditions. Other details are as described in the Materials and methods.

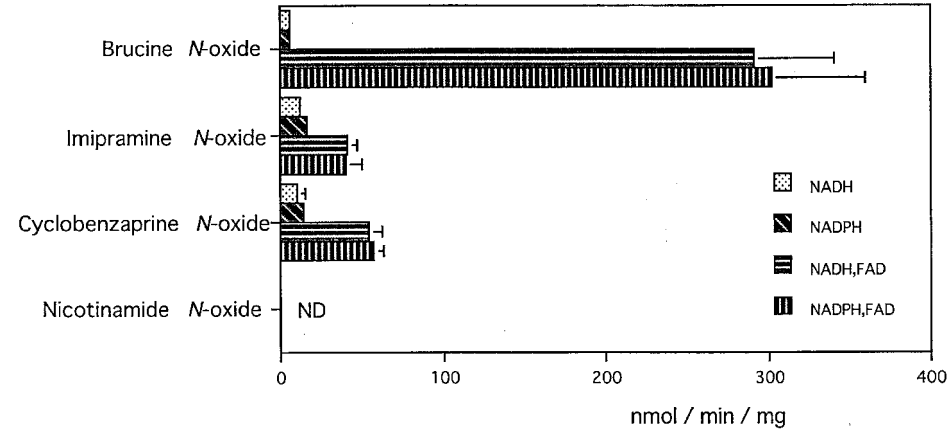


Figure 4. Reduction of *N*-oxide compounds by catalase. Each value represents the mean $\pm$ SD of four experiments. Reactions were conducted with 0.5 nmol catalase in the presence of 2 μ mol of NAD(P)H and 0.1 μ mol FAD at 37°C under anaerobic conditions. Amines formed were determined by HPLC. Other details are as described in the Materials and methods. ND, not detected.

Spectral change of reduced FAD by N-oxide compounds in the presence of catalase

Spectral change of reduced FAD was examined after incubation of *N*-oxide compounds with reduced FAD and catalase under anaerobic conditions. The absorbance at 450 nm due to oxidized FAD was markedly increased after the addition of brucine *N*-oxide, and the time-course was linear for the initial 30 s (figure 5). The absorbance also increased slowly by the addition of imipramine *N*-oxide. However, the addition of nicotinamide *N*-oxide did not produce such an

Table 4. Reduction of brucine N-oxide by haemoproteins and FAD reduced by irradiation with a W lamp in the presence of EDTA.

Addition	Brucine formed ^a (nmol min ⁻¹ mg ⁻¹)
None	1 ± 1
Haemoglobin	164 ± 27
Myoglobin	152 ± 21
Catalase	1780 ± 220
Catalase, CO ^b	193 ± 56

Each value represents the mean ± SD of four experiments.

^a Brucine N-oxide was incubated with haemoprotein in the presence of cofactors for 10 min under anaerobic conditions. Brucine formed was determined by HPLC. Other details are as described in the Materials and methods.

^b Incubation was performed in an atmosphere of carbon monoxide.

effect (figure 5). When the incubation mixture in the case of brucine N-oxide was extracted with ethyl acetate, brucine was detected in the mixture and the stoichiometry of formation of brucine and FAD was about 1:1 (data not shown). This suggests that the oxidation of reduced FAD is involved in the reduction of brucine N-oxide catalyzed by catalase.

These results indicate that brucine N-oxide is enzymatically reduced to brucine by aldehyde oxidase in rabbit liver cytosol and by the non-enzymatic catalytic action of the haem group of catalase in the presence of both FAD and a reduced pyridine nucleotide.

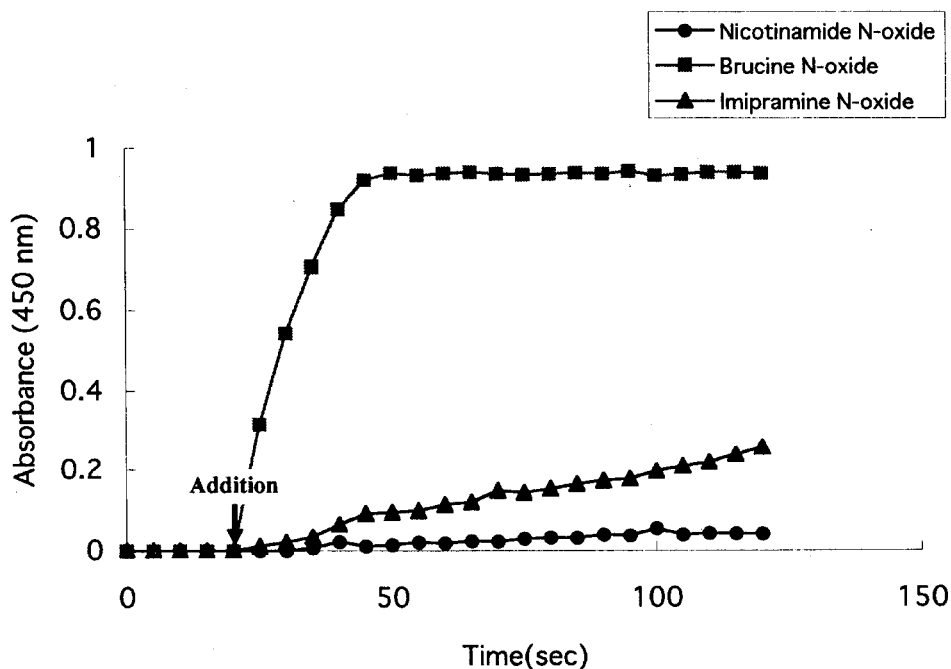


Figure 5. FADH₂ oxidation by brucine N-oxide, imipramine N-oxide and nicotinamide N-oxide in the presence of catalase. The reaction was conducted in a Thunberg-type cuvette containing N-oxide compound, reduced FAD and catalase as described in the Materials and methods.

Discussion

This study has shown that brucine *N*-oxide reduction proceeds via two routes in liver cytosol and blood. One is enzymatic reduction by aldehyde oxidase in liver cytosol, the other is non-enzymatic reduction catalysed by the haem group of catalase in the presence of reduced flavin. Recently, we reported that aliphatic tertiary amine *N*-oxides were reduced non-enzymatically by rat liver microsomes in the presence of NAD(P)H and a flavin (Takekawa *et al.* 2001). It was suggested that a reduced flavin was enzymatically or non-enzymatically formed from a flavin, and then non-enzymatically converted cytochrome P450 to its reduced form, which in turn reduced the tertiary amine *N*-oxides to the corresponding amines. The microsomal activity closely resembled the non-enzymatic reduction in liver cytosol and blood described here. Thus, aliphatic tertiary amines appear to be similarly reduced by catalase in liver cytosol and blood, and by cytochrome P450 in liver microsomes.

Aldehyde oxidase, a cytosolic enzyme, contains FAD, molybdenum and an iron-sulphur centre, and is closely related to xanthine oxidase. The enzyme can catalyse the oxidation of a number of aldehydes and nitrogenous heterocyclic compounds. Moreover, in the presence of an electron donor, it mediates the reduction of a variety of compounds (Beedham 1985). In the current study, brucine *N*-oxide was identified as a compound reducible by the enzyme (figure 6).

The other reduction of brucine *N*-oxide by blood and liver cytosol appears to proceed in two steps. The first step is the reduction of a flavin by NADPH-flavin reductase with NADPH, or by NADH-cytochrome-*b*₅ reductase with NADH in native blood (Yubisui *et al.* 1977, Yubisui and Takeshita 1980), or the non-enzymatic reduction of a flavin by NADPH or NADH as reported by Singer and Kearney (1950). The non-enzymatic reduction of flavins by NAD(P)H may involve trace amounts of metal ions, as suggested by Crichton *et al.* (1975). The second step is the conversion of the haem group of catalase to its reduced form by a reduced flavin, followed by the reduction of brucine *N*-oxide to brucine (figure 6). In this step, the reduced flavins function as reducing agents of haem. The reduction is assumed to proceed by two-electron reduction, as demonstrated by Yubisui (1980 *et al.*) in the reduction of methaemoglobin by FADH₂ and is very rapid. The reduction of flavins by flavin reductase in the first step is assumed to be the rate-limiting step of *N*-oxide reduction. In the spectral study, it was found that when brucine *N*-oxide was incubated with a reduced flavin in the presence of catalase, the UV absorption at 450 nm due to the oxidized form of a flavin was immediately increased by the oxidation of a reduced flavin, and concomitantly

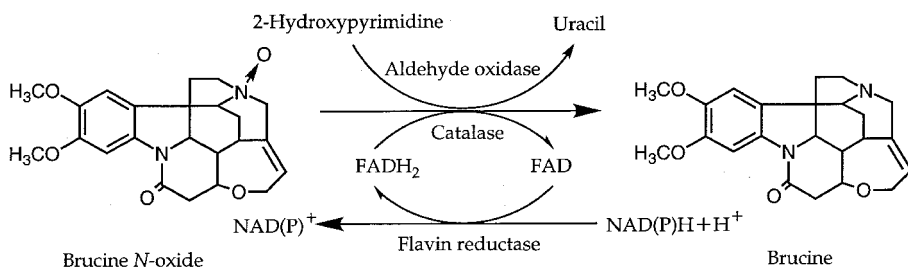


Figure 6. Reduction of brucine *N*-oxide by aldehyde oxidase and catalase.

brucine *N*-oxide was reduced. This supports the above mechanism of brucine *N*-oxide reduction by haem. The reducing activity by aldehyde oxidase shows marked species differences (Beedham 1985). In contrast, the reduced flavin-dependent brucine *N*-oxide reduction in blood was observed widely among species, i.e. rat, mouse, guinea pig, hamster and rabbit (data not shown).

Non-enzymatic reduction of some nitro and azo compounds by reduced flavins has been reported (Kamm and Gillette 1963, Gingell and Walker 1971) and reduced flavin can directly reduce those compounds. In the current study, brucine *N*-oxide was not directly reduced by reduced flavins without haem. Recently, it was demonstrated that halogenated compounds α -bromoisovalerylurea and *p,p'*-DDT were reductively dehalogenated by a haem-catalysed system in rat blood (Sugihara *et al.* 1998, Kitamura *et al.* 1999a). Hydroxamic acids, such as *N*-hydroxy-2-acetylaminofluorene and *N*-hydroxyphenacetin, were also non-enzymatically reduced by cytochrome P450 in rat liver microsomes and haemoglobin in rabbit blood (Kitamura *et al.* 1999c, Sugihara *et al.* 2000). It was also demonstrated that imipramine *N*-oxide was reduced by a quinone-dependent system catalysed by haemoproteins in the liver cytosol of rat (Kitamura *et al.* 1999b). However, the reduced flavin-dependent tertiary amine *N*-oxide reduction in liver cytosol and blood demonstrated in the current study has not previously been reported. This non-enzymatic reduction catalysed by catalase appears to function as a major *N*-oxide reducing system for various tertiary amine *N*-oxides. However, sulphoxide and epoxide compounds were not reduced by this catalase-catalysed system (data not shown). The substrate specificity of this reducing system is complex and further examination is necessary to establish its significance for various types of xenobiotics.

Takekawa *et al.* (2001) demonstrated that reduced flavins non-enzymatically reduced aliphatic tertiary amine *N*-oxides with cytochrome P450. Patterson and Gorrod (1978) also reported that some 4-substituted *N*-ethyl-*N*-methylaniline *N*-oxides were reduced by boiled liver microsomes under anaerobic conditions. The reduced flavin-dependent *N*-oxide reducing activity toward brucine *N*-oxide catalysed by catalase found in the present study was of the same order of magnitude as the microsomal *N*-oxide reductase activity. Thus, the reduced flavin-dependent reduction appears to function as a major system in the reduction of tertiary amine *N*-oxides in the liver and blood of animals. However, nicotinamide *N*-oxide, an aromatic type *N*-oxide, was not reduced by this catalase-involved system. It has been reported that nicotinamide *N*-oxide was also not reduced by the cytochrome P450 system (Kitamura and Tatsumi 1984a). In contrast, aldehyde oxidase exhibited reductase activities toward both an aliphatic tertiary amine *N*-oxide, imipramine *N*-oxide, and an aromatic type *N*-oxide, nicotinamide *N*-oxide, and the reductase activity toward nicotinamide *N*-oxide was extraordinarily high (Kitamura and Tatsumi 1984a). The high water solubility of the *N*-oxide may be one reason for this difference of substrate specificity between tertiary amine *N*-oxides and aromatic type *N*-oxides.

Surprisingly, catalase (a major protein in blood and liver) played a major role in the brucine *N*-oxide reduction by blood, but haemoglobin did not. There may be little difference in the ability of a reduced flavin to reduce catalase and haemoglobin to the reduced forms; rather, the major role of catalase may be due to the high affinity of brucine *N*-oxide for the haem site of the enzyme. There are some reports of minor activity to reduce tertiary amine *N*-oxides by haemoglobin and include

the non-enzymatic reduction of trimethylamine *N*-oxide, indicine *N*-oxide, 4-dimethylaminoazobenzene *N*-oxide, 4-halogeno-*N,N*-dimethylaniline-*N*-oxide and *N,N*-dimethylaniline-*N*-oxide (Vaisey 1956, Terayama 1963, Kiese *et al.* 1971, Renner 1975, Powis and de Graw 1980). However, the reduction rate of *N*-oxide by catalase is one or two order of magnitude greater than that of haemoglobin. Powis *et al.* (1982) proposed that a Fe(III)–EDTA complex stimulated microsomal NAD(P)H-dependent reduction of indicine *N*-oxide. However, no enhancing effect of ferric chloride and EDTA on NAD(P)H and FAD-dependent brucine *N*-oxide reducing activity by catalase was observed in the present study (data not shown). Gillespie and Duffel (1989) reported that horseradish peroxidase catalysed *N*-oxide reduction of indicine *N*-oxide in the presence of high concentrations of NADH (20 mM) and FMN (1 mM) under anaerobic conditions. However, we observed very low brucine *N*-oxide reductase activity with horseradish peroxidase in the presence of NADH and FAD.

It is well known that tertiary amines are oxidized to the corresponding *N*-oxides in liver microsomes (Ziegler 1980). Thus, the reduction kinetics of tertiary amine *N*-oxides to the amines is complex because of the reoxidation of tertiary amine to the parent *N*-oxide. When brucine was incubated with liver cytosol or blood in the presence of NADPH under aerobic conditions, it was not oxidized to brucine *N*-oxide. Furthermore, the microsomal oxidase activity was observed to be 0.34 nmol min⁻¹ mg protein⁻¹ (Kitamura *et al.* 1999b), which is much lower than the reducing activity observed in the present study. High reductase activity is of major relevance for the maintenance of effective concentrations of active tertiary amines in the body. If this condition is met, the system demonstrated in this study would result in the pharmacological and toxicological reactivation of tertiary amines from the *N*-oxides. High reducing activity has also been reported in the interconversion of amidines, amidinohydrazones and guanidines, and their *N*-hydroxyl derivatives (Clement *et al.* 1993, 1994, Clement and Jung, 1994, Clement and Demesmaeker 1997). Other drug-reducing activities of catalase are under investigation in our laboratory.

References

- BEEDHAM, C., 1985, Molybdenum hydroxylases as drug-metabolizing enzymes. *Drug Metabolism Reviews*, **16**, 119–156.
- BICKEL, M. H., 1969, The pharmacology and biochemistry of *N*-oxides. *Pharmacological Reviews*, **21**, 325–355.
- CLEMENT, B. and DEMESMAEKER, M., 1997, Formation of guanoxabenz from guanabenz in human liver. A new metabolic marker for CYP1A2. *Drug Metabolism and Disposition*, **25**, 1266–1271.
- CLEMENT, B. and JUNG, F., 1994, *N*-hydroxylation of the antiprotozoal drug pentamidine catalyzed by rabbit liver cytochrome P-450 2C3 or human liver microsomes, microsomal retroreduction, and further oxidative transformation of the formed amidoximes. *Drug Metabolism and Disposition*, **22**, 486–497.
- CLEMENT, B., LOMB, R. and MÖLLER, W., 1997, Isolation and characterization of the protein components of the liver microsomal O₂-insensitive NADH-benzamidoxime reductase. *Journal of Biological Chemistry*, **272**, 19615–19620.
- CLEMENT, B., SCHULTZE-MOSGAU, M. H., RICHTER, P. H. and BESCH, A., 1994, Cytochrome P450-dependent *N*-hydroxylation of an aminoguanidine (amidinohydrazone) and microsomal retroreduction of the *N*-hydroxylated product. *Xenobiotica*, **24**, 671–688.
- CLEMENT, B., SCHULTZE-MOSGAU, M. H. and WOHLERS, H., 1993, Cytochrome P450 dependent *N*-hydroxylation of a guanidine (debrisoquine), microsomal catalysed reduction and further oxidation of the *N*-hydroxy-guanidine metabolite to the urea derivative. *Biochemical Pharmacology*, **46**, 2249–2267.

- CRICHTON, R. R., ROMAN, F. and WAUTERS, M., 1975, Reductive mobilization of ferritin iron by reduced nicotinamide-adenine dinucleotide flavin mononucleotide. *Biochemical Society Transactions*, **3**, 946-948.
- DAMANI, L. A., 1982, Oxidation at nitrogen centers. In W. B. Jakoby, J. R. Bend and J. Caldwell (eds), *Metabolic Basis of Detoxication* (New York: Academic Press), pp. 127-149.
- FELSTED, R., CHU, A.E.-Y. and CHAYKIN, S., 1973, Purification and properties of the aldehyde oxidases from hog and rabbit livers. *Journal of Biological Chemistry*, **248**, 2580-2587.
- GILLESPIE, S. G. and DUFFEL, M.W., 1989, Peroxidase as a model for reduction of tertiary amine oxides catalyzed by rat hepatic supernatant and microsomal fractions. *Biochemical Pharmacology*, **38**, 573-579.
- GINGELL, R. and WALKER, R., 1971, Mechanisms of azo reduction by *Streptococcus faecalis*. II. The role of soluble flavins. *Xenobiotica*, **1**, 231-239.
- IWASAKI, K., NOGUCHI, H., KATO, R., IMAI, Y. and SATO, R., 1977, Reduction of tertiary amine N-oxide by purified cytochrome P-450. *Biochemical and Biophysical Research Communications*, **77**, 1143-1149.
- KAMM, J. J. and GILLETTE, J. R., 1963, Mechanism of stimulation of mammalian nitro reductase by flavins. *Life Sciences*, **4**, 254-260.
- KIESE, M., RENNER, G. and SCHLAEGER, R., 1971, Mechanism of the autocatalytic formation of ferrihemoglobin by N,N-dimethylaniline N-oxide. *Naunyn-Schmiedeberg's Archives of Pharmacology*, **268**, 247-263.
- KITAMURA, S., KUWASAKO, M., SUGIHARA, K. and TATSUMI, K., 1999a, Debromination of α -(bromoisovaleryl)urea catalyzed by rat blood. *Journal of Pharmacy and Pharmacology*, **51**, 73-78.
- KITAMURA, S., SUGIHARA, K. and TATSUMI, K., 1999b, A unique tertiary amine N-oxides reduction system composed of quinone reductase and heme in rat liver preparations. *Drug Metabolism and Disposition*, **27**, 92-97.
- KITAMURA, S., TAKEKAWA, K., SUGIHARA, K., TATSUMI, K. and OHTA, S., 1999c, Pseudoenzymatic reduction of N-hydroxy-2-acetylaminofluorene to 2-acetylaminofluorene mediated by cytochrome P450. *Carcinogenesis*, **20**, 347-350.
- KITAMURA, S. and TATSUMI, K., 1984a, Involvement of liver aldehyde oxidase in the reduction of nicotinamide N-oxide. *Biochemical and Biophysical Research Communications*, **120**, 602-606.
- KITAMURA, S. and TATSUMI, K., 1984b, Reduction of tertiary amine N-oxides by liver preparations: function of aldehyde oxidase as a major N-oxide reductase. *Biochemical and Biophysical Research Communications*, **121**, 749-754.
- KITAMURA, S., WADA, Y. and TATSUMI, K., 1984, NAD(P)H-dependent reduction of nicotinamide N-oxide by unique enzyme system consisting of liver microsomal NADPH-cytochrome c reductase and cytosolic aldehyde oxidase. *Biochemical and Biophysical Research Communications*, **125**, 1117-1122.
- LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. and RANDALL, R. J., 1951, Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry*, **193**, 265-275.
- PATTERSON, L. H. and GORROD, J. W., 1978, The metabolism of some 4-substituted N-ethyl-N-methylaniline N-oxides. In J. W. Gorrod (ed.), *Biological Oxidation of Nitrogen* (Amsterdam: Elsevier), pp. 471-478.
- PHILLIPSON, J. D., 1971, Alkaloid N-oxides. *Xenobiotica*, **1**, 419-447.
- PHILLIPSON, J. D. and HANDA, S. S., 1978, Alkaloid N-oxides. A review of recent developments. *Lloydia*, **41**, 385-431.
- POWIS, G. and DE GRAW, C. L., 1980, N-Oxide reduction by hemoglobin, cytochrome c and ferrous ions. *Research Communications in Chemical Pathology and Pharmacology*, **30**, 143-150.
- POWIS, G., SVINGEN, B. A. and DE GRAW, C., 1982, Iron-EDTA simulated reduction of indicine N-oxide by hepatic microsomal fraction, isolated hepatocytes, and the intact rat. *Biochemical Pharmacology*, **31**, 293-299.
- POWIS, G. and WINCENTSEN, L., 1980, Pyridine nucleotide cofactor requirements of indicine N-oxide reduction by hepatic microsomal cytochrome P-450. *Biochemical Pharmacology*, **29**, 347-351.
- RENNER, G., 1975, Reactions of 4-halogeno-N,N-dimethylaniline-N-oxide with hemoglobin. *Naunyn-Schmiedeberg's Archives of Pharmacology*, **291**, 31-45.
- SINGER, T. P. and KEARNEY, F. B., 1950, The non-enzymatic reduction of cytochrome c by pyridine nucleotides and its catalysis by various flavins. *Journal of Biological Chemistry*, **183**, 409-429.
- SUGIHARA, K., KITAMURA, S. and OHTA, S., 1998, Reductive dechlorination of DDT to DDD by rat blood. *Biochemistry and Molecular Biology International*, **45**, 85-91.
- SUGIHARA, K., KITAMURA, S., OHTA, S. and TATSUMI, K., 2000, Reduction of hydroxamic acids to the corresponding amides catalyzed by rabbit blood. *Xenobiotica*, **30**, 457-467.
- SUGIHARA, K., KITAMURA, S. and TATSUMI, K., 1996, S(-)-nicotine-1'-N-oxide reductase activity of rat liver aldehyde oxidase. *Biochemistry and Molecular Biology International*, **40**, 535-541.
- SUGIURA, M., IWASAKI, K. and KATO, R., 1976, Reduction of tertiary amine N-oxides by liver microsomal cytochrome P-450. *Molecular Pharmacology*, **12**, 322-334.

- TAKEKAWA, K., KITAMURA, S., SUGIHARA, K. and OHTA, S., 2001, Non-enzymatic reduction of aliphatic tertiary amine *N*-oxides mediated by the haem moiety of cytochrome P450. *Xenobiotica*, **31**, 11–23.
- TERAYAMA, H., 1963. Aminoazo dye amine-*N*-oxide as a possible intermediate metabolite preceding *N*-demethylation and *ortho*-hydroxylation, as well as azo dye-protein binding. *Gann*, **55**, 195–204.
- VAISEY, E. B., 1956, The non-enzymic reduction of trimethylamine oxide to treimethylamine, dimethylamine, and formaldehyde. *Canadian Journal of Biochemistry and Physiology*, **34**, 1085–1090.
- YUBISUI, T., MATSUKAWA, S. and YONEYAMA, Y., 1980, Stopped flow studies on the nonenzymatic reduction of methemoglobin by reduced flavin mononucleotide. *Journal of Biological Chemistry*, **255**, 11694–11697.
- YUBISUI, T., MATSUKI, T., TANISHIMA, K., TAKESHITA, M. and YONEYAMA, Y., 1977, NADPH-flavin reductase in human erythrocytes and the reduction of methemoglobin through flavin by the enzyme. *Biochemical and Biophysical Research Communications*, **76**, 174–182.
- YUBISUI, T. and TAKESHITA, M., 1980, Characterization of the purified NADH-cytochrome *b*₅ reductase of human erythrocytes as a FAD-containing enzyme. *Journal of Biological Chemistry*, **255**, 2454–2456.
- ZIEGLER, D. M., 1980, Microsomal flavin-containing monooxygenase. In W. B. Jakoby (ed.), *Enzymatic Basis of Detoxication I* (New York: Academic Press), pp. 201–227.

Preclinical pharmacokinetics of SB-203580, a potent inhibitor of p38 mitogen-activated protein kinase

K. W. WARD[†], J. W. PROKSCH[†], L. M. AZZARANO[†], J. A. MUMAW[†], T. J. ROETHKE[†], G. J. STELMAN[†], M. J. WALSH[†], K. S. ZEIGLER[†], J. E. MCSURDY-FREED[†], J. R. KEHLER[†], J. CHOKSHI[†], M. A. LEVY[‡] and B. R. SMITH[†]

[†]Preclinical Drug Discovery, Cardiovascular, Urogenitary, and Oncology Centre for Excellence in Drug Discovery, and [‡]Drug Metabolism and Pharmacokinetics, GlaxoSmithKline R&D, UW 2720, 709 Swedeland Road, King of Prussia, PA 19406, USA

Received 28 February 2001

1. SB-203580 (4-(4-fluorophenyl)-2-(4-methylsulphonylphenyl)-5-(4-pyridyl)imidazole) is a potent, selective inhibitor of p38 MAP kinase used extensively as a tool inhibitor in various pharmacological and toxicological models. This study was designed to evaluate the pharmacokinetics of SB-203580 in several preclinical species, both to assist with the interpretation of existing studies and to aid in the design of future studies with this inhibitor.

2. *In vitro*, SB-203580 was stable in mouse, rat, dog, monkey and human plasma over 24 h. However, species differences in plasma protein binding were observed; SB-203580 was 96–97% bound in human plasma and 78–92% bound in other species. These data suggest that protein binding may influence the results of *in vitro* studies using SB-203580, particularly when comparing results from different *in vitro* systems that incorporate plasma components. *In vivo*, SB-203580 demonstrated moderate to high clearance in all species tested, with non-linear elimination observed in the rat at plasma concentrations > 1000 ng ml⁻¹. Although good solution bioavailability was observed in non-rodents (78% in dog, 32% in monkey), lower and more variable bioavailability was observed in the rat and mouse (3–48%).

3. These interspecies differences in bioavailability, and the non-linear pharmacokinetics observed in rat, highlight the importance of monitoring SB-203580 systemic exposure in parallel with the pharmacological endpoint during *in vivo* pharmacology studies.

Introduction

The mitogen-activated protein (MAP) kinases is a family of serine/threonine kinases that regulates a wide variety of cellular signal transduction processes. The various members of the MAP kinase family, including p38 MAP kinase, are important signal transduction components of many critical cellular events, and regulate a variety of proliferation, differentiation and protein synthesis pathways (Cobb 1999). Furthermore, MAP kinases are implicated in numerous pathways that may play integral roles in human diseases, including oncogenesis/metastasis, immune diseases and inflammatory processes (Stein and Anderson 1996). The

* Author for correspondence; e-mail: KEITH_W_WARD@GSK.COM

pivotal role of MAP kinases in such numerous and diverse biological processes has resulted in extensive investigation of this kinase family that will likely remain an ongoing area of active research interest.

Pharmacological inhibition of MAP kinase activity is a common experimental manipulation in the study of their role in biological processes. Of the different pathway inhibitors available, one of the most widely used is SB-203580 (4-(4-fluorophenyl)-2-(4-methylsulphonylphenyl)-5-(4-pyridyl)imidazole; figure 1), which targets p38 MAP kinase. Since its preparation and initial description as a potent and reasonably selective p38 MAP kinase inhibitor in 1995 (Gallagher *et al.* 1995), SB-203580 has been used as a 'tool compound' in hundreds of published investigations. These investigations span a wide range of studies, including *in vitro*, *ex vivo* and *in vivo* models under a variety of different conditions, species and cell lines. However, to date, no published data have been available concerning the interspecies preclinical pharmacokinetics of SB-203580. Delineation of the dispositional properties of this compound would be useful in the interpretation of the studies published to date with SB-203580, and also would likely aid in future study design. Therefore, the present study was conducted to evaluate the *in vivo* pharmacokinetic properties of SB-203580 in several preclinical species, including the mouse, rat, dog and monkey; *in vitro* parameters including plasma stability and plasma protein binding also were determined.

Materials and methods

Materials

SB-203580 was synthesized by the Department of Medicinal Chemistry at GlaxoSmithKline Pharmaceuticals (King of Prussia, PA, USA) and was determined to be at least 99% pure. All other materials were purchased from standard vendors, and were of the highest available purity.

Animals

Male outbred mice (CrI:CD-1(ICR) BR) weighing 31–34 g, male outbred rats (CrI:CD(SD)IGS BR; all Charles River, Raleigh, NC, USA) weighing 290–360 g, male pure-bred Beagle dogs (Marshall Research, North Rose, NY, USA) weighing 9.0–10.9 kg, and male Cynomolgus monkeys (*Macaca fascicularis*; Charles River Primate Labs, Houston, TX, USA) weighing 3.3–5.0 kg were used for the pharmacokinetic studies. All animals were housed according to the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* in individual cages in unidirectional airflow rooms with controlled temperature ($22 \pm 2^\circ\text{C}$) and relative humidity ($50 \pm 10\%$) and 12-h light/dark cycles (06:00–18:00 hours). Filtered tap water was available *ad libitum*. Animals were fed a standard animal diet (Purina Mills, St Louis, MO, USA); food was available *ad libitum* except for certain overnight periods before dosing, as described below. Whenever overnight fasting was employed, food was provided after the 240-min blood sample was obtained on the study day. All animal use was conducted according to

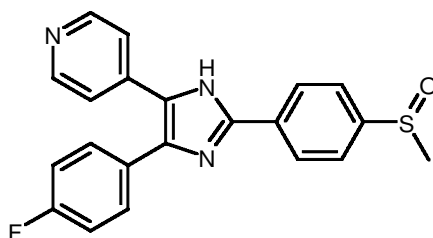


Figure 1. Chemical structure of SB-203580 (4-(4-fluorophenyl)-2-(4-methylsulphonylphenyl)-5-(4-pyridyl)imidazole).

protocols reviewed and approved by the Institutional Animal Care and Use Committee, and all surgical procedures were conducted using aseptic techniques in special-purpose operating suites.

In vivo pharmacokinetic studies

All dosages were administered as solutions in either 20% (i.v.) or 6% (p.o.) aqueous hydroxypropyl- β -cyclodextrin (Encapsin; Cerestar, Hammond, IN, USA), and contained $\leq 2\%$ dimethyl sulphoxide (v/v) to aid in dissolution of SB-203580. Previous experience with this compound class has indicated that these dose vehicles did not alter their pharmacokinetics. Verification of dose solution concentrations of SB-203580 was performed by LC/MS/MS (see below). In all studies, the same animals received the i.v. and p.o. dosage at least two (mouse and rat) or seven (dog and monkey) days apart. A complete blood chemistry panel was performed on all non-rodents before each study day to obtain baseline values and ensure haematological recovery. Blood samples were collected at various times during and after drug administration, as noted in the figures; plasma (50 μ l) was isolated by centrifugation and stored at -70°C until analysis. For mouse, to facilitate sequential sampling while minimizing the total volume of blood withdrawn, 25 μ l whole blood was collected and mixed with 25 μ l analytical-grade water; samples were stored frozen until analysis. All doses below are nominal doses; actual doses are noted in the tables.

Mouse. Four mice received a surgically implanted jugular vein catheter each for i.v. drug administration. The surgery was performed under ketamine/xylazine (80/50 mg kg^{-1} , i.m.) anaesthesia at least 3 days before the first day of the experiment; each catheter was filled with heparinized saline when not in use to maintain patency. Mice were placed in plastic restrainers to facilitate drug administration and blood sampling (from a lateral tail vein) for up to 2 h before being returned to their cages. On study day 1, mice received SB-203580 (4 mg kg^{-1}) as a 30-min i.v. infusion (4.0 ml kg^{-1}). On a different study day, after an overnight fast, the mice received SB-203580 (8 mg kg^{-1}) as an p.o. gavage (16 ml kg^{-1}).

Rat. Three rats received surgically implanted femoral vein catheters for drug administration, as described for the mouse above, 4–5 days before the first day of the experiment. Rats were held in restrainers to facilitate drug administration and blood sampling (from a lateral tail vein) for up to 2 h before being returned to their cages. On study day 1, rats received SB-203580 (2 mg kg^{-1}) as a 30-min i.v. infusion (4.0 ml kg^{-1}). On a different study day, after an overnight fast, rats received SB-203580 (4 mg kg^{-1}) as an p.o. gavage (16 ml kg^{-1}). To investigate the pharmacokinetic linearity of SB-203580 in rat, three additional rats received both femoral vein (for dosing) and contralateral femoral artery (for blood sampling) catheters. These dual-cannulated rats received SB-203580 as an accelerated i.v. infusion (0.43 $\mu\text{g kg}^{-1} \text{ min}^{-2}$; 0.22 $\mu\text{l kg}^{-1} \text{ min}^{-2}$) designed to investigate the pharmacokinetic linearity of SB-203580 over an approximate plasma concentration range of 0–5000 ng ml^{-1} (Ward *et al.* 2000). Finally, to confirm the results of the accelerated infusion study, six additional rats received both femoral vein (for dosing) and contralateral femoral artery (for blood sampling) catheters; these rats received SB-203580 as a 16-h continuous rate i.v. infusion at either 12.63 or 90 $\mu\text{g kg}^{-1} \text{ min}$ ($n = 3$ per group; 2.0 $\text{ml kg}^{-1} \text{ h}^{-1}$).

Dog. Dogs had a previously implanted chronic vascular access port in the portal vein used for blood sampling. Furthermore, a cephalic vein catheter was placed temporarily in each dog for blood sampling; on study day 1, a catheter was also placed in the saphenous vein for drug administration. Dogs were restrained in slings to facilitate drug administration and blood sampling for up to 2 h before being returned to their cages. On study day 1, dogs received SB-203580 (2 mg kg^{-1}) as a 60-min i.v. infusion (4.0 ml kg^{-1}). On study day 2, after an overnight fast, dogs received SB-203580 (4 mg kg^{-1}) as an p.o. gavage (4 ml kg^{-1}).

Monkey. Monkeys had a previously implanted chronic vascular access port in a femoral vein and the portal vein used for blood sampling. On study day 1, a catheter was also placed in a saphenous vein for drug administration. Monkeys were fasted overnight before each study day. The monkeys were placed in restraining chairs for the first 4 h of the study to facilitate drug administration and blood sampling. On study day 1, monkeys received SB-203580 (2 mg kg^{-1}) as a 60-min i.v. infusion (4 ml kg^{-1}). On study day 2, monkeys received SB-203580 (4 mg kg^{-1}) as an p.o. gavage (4 ml kg^{-1}). On study day 3, monkeys received SB-203580 (6 mg kg^{-1}) as a 60-min i.v. infusion (4 ml kg^{-1}).

In vitro plasma stability and plasma protein binding

Plasma protein binding of SB-203580 was investigated in each of the species in which *in vivo* pharmacokinetic parameters were determined, as well as in foetal calf serum (FCS) and human plasma, by equilibrium dialysis (Pacifi and Viani 1992). Briefly, whole blood was collected by cardiac puncture

from mouse and rat, and by peripheral venipuncture from dog, monkey and a male human volunteer; plasma was obtained by centrifugation. Fetal calf serum was obtained commercially (Gibco BRL, Gaithersburg, MD, USA). Plasma protein binding was determined using Spectra/Por[®] 4 dialysis membranes (Spectrum Laboratories, Rancho Dominguez, CA, USA; molecular weight cut-off = 12–14 kDa). Membranes were soaked overnight in 0.1 M phosphate-buffered saline and blotted on tissue paper before cell assembly. Dialysates were prepared in plasma of each species, FCS, and buffer at final concentrations of 1000 and 10 000 ng ml⁻¹. Dialysates were vortexed gently to ensure uniform mixing, and triplicate 50- μ l aliquots collected and analysed to verify initial concentrations. Equilibrium dialysis cells were rotated at $\sim$ 15 rpm for 24 h at 37°C. Following incubation, 50- μ l aliquots of both plasma and buffer were withdrawn from each cell and analysed for SB-203580 concentration. Plasma stability of SB-203580 was also investigated in the species in which *in vitro* protein-binding measurements were performed. For these studies, SB-203580 was incubated for up to 24 h in buffer and plasma (1 ml incubation volume) from each species at a concentration of 1500 ng ml⁻¹. Samples (50 μ l) were obtained periodically and analysed for concentrations of SB-203580.

Analytical procedure

SB-203580 was isolated from samples by precipitation with acetonitrile and quantified by LC/MS/MS using a Turbo IonSpray interface. Briefly, internal standard (150 μ l 200 ng ml⁻¹ of a dimethylphenyl analogue of SB-203580 in acetonitrile/10 mM ammonium formate pH 3.0, 85/15 v/v) was added to 50 μ l of sample, and the mixture was vortex-mixed and centrifuged for 10 min at 15 000g, with the supernatant (0.5 μ l) injected onto the LC/MS/MS system using an HTS PAL autosampler (Leap Technologies). The mobile phase was composed of a 60/40 (v/v) mixture of acetonitrile and 10 mM ammonium formate, pH 3.0. A quaternary pumping system (Rheos 4000) was used to deliver the mobile phase to a 3 $\times$ 50 mm, 3 μ , Genesis C18 analytical column. The flow rate was set at 450 μ l min⁻¹ and was introduced into the source of a Sciex API 365 mass spectrometer with no post-column split. The mass spectrometer was coupled to the HPLC via a Turbo IonSpray interface (500°C). Positive-ion multiple reaction monitoring was employed for the detection of SB-203580 and internal standard. The selected precursor ions were m/z 378 for SB-203580 and 388 for the internal standard. The product ions monitored were at m/z 363 and 373 for SB-203580 and the internal standard. Using a (1/ x) weighted linear regression analysis of the calibration curve, linear responses in the analyte/internal standard peak area ratios were observed for SB-203580 concentrations ranging from 5 to 2500 ng ml⁻¹.

Pharmacokinetic analysis

Non-compartmental pharmacokinetic analysis was performed using WinNonlin Professional v.2.1 (Scientific Consultants, Inc., Mountain View, CA, USA). For the i.v. data, several different compartmental models were applied. The most appropriate model was chosen based on Akaike's Information Criterion and lack of bias in residual error (Gabrielsson and Weiner 1997). All area under the curve (AUC) calculations utilized area to the last observable plasma concentration (AUC_{0- t}). Bioavailability was estimated by dividing the dose-normalized area under the concentration-time curve (DNAUC) resulting from p.o. administration by the DNAUC resulting from i.v. administration. Individual parameters were generated for each animal; parameter estimates were averaged and reported as mean $\pm$ SD. For plasma protein binding, the unbound fraction (f_u) of SB-203580 was determined by comparing the post-dialysis concentrations on both sides of the dialysis membrane, with a correction for fluid shifts (Boudinot and Jusko 1984). Values are reported as a mean $\pm$ SD for three determinations.

Statistical analysis

An unpaired or paired Student's *t*-test assessed the significance of differences between the various parameters determined for SB-203580, as described in the Results. One-way ANOVA with a Bonferroni *post-hoc* comparison assessed the significance of differences in parameters for SB-203580 across groups, where appropriate. In all cases, $p < 0.05$ was predetermined as the criterion of significance.

Results

The pharmacokinetic parameters and concentration versus time profiles for SB-203580 in the mouse are shown in table 1 and figure 2, respectively. SB-203580 demonstrated very high plasma clearance (2.4 times hepatic plasma flow in the mouse; Davies and Morris 1993) with a volume of distribution $\sim$ 10 times total body water and a terminal half-life of $\sim$ 55 min. Following oral administration of

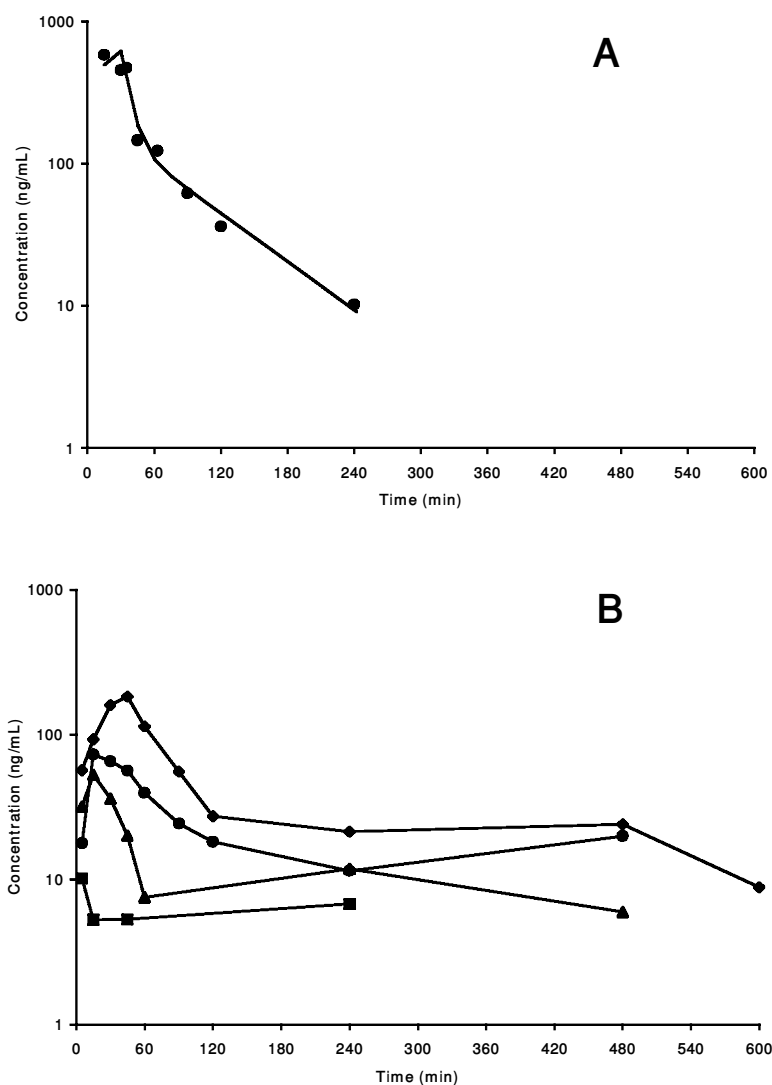


Figure 2. Plasma concentration–time profiles for SB-203580 in mouse. Symbols represent measured plasma concentrations after 4.5 mg kg⁻¹ i.v. (A) or 8.3 mg kg⁻¹ p.o. (B) administration. Lines represent either the best-model fit to the observed data (A) or straight-line interpolation between data points (B). Data from a representative mouse are shown in (A); data from individual mice are presented in (B) due to variability.

SB-203580 as a solution, variable systemic exposure was observed in the mouse, with oral bioavailability ranging from 3.4 to 48.2%. Secondary input peaks were observed in the mouse following oral administration (figure 2); these later increases in concentration, coupled with the large variability, precluded calculation of additional oral pharmacokinetic parameters such as half-life or MRT for SB-203580 in mouse.

The results from the initial rat pharmacokinetic study are displayed in table 1 and figure 3. SB-203580 demonstrated moderate to high clearance in rat with a distributional volume ~ 4 times total body water. The apparent terminal elim-

ination half-life was long (~ 3.5 h), but concentrations in the terminal phase were low ($< 10\text{ ng ml}^{-1}$). Following oral solution administration in rat, SB-203580 demonstrated bioavailability of $\sim 12\%$; the presence of apparent secondary input peaks at later time points precluded the derivation of additional oral pharmacokinetic parameters (figure 3).

The results of pharmacokinetic linearity experiments for SB-203580 in the rat are given in table 2 and figure 4. Initially, an accelerated infusion study design was employed to define the range of linear kinetics of SB-203580 in rat. Linear

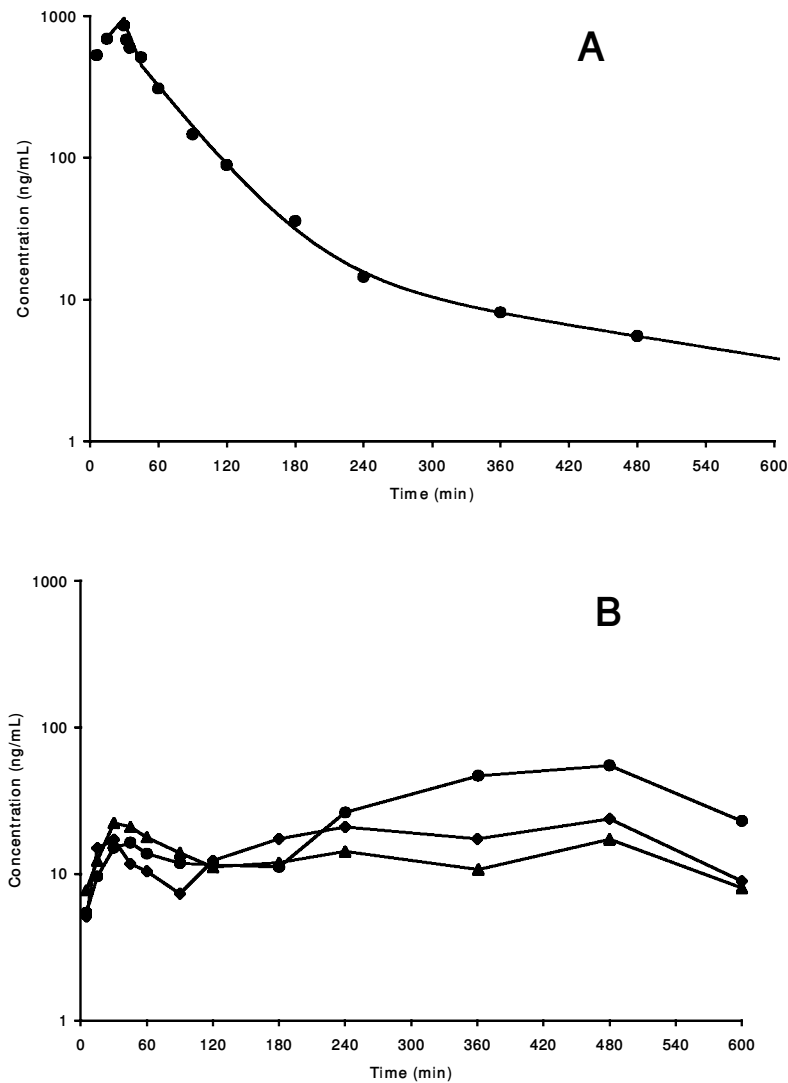


Figure 3. Plasma concentration–time profiles for SB-203580 in rat. Symbols represent measured plasma concentrations after 2.1 mg kg^{-1} i.v. (A) or 4.1 mg kg^{-1} p.o. (B) administration. Lines represent either the best-model fit to the observed data (A) or straight-line interpolation between data points (B). Data from a representative rat are shown in (A); data from individual rats are presented in (B) due to variability.

Table 1. Pharmacokinetic parameters defining systemic disposition of SB-203580 in mouse and rat. Data are presented as mean $\pm$ SD (n = four mice or three rats), and were derived from compartmental analysis unless otherwise noted.

Parameter	Mouse		Rat	
	4.5 mg kg ⁻¹ , i.v.	8.3 mg kg ⁻¹ , p.o. ^a	2.1 mg kg ⁻¹ , i.v.	4.1 mg kg ⁻¹ , p.o. ^a
$C_{\max}$ (ng ml ⁻¹) ^b	587 $\pm$ 173	73.1, 183, 53.0, 10.2	899 $\pm$ 82	54.8, 23.7, 22.2
$T_{\max}$ (min) ^b	30 $\pm$ 0	15, 45, 15, 5	60 $\pm$ 0	480, 480, 30
$t_{1/2\alpha}$ (min)	3.8 $\pm$ 2.2	— ^c	1.14 $\pm$ 0.22	—
$t_{1/2\beta}$ (min)	55.1 $\pm$ 7.7	—	29.5 $\pm$ 3.8	—
$t_{1/2\gamma}$ (min)	—	—	209 $\pm$ 95	—
MRT (min)	50.1 $\pm$ 14.8	—	71.9 $\pm$ 17.9	—
Cl (ml min ⁻¹ kg ⁻¹)	138 $\pm$ 39	—	42.1 $\pm$ 6.3	—
$V_{d_{ss}}$ (l kg ⁻¹) ^b	7.04 $\pm$ 3.26	—	2.96 $\pm$ 0.34	—
Oral f (%) ^b	—	14.5, 48.2, 7.3, 3.4	—	16.9, 10.2, 9.5

^a Data are presented as individual values due to large variability; animals are presented in the same order.

^b Measured (not fitted) parameter.

^c Not determined for this route of administration.

regression analysis of the slope of the concentration-versus time profile from 0 to 180 and from 180 to 480 min (table 2) suggested that the clearance of SB-203580 was initially constant at 36.9 ± 2.8 ml min⁻¹ kg⁻¹, but that at concentrations exceeding ~ 1000 ng ml⁻¹, clearance decreased to 16.1 ± 1.6 ml min⁻¹ kg⁻¹ ($p = 0.0004$; Student's paired two-tailed t -test). To confirm this observation, a follow-up experiment was performed in which SB-203580 was infused in two different groups of rat to target steady-state concentrations below and above this apparent region of non-linearity. As shown in table 2, plasma clearance of SB-203580 was non-linear in rat, with clearances of 44.3 ± 11.6 and 16.9 ± 1.3 ml min⁻¹ kg⁻¹ at plasma concentrations of ~ 300 and 5400 ng ml⁻¹, respectively ($p = 0.015$; Student's unpaired two-tailed t -test).

The data describing the disposition of SB-203580 in non-rodent species are displayed in tables 3 and 4 and figures 5–7. In dog, SB-203580 exhibited a moderate plasma clearance (15.3 ± 2.2 ml min⁻¹ kg⁻¹), with an elimination half-life of ~ 1 h and a distributional volume approximately twice total body water (table 3 and figure 5). Solution oral bioavailability of SB-203580 in dog was excellent, at $\sim 79\%$. Comparison of the systemic (46.5 ± 10.0 kg min l⁻¹) and portal (45.5 ± 7.9 kg min l⁻¹) dose-normalized AUC after oral administration suggested

Table 2. Estimation of plasma clearance during either an accelerated infusion or two separate infusions to steady-state of SB-203580 in rat.

Experiment	Concentration range (ng ml ⁻¹)	Apparent steady-state concentration (ng mg ⁻¹)	Plasma clearance (ml min ⁻¹ kg ⁻¹)
Accelerated infusion, 0–180 min	0–1824 $\pm$ 334	not applicable	36.9 $\pm$ 2.8
Accelerated infusion, 180–480 min	1824 $\pm$ 334 to 9530 $\pm$ 539	not applicable	16.1 $\pm$ 1.6 ^a
Low steady-state infusion	not applicable	297 $\pm$ 68	44.3 $\pm$ 11.6
High steady-state infusion	not applicable	5360 $\pm$ 406	16.9 $\pm$ 1.3 ^b

Data are presented as mean $\pm$ SD ($n = 3$).

^a Significantly different from that derived from 0 to 180 min data ($p = 0.0004$).

^b Significantly different from that derived from low infusion rate ($p = 0.015$).

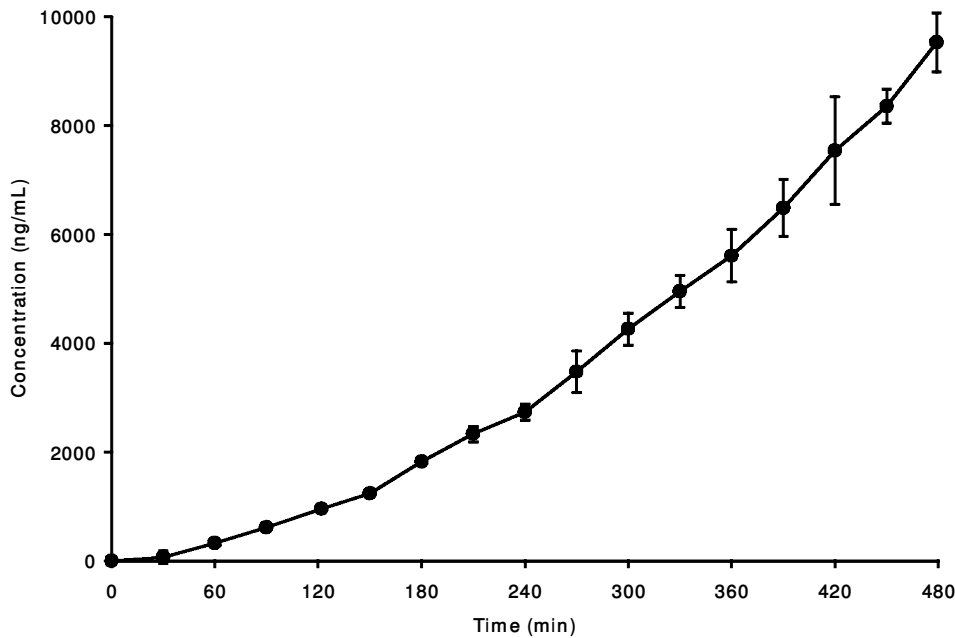


Figure 4. Plasma concentration–time profiles for SB-203580 in rat during an 8-h accelerated infusion. Symbols represent measured plasma concentrations (mean $\pm$ SD from $n = 3$ rats); lines are a straight-line interpolation between data points.

that SB-203580 did not undergo substantial first-pass hepatic extraction in dog. In monkey, SB-203580 demonstrated low clearance with a volume of distribution about twice total body water and a half-life of ~ 1.5 h (table 4 and figure 6). After oral administration, bioavailability of SB-203580 in the monkey was 36–41% (one of the monkeys did not successfully complete the oral part of the experiment due to sampling difficulties; data are not shown for this animal). Comparison of the systemic (23.3 and $28.2 \text{ kg min l}^{-1}$) and portal (35.6 and $39.5 \text{ kg min l}^{-1}$) dose-normalized AUC after oral administration suggested that SB-203580 underwent at least moderate first-pass extraction in the monkey. To investigate the linearity of SB-203580 elimination in the monkey, a 3-fold higher i.v. dose was also administered. Following this higher dose, C_{max} increased in an approximately dose-proportional fashion (1729 ± 380 for the low dose versus 4709 ± 1119 for the higher dose; dose-normalized C_{max} not different, $p = 0.73$, Student’s paired two-tailed t -test). However, plasma clearance was only approximately half that obtained following the lower i.v. dose (13.2 ± 3.9 versus 7.67 ± 0.86 for the higher dose; $p = 0.039$, Student’s paired two-tailed t -test). Additional pharmacokinetic parameters were not calculated for this i.v. dose, because the low and variable concentrations in the terminal elimination phase precluded accurate model fitting for this dose (figure 7).

In addition to the *in vivo* pharmacokinetic studies, the *in vitro* plasma protein binding and plasma stability of SB-203580 also were examined. SB-203580 was stable in mouse, rat, dog, monkey and human plasma, as well as in foetal calf serum, over the course of 24 h (data not shown). Plasma protein binding data for SB-203580 are summarized in table 5. In preclinical species, including mouse, rat,

Table 3. Pharmacokinetic parameters defining systemic disposition of SB-203580 in dog after various dosages and routes of administration. Data are presented as mean ±SD (*n* = 3), and were derived from compartmental analysis unless otherwise noted.

Parameter	Dosing Regimen		
	2.1 mg kg ⁻¹ , i.v.	4.0 mg kg ⁻¹ , p.o., portal samples	4.0 mg kg ⁻¹ , p.o., systemic samples
<i>C</i> _{max} (ng ml ⁻¹) ^a	1729 ± 380	1967 ± 240	1616 ± 374
<i>T</i> _{max} (min) ^a	60 ± 0	16.7 ± 12.6	35.0 ± 8.7
<i>t</i> _{1/2} (min)	58.9 ± 4.0	— ^b	—
MRT (min)	85.0 ± 5.7	—	—
Cl (ml min ⁻¹ kg ⁻¹)	15.3 ± 2.2	—	—
<i>V</i> _{dss} (l kg ⁻¹)	1.30 ± 0.16	—	—
Oral <i>F</i> (%) ^a	—	—	79.3 ± 6.1

^a Measured (not fitted) parameter.
^b Not determined for this route of administration.

dog and monkey, plasma protein binding of SB-203580 was moderate to high, with unbound fractions ranging from 7 to 10%. Plasma protein binding was substantially higher in human than in the preclinical species, with 3.2% unbound, and was much lower in foetal calf serum (20% unbound). No evidence for substantial concentration-dependence in protein binding was observed over the concentration range evaluated in this study (1000–10 000 ng ml⁻¹).

Discussion

SB-203580 is used extensively in many research fields as an investigative tool compound for studying the effects of p38 inhibition in biological systems both *in vitro* and *in vivo*. For example, SB-203580 has been used in experiments evaluating such wide-ranging phenomena as bacterial sepsis (Chakravortty *et al.* 2000), response to CNS trauma (Behrens *et al.* 1999), cardiac ischaemia/reperfusion injury (Ma *et al.* 1999, Barancik *et al.* 2000, Yue *et al.* 2000), and inflammatory

Table 4. Pharmacokinetic parameters defining systemic disposition of SB-203580 in monkey after various dosages and routes of administration. Data are presented as mean ±SD (*n* = 3), and were derived from compartmental analysis unless otherwise noted.

Parameter	Dosing regimen		
	2.1 mg kg ⁻¹ , i.v.	4.0 mg kg ⁻¹ , p.o., portal samples ^c	4.0 mg kg ⁻¹ , p.o. systemic samples ^c
<i>C</i> _{max} (ng mg ⁻¹) ^a	1174 ± 110	1090, 1279	338, 547
<i>T</i> _{max} (min) ^a	60 ± 0	15, 37	45, 45
<i>t</i> _{1/2α} (min)	2.84 ± 2.03	— ^b	—
<i>t</i> _{1/2β} (min)	90.4 ± 11.8	—	—
MRT (min)	94.5 ± 14.6	—	—
Cl (ml min ⁻¹ kg ⁻¹)	13.2 ± 3.9	—	—
<i>V</i> _{dss} (l/kg ⁻¹)	1.21 ± 0.23	—	—
Oral <i>F</i> (%) ^a	—	—	36.0, 40.5

^a Measured (not fitted) parameter.
^b Not determined for this route of administration.
^c Only two monkeys successfully completed the oral leg of the experiment; data are reported as individual values for those two animals.

Table 5. Plasma protein binding of SB-203580 in preclinical species and human. Data are presented as mean $\pm$ SD ($n = 3$ determinations per species per concentration).

Concentration (ng ml ⁻¹)	f_u (%)					
	Mouse	Rat	Dog	Monkey	Foetal calf serum	Human
1000	9.39 $\pm$ 1.59	8.05 $\pm$ 0.49	6.45 $\pm$ 0.20	7.28 $\pm$ 0.68	18.6 $\pm$ 1.1	2.66 $\pm$ 0.23
10 000	11.44 $\pm$ 0.93	8.90 $\pm$ 1.01	7.44 $\pm$ 0.32	7.44 $\pm$ 0.29	22.0 $\pm$ 0.2	3.75 $\pm$ 0.36
Average data	10.4 $\pm$ 1.6	8.47 $\pm$ 0.85	6.94 $\pm$ 0.59	7.36 $\pm$ 0.47	20.3 $\pm$ 2.0	3.20 $\pm$ 0.65

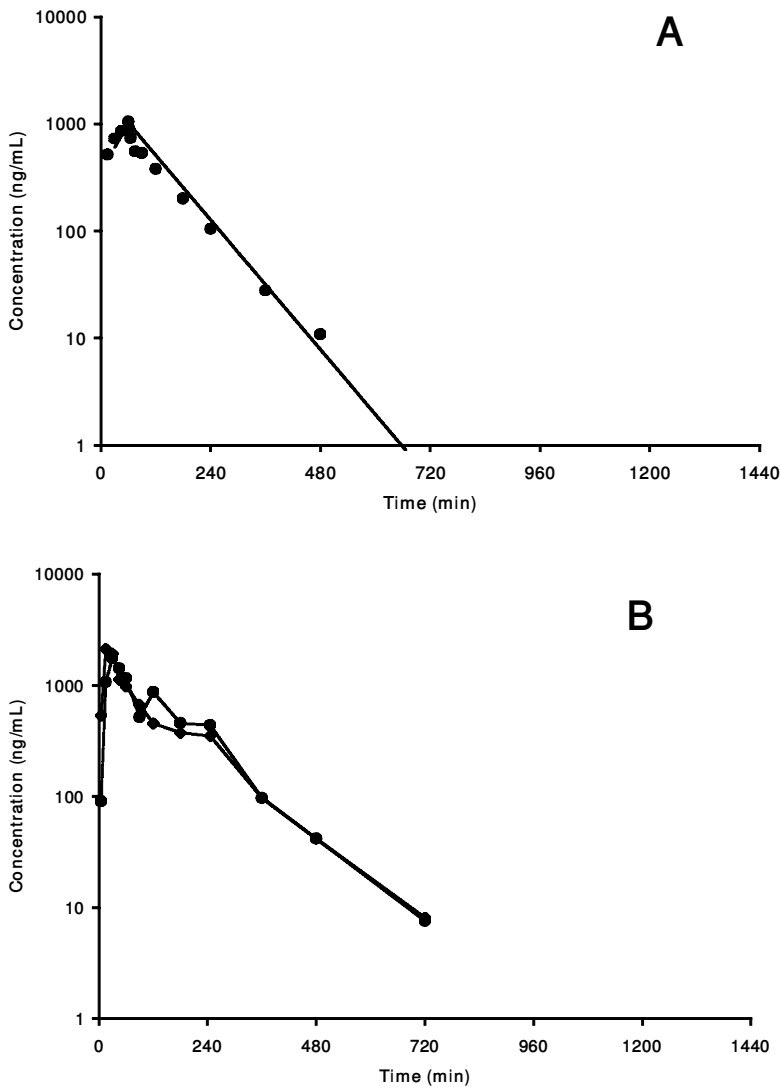


Figure 5. Representative plasma concentration-time profiles for SB-203580 in dog. Symbols represent measured plasma concentrations after 2.1 mg kg⁻¹ i.v. (A) or 4.0 mg kg⁻¹ p.o. (B) administration. Lines represent either the best-model fit to the observed data (A) or a straight-line interpolation between portal (diamonds) or systemic (circles) data points (B).

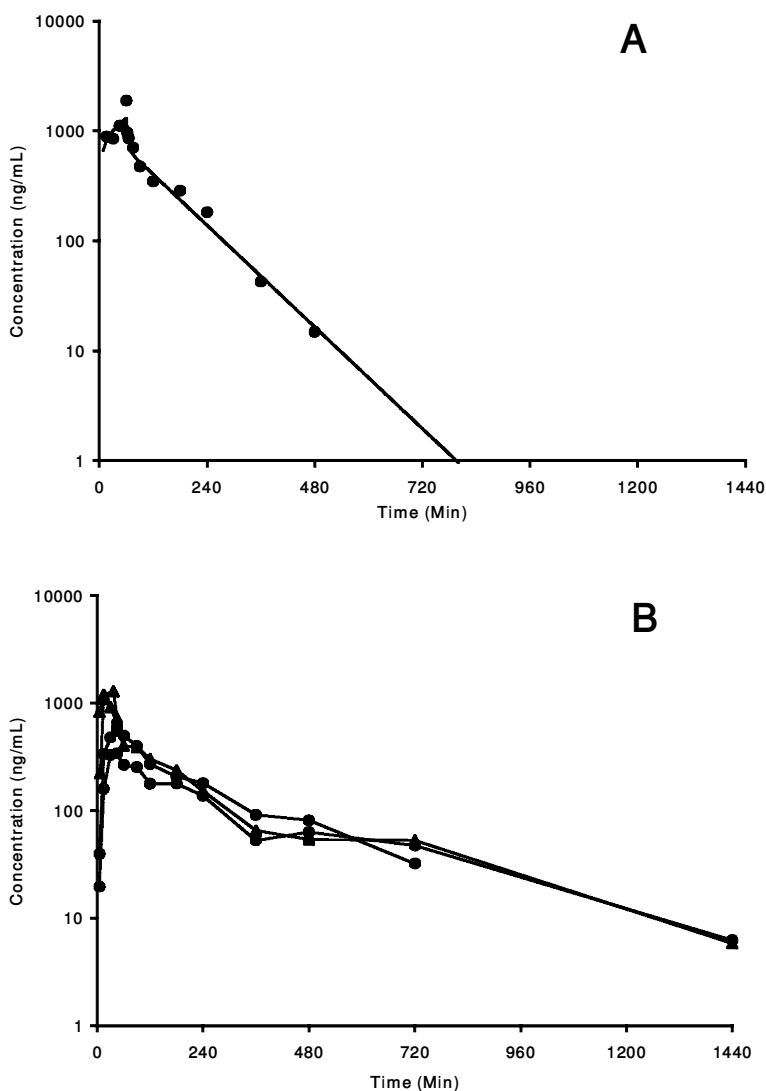


Figure 6. Plasma concentration-time profiles for SB-203580 in monkey. Symbols represent measured plasma concentrations after 2.1 mg kg⁻¹ i.v. (A) or 4.0 mg kg⁻¹ p.o. (B) administration. Lines represent either the best-model fit to the observed data (A) or a straight-line interpolation between portal (triangles) or systemic (circles) data points (B). Data from a representative monkey are shown in (A); data from individual monkeys are presented in (B) due to variability.

disease including collagen- and adjuvant-induced arthritis (Badger *et al.* 1996). The pharmacological activities of SB-203580 are thus well described in the literature. Before this work, however, no published reports of the pharmacokinetics of SB-203580 have been presented. Furthermore, few, if any, researchers appear to have quantified systemic exposure of SB-203580 in their pharmacological models.

The *in vivo* pharmacokinetic data generated in this study that describe the disposition of SB-203580 may allow for more thorough data interpretation/interrogation for experiments employing this compound, as well as improved

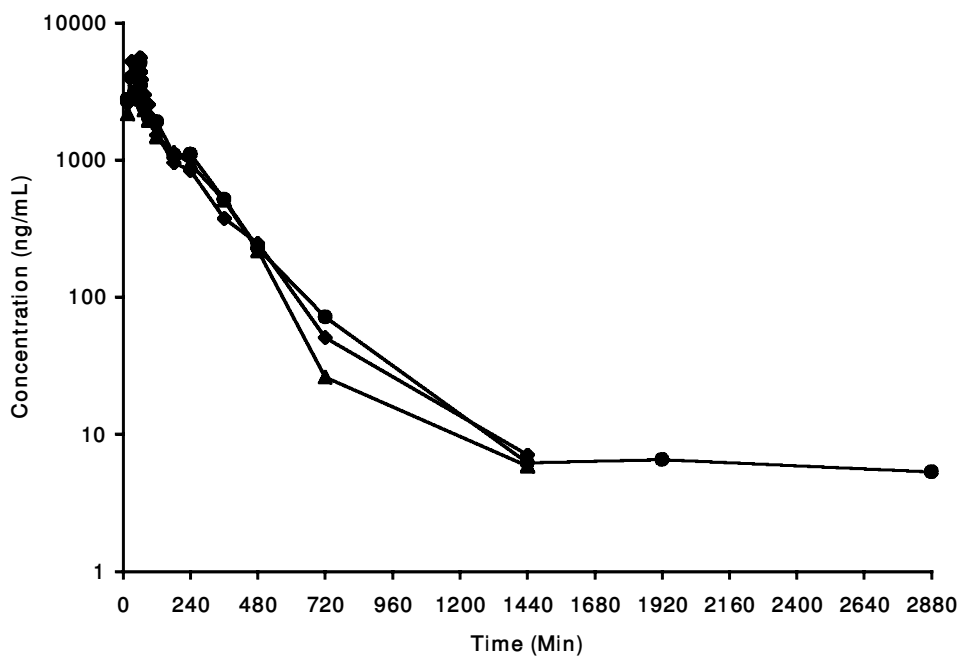


Figure 7. Plasma concentration–time profiles for SB-203580 in monkey. Symbols represent measured plasma concentrations after 6.1 mg kg^{-1} i.v. administration. Lines represent a straight-line interpolation between data points. Data from individual monkeys are presented due to variability.

study designs for future studies. For example, in a recent *in vivo* study, rat doses were selected based upon the potency of SB-203580 *in vitro*, and a presumed volume of distribution of 0.751 kg^{-1} for SB-203580 in rat (Joyeux *et al.* 2000). Although this rationale is reasonable in the absence of pharmacokinetic data, the data from the present set of studies reveal that the actual distributional volume of SB-203580 in the rat was more than 4-fold higher, at nearly 31 kg^{-1} . Thus, *in vivo* plasma concentrations were likely substantially lower than anticipated in the study by Joyeux and colleagues. Although this would not have impacted the conclusions drawn by these investigators, this example highlights the potential utility of SB-203580 pharmacokinetic data. The present data should allow a more precise definition of the dose versus concentration relationship, allowing for increased accuracy in dose selection. Furthermore, although oral administration has been employed for SB-203580 *in vivo*, the present data clearly demonstrate that even when dosed as a solution, substantial variability in oral exposure results in the rodent. Such large variability will obviously impact the pharmacological outcome from these studies; this observation highlights the importance of measuring at least a partial concentration versus time profile *in vivo* to generate pharmacokinetic data that correspond with the pharmacodynamic endpoint of interest.

The *in vitro* data generated in the present investigation should also be valuable in the interpretation of studies with SB-203580. It is generally recognized that only the free fraction of a drug is available to establish a transcellular equilibrium, and thus to achieve a pharmacological effect (Benet *et al.* 1990). Drugs with $< 10\%$ free fraction are generally considered to be highly protein bound (Rowland and Tozer

1995). The data in the current study demonstrate that SB-203580 is highly bound to plasma proteins *in vitro* in mouse, rat, dog, monkey and human plasma. Consequently, any *in vitro* studies using SB-203580 that include some plasma or serum component in the culture medium from these species will likely be influenced by a decreased concentration of unbound active drug due to protein binding. Furthermore, a similar effect was observed for foetal calf serum (81% bound), though to a lesser degree than with plasma from other species. These data suggest that a comparison of the *in vitro* potency of SB-203580 for a given pharmacological effect must carefully take into consideration the potential influence of plasma or serum proteins in the culture systems being studied. For example, recent *in vitro* studies by Liang *et al.* (2000) employed medium containing 10% foetal bovine serum, and evaluated the effects of SB-203580 and PD98059 (an inhibitor of ERK MAP kinase) on strain-activated brain natriuretic peptide gene transcription activity. Among other findings, this study suggested that 10 μ M SB-203580 produced a 35% reduction in activity, that 10 μ M PD98059 produced 75% reduction, and that a combination of both was more effective than either alone (85% reduction). Whereas the authors conclude from these data that there is functional overlap between p38 and ERK MAP kinases in the regulation of brain natriuretic peptide transcriptional regulation, an alternate hypothesis could be that differences in plasma protein binding of the two compounds resulted in different free fractions in the cell culture system, and that co-administration of both together altered the protein binding of one or the other. No data are available describing the protein binding of PD98059, however, the relatively high plasma protein binding of SB-203580 suggests that consideration of this potential confounding factor may be important in interpreting such studies.

Although the pharmacokinetics of SB-203580 have not previously been published, some data relating to the metabolism of SB-203580 analogues are available. Previously, the metabolism of SK&F-86002 (6-(4'-fluorophenyl)-5-(4'-pyridyl)-2,3-dihydroimidazo-[2,1-*b*]-thiazole), a less potent and non-selective cytokine biosynthesis inhibitor, was investigated *in vitro* (Howard *et al.* 1990) and *in vivo* (Newton *et al.* 1989) in the rat. SK&F-86002 was extensively metabolized, but the metabolism was primarily cytochrome P450-mediated sulfoxidation on the thiazole moiety; metabolites arising from the 4-fluorophenyl or the pyridine groups were not detected. These data suggest that metabolism of SB-203580 would likely occur around the phenyl sulfoxide moiety, rather than on the pyridine or 4-fluorophenyl portions of the molecule; further experiments would be required to more definitively identify sites of metabolism for SB-203580.

The present investigation has provided evidence for the existence of non-linear elimination kinetics *in vivo* for SB-203580. This observation was not unexpected, based on the available information in the literature for SB-203580. For example, SB-203580 is a potent inhibitor of the major human cytochrome P450 enzymes (CYP1A2, CYP2C9, CYP2C19, CYP3A4 and CYP2D6; Adams *et al.* 1998). Furthermore, upon chronic administration of SB-203580 (10-day dose-ranging safety studies) in the rat, elevated liver weights and significant hepatic P450 induction were observed (Adams *et al.* 1998). More recently, SB-203580 has been shown to enhance the induction of some Phase II drug metabolizing enzymes (Yu *et al.* 2000). Together, these observations are consistent with the non-linear elimination kinetics observed for SB-203580 in the rat and the monkey in the present study. The non-linear behaviour of SB-203580 has obvious potential to

confound the interpretation of data from *in vivo* pharmacology studies employing this p38 inhibitor as a tool compound. For example, the present investigation demonstrates that in the rat, concentrations associated with non-linear elimination ($> 1000 \text{ ng ml}^{-1}$) would not be difficult to attain, particularly with parenteral routes of administration. In this region, dose-proportionality of exposure, and thus likely of pharmacological effect, may not be observed, which could be an important consideration in the understanding of apparent dose-response relationships for SB-230580.

In summary, the pharmacokinetics of SB-203580, a potent and widely used p38 MAP kinase inhibitor, are described in the mouse, rat, dog, and monkey. Furthermore, the *in vitro* plasma stability and plasma protein binding also has been investigated in various species. When dosed as a solution, SB-203580 demonstrated low and variable oral bioavailability in the rodent, with improved absorption in the dog and monkey. Evidence for non-linear elimination was observed in both the rat and the monkey. SB-203580 was moderately to highly bound to plasma proteins *in vitro* in all species examined. These observations should be taken into consideration when designing studies using SB-203580, and when interpreting the resulting data.

Acknowledgements

The authors gratefully acknowledge the expert technical assistance of members of the Laboratory Animal Sciences Department, including Guy Vaden, Michelle Schaeffer and Marianne Eybye, as well as additional members of Preclinical Pharmacokinetics (Rita Truongcao and Jeff Orban) in the conduct of the in-life portions of the experiments described here. The supportive assistance of Jerry Adams (GSK Medicinal Chemistry) in supplying SB-203580 and in helpful discussions of the manuscript is also acknowledged. This work presented in part at the 2001 Annual Meeting of the Society of Toxicology, 25–29 March, San Francisco, CA.

References

- ADAMS, J. L., BOEHM, J. C., KASSIS, S., GORYCKI, P. D., WEBB, E. F., HALL, R., SORENSON, M., LEE, J. C., AYRTON, A., GRISWOLD, D. E. and GALLAGHER, T. F., 1998, Pyrimidinylimidazole inhibitors of CSBP/p38 kinase demonstrating decreased inhibition of hepatic cytochrome P450 enzymes. *Bioorganic Medicine and Chemistry Letters*, **8**, 3111–3116.
- BADGER, A. M., BRADBEER, J. N., VOTTA, B., LEE, J. C., ADAMS, J. L. and GRISWOLD D. E., 1996, Pharmacological profile of SB 203580, a selective inhibitor of cytokine suppressive binding protein/p38 kinase, in animal models of arthritis, bone resorption, endotoxin shock and immune function. *Journal of Pharmacology and Experimental Therapeutics*, **279**, 1453–1461.
- BARANCIK, M., HTUN, P., STROHM, C., KILIAN, S. and SCHAPER, W., 2000, Inhibition of the cardiac p38-MAPK pathway by SB203580 delays ischemic cell death. *Journal of Cardiovascular Pharmacology*, **35**, 474–483.
- BEHRENS, M. M., STRASSER, U., KOH, J.Y., GWAG, B. J. and CHOI, D. W., 1999, Prevention of neuronal apoptosis by phorbol ester-induced activation of protein kinase C: Blockade of p38 mitogen-activated protein kinase. *Neuroscience*, **94**, 917–927.
- BENET, L. Z., MITCHELL, J. R. and SHEINER, L. B., 1990, General principles: introduction. In *Goodman and Gilman's The Pharmacological Basis of Therapeutics* (New York: Pergamon).
- BOUDINOT, F. D. and JUSKO, W. J., 1984, Fluid shifts and other factors affecting plasma protein binding of prednisolone by equilibrium dialysis. *Journal of Pharmaceutical Sciences*, **73**, 774–780.

- CHAKRAVORTTY, D., KATO, Y., KOIDE, N., SUGIYAMA, T., KAWAI, M., FUKADA, M., YOSHIDA, T. and YOKOCHI, T., 2000, Extracellular matrix components prevent lipopolysaccharide-induced bovine arterial endothelial cell injury by inhibiting p38 mitogen-activated protein kinase. *Thrombosis Research*, **98**, 187–193.
- COBB, M. H., 1999, MAP kinase pathways. *Progress in Biophysics and Molecular Biology*, **71**, 479–500.
- DAVIES, B. and MORRIS, T., 1993, Physiological parameters in laboratory animals and humans. *Pharmaceutical Research*, **10**, 1093–1095.
- GABRIELSSON, J. and WEINER, D., 1997, *Pharmacokinetic and Pharmacodynamic Data Analysis: Concepts and Applications* (Stockholm: Apotekarsocieteten).
- GALLAGHER, T. F., FIER-THOMPSON, S. M., GARIGIPATI, R. S., SORENSON, M. E., SMITANA, J. M., LEE, D., BENDER, P. E., LEE, J. C., LAYDON, J. T., GRISWOLD, D. E., CHABOT-FLETCHER, M. C., BRETON, J. C. and ADAMS, J. L., 1995, 2,4,5-triarylimidazole inhibitors of IL-1 biosynthesis. *Bioorganic Medicine and Chemistry Letters*, **5**, 1171–1176.
- HOWARD, M. O., NEWTON, J. F., KOHANE, D. J., YODIS, L. P., SAVERINO, C. M., QUALLS, C. W., JR and SCHWARTZ, L. W., 1990, In vitro metabolism of the novel antiinflammatory agent 6-(4-fluorophenyl)-5-(4-pyridyl)-2,3-dihydroimidazo-[2,1-b]-thiazole. *Drug Metabolism and Disposition*, **18**, 607–612.
- JOYEUX, M., BOUMENDJEL, A., CARROLL, R., RIBOUT, C., GODIN-RIBOUT, D. and YELLON, D. M., 2000, SB 203580, a mitogen-activated protein kinase inhibitor, abolishes resistance to myocardial infarction induced by heat stress. *Cardiovascular and Drugs Therapy*, **14**, 337–343.
- LIANG, F., LU, S. and GARDNER, D. G., 2000, Endothelin-dependent and -independent components of strain-activated brain natriuretic peptide gene transcription require extracellular signal regulated kinase and p38 mitogen-activated protein kinase. *Hypertension*, **35**, 188–192.
- MA, X. L., KUMAR, S., GAO, F., LOUDEN, C. S., LOPEZ, B. L., CHRISTOPHER, T. A., WANG, C., LEE, J. C., FEUERSTEIN, G. Z. and YUE, T. L., 1999, Inhibition of p38 mitogen-activated protein kinase decreases cardiomyocyte apoptosis and improves cardiac function after myocardial ischemia and reperfusion. *Circulation*, **99**, 1685–1691.
- NEWTON, J. F., YODIS, L. P., KEOHANE, D., ECKARDT, R., DEWEY, R., DENT, J. and MICO, B., 1989, Pharmacokinetics and metabolism of SK&F 86002 in male and female Sprague–Dawley rats. *Drug Metabolism and Disposition*, **17**, 174–179.
- PACIFICI, G. M. and VIANI, A., 1992, Methods of determining plasma and tissue binding of drugs. *Clinical Pharmacokinetics*, **23**, 449–468.
- ROWLAND, M. and TOZER, T. N. (eds), 1995, Distribution. In *Clinical Pharmacokinetics* (Philadelphia: Lippincott Williams & Wilkins), pp. 137–155.
- STEIN, B. and ANDERSON, D., 1996, The MAP kinase family: New ‘MAPs’ for signal transduction pathways and novel targets for drug discovery. *Annual Reports in Medicine and Chemistry*, **31**, 289–298.
- WARD, K. W., GRIFFITHS, R., LEVY, M. A. and SMITH, B. R., 2000, Evaluation of the use of accelerated infusions for the determination of pharmacokinetic linearity. *Journal of Pharmacology and Experimental Therapeutics*, **293**, 468–479.
- YU, R., MANDELKAR, S., LEI, W., FAHL, W. E., TAN, T. H. and KONG, A.-N. T., 2000, p38 mitogen-activated protein kinase negatively regulates the induction of Phase II drug-metabolizing enzymes that detoxify carcinogens. *Journal of Biological Chemistry*, **275**, 2322–2327.
- YUE, T. L., WANG, C., GU, J. L., MA, X. L., KUMAR, S., LEE, J. C., FEUERSTEIN, G. Z., THOMAS, H., MALEE, B. and OHLSTEIN, E. H., 2000, Inhibition of extracellular signal-regulated kinase enhances ischemia/reoxygenation-induced apoptosis in cultured cardiac myocytes and exaggerates reperfusion injury in isolated perfused heart. *Circulation Research*, **86**, 629–699.

Oxidation of phenothiazine and phenoxazine by *Cunninghamella elegans*

J. B. SUTHERLAND^{†*}, J. P. FREEMAN[†], T. M. HEINZE[†],
J. D. MOODY[†], I. A. PARSHIKOV[‡], A. J. WILLIAMS[‡]
and D. ZHANG[§]

[†] National Center for Toxicological Research, Food and Drug Administration, Jefferson, AR 72079, USA

Received 19 February 2001

1. To determine the ability of fungi to metabolize sulphur- and oxygen-containing azaarenes, *Cunninghamella elegans* ATCC 9245 was grown in 125-ml flasks containing fluid Sabouraud medium. The cultures and controls were incubated at 28°C with shaking and dosed with 16.7 mM phenothiazine or phenoxazine. After incubation for 72 h, the mycelia and filtrates were extracted with ethyl acetate and the combined residues analysed by high-performance liquid chromatography. Residual phenothiazine and phenoxazine were 21 and 22%, respectively, of the total UV absorbance at 254 nm.

2. The metabolites were identified by mass spectrometry and proton nuclear magnetic resonance spectroscopy. The fungus oxidized phenothiazine to phenothiazine sulphoxide, 3-hydroxyphenothiazine sulphoxide, phenothiazin-3-one, and 3-hydroxyphenothiazine and oxidized phenoxazine to phenoxazin-3-one.

3. Three of the four compounds produced by *C. elegans* from phenothiazine were identical to those produced by mammals, supporting the use of the fungus as a microbial model for drug metabolism.

Introduction

Phenothiazine has a tricyclic chemical structure found in dyes, such as methylene blue and Azure A, and in numerous synthetic drugs. It is used as an anthelmintic for cattle and sheep (Kelly *et al.* 1981, Somers *et al.* 1987). Phenothiazine has been reported to inhibit the mutagenicity of benzo[*a*]pyrene (Kittle *et al.* 1981), act as a calmodulin antagonist (Bindra *et al.* 1995), and inhibit bone resorption by osteoclasts (Hall *et al.* 1996). It also induces P-glycoproteins and several cytochromes P450 in rat (Burt and Thorgeirsson 1988, Tateishi *et al.* 1999). The metabolism of phenothiazine has been studied in mammals (Mitchell and Waring 1979, 1981, Mitchell 1980, 1982, Waring and Mitchell 1985). Phenothiazine is a minor metabolite produced during the biotransformation of methdilazine by *Cunninghamella elegans* (Zhang *et al.* 1996a) and of *N*-acetylphenothiazine by *Penicillium simplicissimum* (Parshikov *et al.* 1999).

Phenoxazine has a tricyclic chemical structure found in the antibiotic dactinomycin and dyes such as Nile Blue and Brilliant Cresyl Blue. It functions as a modulator allowing the accumulation of vincristine and vinblastine in otherwise multi-drug-resistant human cancer cell lines (Thimmaiah *et al.* 1990, Wadkins and

* Author for correspondence: e-mail: jsutherland@nctr.fda.gov

[‡] Present address: Department of Medicinal Chemistry, University of Mississippi, University, MS 38677, USA.

[§] Present address: Department of Metabolism and Pharmacokinetics, Bristol-Myers Squibb, Princeton, NJ 08502, USA.

Houghton 1993) and it inhibits lipid peroxidation in rat brain homogenates (Cini *et al.* 1994). Little is known about the metabolism of phenoxazine in biological systems.

Although some bacteria co-metabolize phenothiazine during growth on carbazole (Gieg *et al.* 1996), the ability of fungi to transform unsubstituted phenothiazine and phenoxazine is unknown. *C. elegans* biotransforms a variety of drugs and xenobiotic compounds (Sutherland *et al.* 1995, Zhang *et al.* 1996a). Among them are *N*-heterocyclic compounds, such as acridine, quinoline and isoquinoline (Sutherland *et al.* 1994a, b), and *S*-heterocyclic compounds, such as dibenzothio-*phene* (Crawford and Gupta 1990, Schlenk *et al.* 1994). *C. elegans* also oxidizes two substituted phenothiazine drugs, chlorpromazine and methdilazine, to sulphoxides and 3-hydroxy derivatives (Zhang *et al.* 1996a). The present paper demonstrates the ability of *C. elegans* to oxidize phenothiazine and phenoxazine and the major products have been identified.

Materials and methods

Mycelium of *Cunninghamella elegans* ATCC 9245 for use as inoculum was grown on potato dextrose agar and macerated aseptically in sterile saline solution. After the addition of 1 ml mycelial suspension (dry weight 0.4 mg) to 125-ml conical flasks containing 30 ml fluid Sabouraud medium (Remel, Inc., Lenexa, KS, USA), triplicate cultures and non-inoculated controls were incubated at 28°C in the dark with shaking at 150 rpm. After 72 h, 1 ml of a filter-sterilized solution of phenothiazine (Sigma Chemical Co., St Louis, MO, USA) or phenoxazine (Fisher Scientific, Pittsburgh, PA, USA) in acetone was added aseptically to the cultures and controls. The final concentration of each substrate was 16.7 mM. All of the flasks were replaced on the shaker. After incubation for another 72 h, the mycelia were collected on glass wool. The mycelia and filtrates were extracted three times with equal volumes of ethyl acetate, which were combined, dried over anhydrous sodium sulphate and removed *in vacuo*. Metabolites were dissolved in 2.0 ml methanol for analysis.

Metabolites were separated by high-performance liquid chromatography (hplc), using a Shimadzu (Kyoto, Japan) LC-600 chromatograph with a UV detector at 254 nm. An Inertsil-5 ODS-3 base-deactivated analytical C₁₈ column (25 cm × 4.6 mm i.d.; MetaChem Technologies, Torrance, CA, USA) was used with a 20-min linear gradient of 55–95% methanol in buffer (50 mM ammonium acetate, pH 5.5) followed by a 10-min hold. The flow rate was 1 ml min⁻¹. Fractions containing metabolites were collected as they eluted from the column, concentrated to dryness *in vacuo* and dissolved in methanol. UV/visible absorption spectra of the metabolites were obtained with a Shimadzu UV-2101PC spectrophotometer.

Direct exposure probe/mass spectrometry (DEP/MS) and gas chromatography/mass spectrometry (GC/MS) were performed with a Finnigan model TSQ 700 triple quadrupole mass spectrometer operated in the single quadrupole mode. The DEP was heated with a linear current ramp of 10 mA s⁻¹ to 800 mA. Electron ionization (EI), positive-ion chemical ionization (PICI) and negative-ion chemical ionization (NICI) were employed with the DEP/MS analyses. For the chemical ionization analyses, the reagent gas was 10% NH₃ in N₂ at ~690 Pa. The quadrupole (Q1) was scanned from *m/z* 50 to 500 with a 0.5 s cycle time. Electron

ionization was used for the GC/MS analyses. The gas chromatograph was a Varian (Walnut Creek, CA, USA) model 3400 with a temperature-programmable interface. The carrier gas was He at 69 kPa; the temperatures of the injector and column were 250°C and that of the interface was 300°C.

Liquid chromatography/electrospray ionization mass spectrometry (LC/ESI MS) was performed with a Hewlett-Packard HP1090L/M hplc system and HP5989B quadrupole mass spectrometer operated in the positive-ion electrospray mode. A Prodigy ODS-3 column (25 cm $\times$ 2.0 mm i.d.; 5 μ m Phenomenex) was used. The mobile phase was acetonitrile/water with constant 3 mM ammonium formate (20:80 v/v, in a 45-min linear gradient to 80:20). The quadrupole was scanned from m/z 50 to 550 at 1.52 scans s^{-1} .

Molecular weights of analytes were determined with a capillary exit voltage of 100 V, where protonated molecules were the base peaks and minor $[M + H - \bullet OH]^+$ ions were observed for the sulphoxides. Samples were reanalysed with a capillary exit voltage of 200 V for fragmentation by in-source collision-induced dissociation (CID).

For proton nuclear magnetic resonance (^1H -nmr) analysis, metabolites were dissolved in acetone- d_6 (Parshikov *et al.* 1999). Typical data acquisition parameters were: data size, 32 K; sweep width, 7042 Hz; filter width, 8900 Hz; acquisition time, 2.33 s; flip angle, 90°; relaxation delay, 0 s; temperature, 301 K. For measurement of coupling constants, the free induction decay (FID) was zero-filled to 64 K, resulting in a final data point resolution of 0.215 Hz/point.

Results

Phenothiazine

The elution profiles of extracts from cultures of *C. elegans* grown with phenothiazine (figure 1A) showed four metabolites (A2–5) as well as residual phenothiazine (A6) and an unidentified peak also found in the non-inoculated controls (A1). When the cultures were harvested at 72 h, the amount of residual phenothiazine in the dosed cultures was only 8.5% as much as that in the non-inoculated controls. The mean areas (A_{254}) of peaks from analyses of triplicate cultures and non-inoculated controls are given in table 1; phenothiazine was 21% of the total for the cultures.

Peak A2, which eluted at 4.2 min (figure 1A), was found only in cultures of *C. elegans* dosed with phenothiazine. The UV/visible absorption spectrum of peak A2 had λ_{max} = 207, 273, 304 and 344 nm. The DEP/EI mass spectrum (figure 2A) showed ions at m/z 231 [M^+], 215 ($M^+ - 16$), 213 ($M^+ - 18$) and 185 ($M^+ - 46$); DEP/NICI shows ions at m/z 214/213; and DEP/PICI shows ions at m/z 216/214. The positive-ion ESI mass spectrum at 100 V (data not shown) had ions at m/z 232 (100% relative abundance) [$M + H$] $^+$ and 215 (8%) ($MH^+ - 17$). The positive-ion ESI mass spectrum with CID (figure 2B) had significant ions at m/z 232 [$M + H$] $^+$, 215 ($MH^+ - 17$), 214 ($MH^+ - 18$), 186 ($MH^+ - 46$), 183 ($MH^+ - 49$), 182 ($MH^+ - 50$) and 154. All mass spectra were consistent with a hydroxyphenothiazine sulphoxide. The ^1H -nmr spectrum of peak A2 (table 2) showed seven resonances in the aromatic region, with a coupling pattern that indicated a single substitution on one ring, and a broad singlet located downfield of the other aromatic resonances. Upon saturation of the broad singlet, nuclear

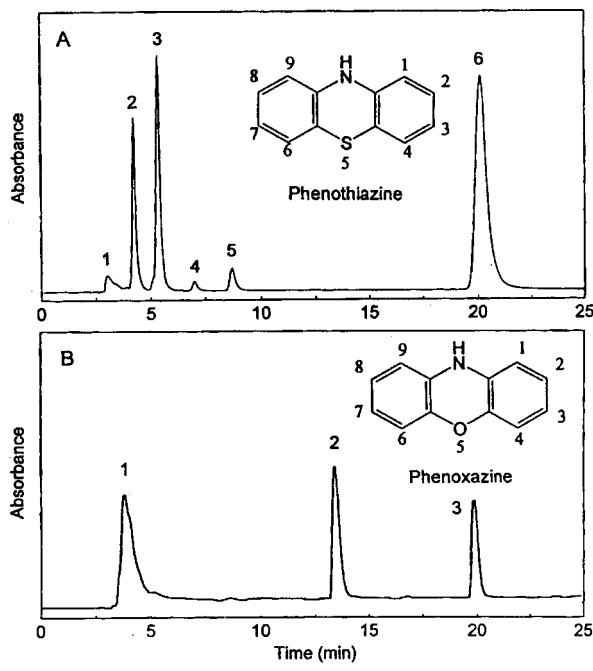


Figure 1. Hplc elution profiles of the ethyl acetate extracts from cultures of *C. elegans* dosed with (A) 16.7 mM phenothiazine (1, unknown; 2, 3-hydroxyphenothiazine sulphoxide; 3, phenothiazine sulphoxide; 4, 3-hydroxyphenothiazine; 5, phenothiazin-3-one; 6, phenothiazine) and (B) 16.7 mM phenoxazine (1, unknown; 2, phenoxazin-3-one; 3, phenoxazine).

Overhauser effects (NOE) were observed at the doublet of doublets at 7.28 ppm (H1) and at the meta-coupled doublet at 7.31 ppm (H9). The substitution was shown to be at the C-3 position, based on homonuclear decoupling experiments that also led to the assignment of the other resonances. From these data, peak A2 was identified as 3-hydroxyphenothiazine sulphoxide (Girard *et al.* 1987).

Table 1. Hplc analysis of the ethyl acetate extracts from 72-h cultures of *C. elegans*, dosed with 16.7 mM phenothiazine or phenoxazine, and from non-inoculated controls.^a

Peak	Hplc peak area (×10 ⁶)			
	<i>C.elegans</i> + phenothiazine	Phenothiazine only	<i>C.elegans</i> + phenoxazine	Phenoxazine only
Unknown (A1, B1) ^b	5.8 ± 0.6	3.7 ± 0.6	3.6 ± 1.3	1.5 ± 0.4
3-Hydroxyphenothiazine sulphoxide (A2)	12.6 ± 1.4	ND ^c	ND	ND
Phenothiazine sulphoxide (A3)	31.0 ± 3.5	3.2 ± 1.3	ND	ND
3-Hydroxyphenothiazine (A4)	trace	ND	ND	ND
Phenothiazin-3-one (A5)	5.3 ± 1.6	ND	ND	ND
Phenothiazine (A6)	14.4 ± 14.3	169.2 ± 53.2	ND	ND
Phenoxazin-3-one (B2)	ND	ND	2.4 ± 1.0	0.5 ± 0.1
Phenoxazine (B3)	ND	ND	1.7 ± 0.8	7.0 ± 0.9

^a Triplicate 10 µl samples from extracts in 2 ml methanol were analysed by hplc (Inertsil-5 ODS-3 analytical column and a 20-min gradient of 55–95% methanol in buffer). Flow rate was 1 ml min⁻¹ and UV detector was at 254 nm.

^b Peak area in control cultures of *C. elegans* was 2.1 ± 0.7 × 10⁶.

^c Not detected.

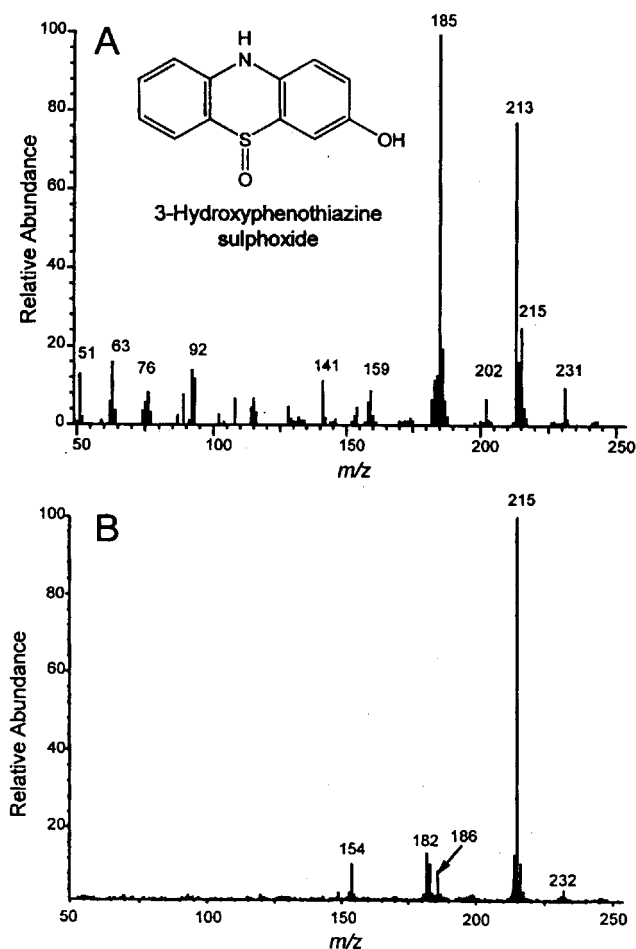


Figure 2. Mass spectra for peak A2, 3-hydroxyphenothiazine sulfoxide, produced from phenothiazine by *C. elegans*. (A) Electron ionization mass spectrum (DEP/MS); (B) positive-ion electrospray mass spectrum (LC/ESI MS) with CID.

Peak A3, which eluted at 5.3 min, was the major metabolite isolated from cultures dosed with phenothiazine (figure 1A); a small amount was produced abiotically in the controls (table 1). The UV absorption spectrum of peak A3, which had $\lambda_{\max} = 228, 271, 303$ and 338 nm, and the EI mass spectrum (figure 3A) were similar to those of phenothiazine sulfoxide (Mitchell and Waring 1979). The positive-ion ESI mass spectrum at 100 V (data not shown) had ions at m/z 216 (100% relative abundance) $[M + H]^+$ and 199 (4%) $(MH^+ - 17)$. The positive-ion ESI mass spectrum with CID (figure 3B) had significant ions at m/z 216 $[M + H]^+$, 199 $(MH^+ - 17)$, 167 $(MH^+ - 49)$ and 166 $(MH^+ - 50)$ and was consistent with phenothiazine sulfoxide. The aromatic region of the ^1H -nmr spectrum of peak A3 (table 2) showed the same type and number of resonances as that of phenothiazine, except that they were shifted downfield. Selective saturation of the broad singlet at 10.00 ppm (H10) resulted in an NOE to 7.38 ppm (H1 and H9). Homonuclear decoupling experiments were used to make the other assignments. Since the aromatic resonances were downfield from those of phenothiazine

Table 2. Proton nmr spectral data for the metabolites produced by *C. elegans* grown in fluid Sabouraud medium and dosed with phenothiazine or phenoxazine.

Assignment	Chemical shifts (ppm) ^a			
	3-Hydroxyphenothiazine sulphoxide (A2) ^b	Phenothiazine sulphoxide (A3) ^c	Phenothiazin-3-one (A5) ^d	Phenoxazin-3-one (B2) ^e
H1	7.28	7.38	7.63	7.49
H2	7.13	7.56	6.86	6.80
H3		7.21		
H4	7.33	7.89	6.76	6.20
H6	7.84	7.89	7.89 ^f	7.43
H7	7.14	7.21	7.58	7.64
H8	7.52	7.56	7.58	7.44
H9	7.31	7.38	7.66 ^f	7.81
H10	9.69	10.00		

^a Metabolites were dissolved in deuterated acetone; coupling constants reported are first-order.
^b $J_{1,2} = 8.8$; $J_{2,4} = 2.8$; $J_{6,7} = 8.0$; $J_{6,8} = 1.5$; $J_{7,9} = 1.1$; $J_{8,9} = 8.4$.
^c $J_{1,2} = 8.2$; $J_{1,3} = 1.1$; $J_{2,3} = 7.7$; $J_{2,4} = 1.5$; $J_{3,4} = 7.7$.
^d $J_{1,2} = 9.9$; $J_{2,4} = 2.2$.
^e $J_{1,2} = 9.9$; $J_{2,4} = 2.1$; $J_{6,7} = 8.2$; $J_{6,8} = 1.3$; $J_{6,9} = 0.5$; $J_{7,8} = 7.6$; $J_{7,9} = 1.6$; $J_{8,9} = 7.9$.
^f Assignments may be reversed.

and the H10 resonance was present, peak A3 was identified as phenothiazine sulphoxide.

Peak A4 eluted at 7.0 min (figure 1A). It was colourless, but upon exposure to air was converted rapidly to the metabolite, peak A5.

Peak A5 was red and eluted at 8.6 min (figure 1A). It had a UV absorption spectrum with $\lambda_{\text{max}} = 236, 272, 288$ (shoulder), 368 and 504 nm. Although no peak was observed at 258 nm, the UV spectrum was otherwise similar to that reported for phenothiazin-3-one (Mitchell and Waring 1979). The EI mass spectrum of peak A5 (figure 4A) had a molecular ion at m/z 213 [M^+] and a major fragment ion at m/z 185 ($M^+ - 28$) and was consistent with the structure of phenothiazin-3-one, but lacked most of the less abundant ions listed by Beckett and Navas (1978) and Mitchell and Waring (1979). The positive-ion ESI mass spectrum at 100 V (data not shown) had only the protonated molecule at m/z 214. The positive-ion ESI mass spectrum with CID (figure 4B) shows ions at m/z 214 [$M + H$]⁺, 186 ($MH^+ - 28$), 182 ($MH^+ - 32$), 170 ($MH^+ - 44$) and 154 ($MH^+ - 60$). The aromatic region of the ¹H-nmr spectrum of peak A5 (table 2) consisted of seven resonances with ring substitution at the C-3 position, as shown by NOE and homonuclear decoupling experiments. There was no broad downfield singlet.

To determine the position of the oxo-substituent in peak A5, aqueous ascorbic acid was used to reduce the metabolite to a colourless compound. After the ascorbate had been removed with a Sep-Pak C₁₈ cartridge (Waters Corp., Milford, MA, USA), the metabolite was reanalysed by hplc. Since it eluted at 7.0 min, like peak A4, and an EI mass spectrum obtained by GC/MS (figure 4C) was nearly identical to that of 3-hydroxyphenothiazine (Beckett and Navas 1978), peak A5 was deduced to be phenothiazin-3-one.

Residual phenothiazine (A6) eluted at 19.5 min (figure 1A); the ¹H-nmr spectrum was consistent with one published previously (Clin and Lemanceau 1969).

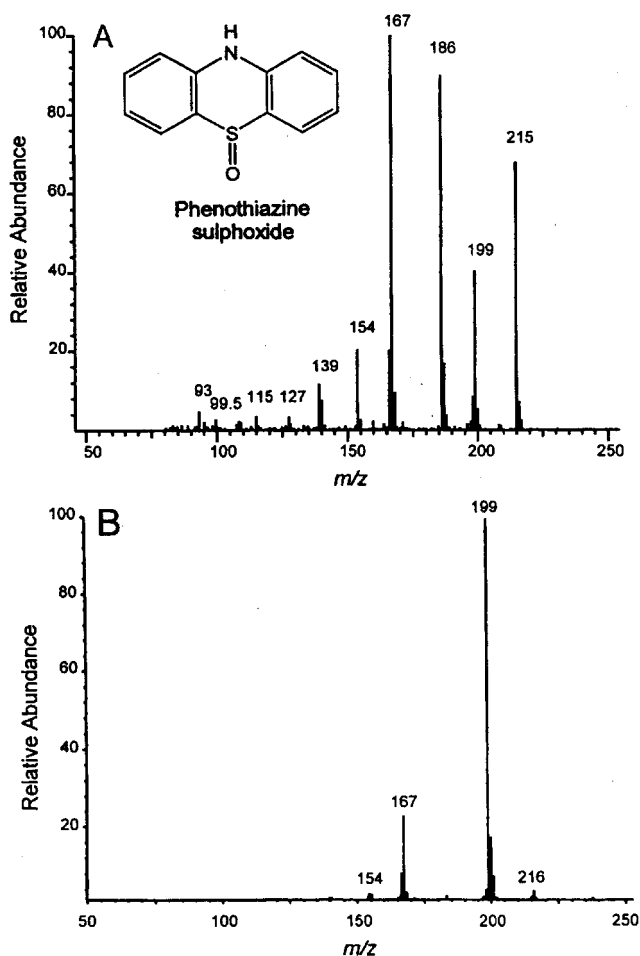


Figure 3. Mass spectra for peak A3, phenothiazine sulphoxide, produced from phenothiazine by *C. elegans*. (A) Electron ionization mass spectrum (DEP/MS); (B) positive-ion electrospray mass spectrum (LC/ESI MS) with CID.

Phenoxazine

The hplc elution profile of the extract from *C. elegans* grown with phenoxazine (figure 1B) shows one major metabolite (B2) at 13.4 min and residual phenoxazine (B3) at 20.0 min. An unidentified major peak (B1) was seen but also found in the non-inoculated controls. When the cultures were harvested at 72 h, the amount of residual phenoxazine in the dosed cultures was only 24% as much as that in the non-inoculated controls. The mean areas (A_{254}) of peaks from analyses of triplicate cultures and non-inoculated controls are given in table 1; phenoxazine was 22% of the total for the cultures.

The major metabolite B2 (table 1) was yellow-brown and had $\lambda_{\max} = 244, 348$ and 447 nm in the UV/visible absorption spectrum. The EI mass spectrum (figure 5A), which had ions at m/z 197 [M^+] and 169 ($M^+ - 28$), the positive-ion ESI mass spectrum at 100 V (data not shown), which had only the protonated molecule at m/z 198, and the positive-ion ESI mass spectrum with CID (figure 5B), which

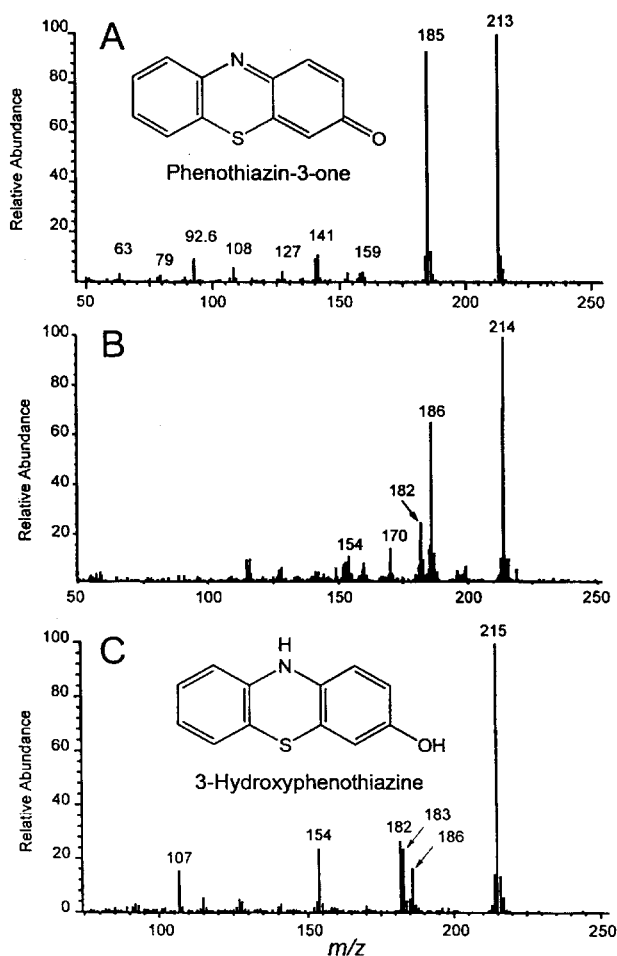


Figure 4. Mass spectra for peak A5, phenothiazin-3-one, produced from phenothiazine by *C. elegans*. (A) Electron ionization mass spectrum (GC/MS); (B) positive-ion electrospray mass spectrum (LC/ESI MS) with CID; (C) electron ionization mass spectrum (GC/MS) for peak A5 after chemical reduction by ascorbic acid.

had ions at m/z 198 $[M + H]^+$, 170 ($MH^+ - 28$), 142 ($MH^+ - 56$) and 115 ($MH^+ - 73$), were all consistent with phenoxazin-3-one. The ^1H -nmr spectrum (table 2) was also consistent with identification of the metabolite as phenoxazin-3-one (Bolognese *et al.* 1986).

Residual phenoxazine (peak B3) eluted at 20.0 min (figure 1B); the ^1H -nmr spectrum was consistent with that of Clin and Lemanceau (1969).

Discussion

Phenothiazin-3-one is formed by the oxidation of 3-hydroxyphenothiazine in air (Beckett and Navas 1978). Both phenothiazin-3-one and phenothiazine sulphoxide can be produced chemically from phenothiazine at low pH and high temperature (Roseboom and Perrin 1977), but the amounts detected in cultures of *C. elegans* were much greater than those produced in the controls. Phenothiazin-

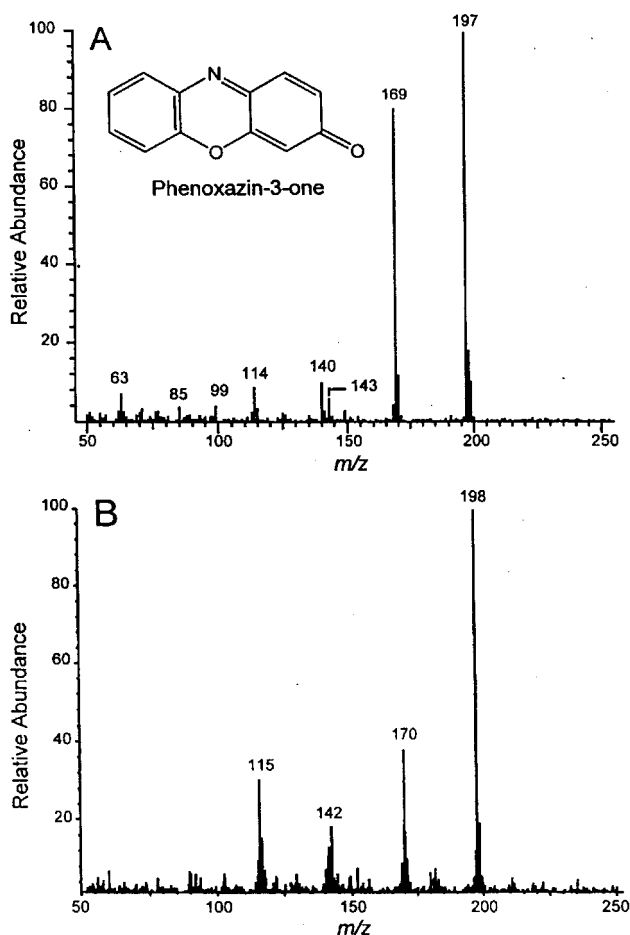


Figure 5. Mass spectra for peak B2, phenoxazin-3-one, produced from phenoxazine by *C. elegans*. (A) Electron ionization mass spectrum (DEP/MS); (B) positive-ion electrospray mass spectrum (LC/ESI MS) with CID.

3-one inhibits NAD-dependent oxidation reactions in liver mitochondria (Gallagher *et al.* 1965).

Although the metabolism of phenoxazine has not been described, phenoxazin-3-one is metabolized by rodent liver, lung and skin microsomes to resorufin (7-hydroxyphenoxazin-3-one) (Burke and Mayer 1983, Lum *et al.* 1986, Rettie *et al.* 1986). This microsomal oxidation, which requires NADPH, is associated with a constitutive cytochrome P450 (Burke and Mayer 1983, Lum *et al.* 1986). Phenoxazine was converted to phenoxazin-3-one by *C. elegans*, but no resorufin was detected in the present study.

A constitutive microsomal cytochrome P450 is produced by *C. elegans* (Zhang *et al.* 1996b, Wang *et al.* 2000) and could have been involved in some of the biotransformations. Acetone, along with other organic solvent vehicles commonly used for experiments with hydrophobic compounds, is a competitive inhibitor of cytochrome P450 activity (Backes and Canady 1981, Kontir *et al.* 1986) and may have affected the rate of biotransformation.

C. elegans oxidizes acridine to a *trans*-dihydrodiol and a phenol (Sutherland *et al.* 1994a) and chlorpromazine and methdilazine to the corresponding phenols (Zhang *et al.* 1996a). It produces a sulphone from dibenzothiophene (Crawford and Gupta 1990, Schlenk *et al.* 1994) and *N*-oxides from quinoline and isoquinoline (Sutherland *et al.* 1994b), but neither a sulphone nor an *N*-oxide was produced from phenothiazine. A related fungus, *C. verticillata*, transforms *N*-acetylphenothiazine to phenothiazine sulfoxide, phenothiazin-3-one and phenothiazine *N*-glucoside (Parshikov *et al.* 1999).

The fact that three of the four compounds produced by *C. elegans* from phenothiazine were identical to those produced during the metabolism of phenothiazine by mammals (Mitchell 1982) lends additional support for the use of this fungus as a microbial model for drug metabolism (Zhang *et al.* 1996a).

Acknowledgements

The authors thank C. E. Cerniglia and Y.-H. Kim for many helpful suggestions. The work was supported in part by an appointment to the Postgraduate Research Program at the National Center for Toxicological Research administered by the Oak Ridge Institute for Science and Education through an interagency agreement between the US Department of Energy and the US Food and Drug Administration.

References

- BACKES, W. L. and CANADY, W. J., 1981, The interaction of hepatic cytochrome P-450 with organic solvents: the effect of organic solvents on apparent spectral binding constants for hydrocarbon substrates. *Journal of Biological Chemistry*, **256**, 7213–7227.
- BECKETT, A. H. and NAVAS, G. E., 1978, Aromatic oxidation of some phenothiazines. *Xenobiotica*, **8**, 721–736.
- BINDRA, A., GIRI, S. and KHULLER, G. K., 1995, Identification, localization and possible role of calmodulin like protein in phospholipid synthesis of *Microsporum gypseum*. *Biochimica et Biophysica Acta*, **1255**, 118–122.
- BOLOGNESE, A., SCHERILLO, G. and SCHÄFER, W., 1986, Reaction of *o*-aminophenol and *p*-benzoquinone in acetic acid. *Journal of Heterocyclic Chemistry*, **23**, 1003–1006.
- BURKE, M. D. and MAYER, R. T., 1983, Differential effects of phenobarbitone and 3-methylcholanthrene induction on the hepatic microsomal metabolism and cytochrome P-450-binding of phenoxazone and a homologous series of its *n*-alkyl ethers (alkoxyresorufins). *Chemico-Biological Interactions*, **45**, 243–258.
- BURT, R. K. and THORGEIRSSON, S. S., 1988, Coinduction of MDR-1 multidrug-resistance and cytochrome P-450 genes in rat liver by xenobiotics. *Journal of the National Cancer Institute*, **80**, 1383–1386.
- CINI, M., FARIELLO, R. G., BIANCHETTI, A. and MORETTI, A., 1994, Studies on lipid peroxidation in the rat brain. *Neurochemical Research*, **19**, 283–288.
- CLIN, B. and LEMANCEAU, B., 1969, Étude par résonance magnétique nucléaire des dibenzothiophène, phénoxathiine, dibenzoparathiazine et phénoxazine. *Journal de Chimie Physique et de Physicochimie Biologique*, **66**, 1327–1329.
- CRAWFORD, D. L. and GUPTA, R. K., 1990, Oxidation of dibenzothiophene by *Cunninghamella elegans*. *Current Microbiology*, **21**, 229–231.
- GALLAGHER, C. H., KOCH, J. H. and MANN, D. M., 1965, The effect of phenothiazine on the metabolism of liver mitochondria. *Biochemical Pharmacology*, **14**, 799–811.
- GIEG, L. M., OTTER, A. and FEDORAK, P. M., 1996, Carbazole degradation by *Pseudomonas* sp. LD2: Metabolic characteristics and the identification of some metabolites. *Environmental Science and Technology*, **30**, 575–585.
- GIRARD, Y., HAMEL, P., THÉRIEN, M., SPRINGER, J. P. and HIRSHFIELD, J., 1987, First synthesis of sulfoxides and sulfones in the 3*H*-phenothiazin-3-one and 5*H*-benzo[*a*]phenothiazin-5-one ring systems. Addition reactions with nucleophiles. *Journal of Organic Chemistry*, **52**, 4000–4006.

- HALL, T. J., NYUGEN, H., SCHAEUBLIN, M., MICHALSKY, M. and MISSBACH, M., 1996, Phenothiazines are potent inhibitors of osteoclastic bone resorption. *General Pharmacology*, **27**, 845–848.
- KELLY, J. D., WHITLOCK, H. V., PORTER, C. J., GRIFFIN, D. and MARTIN, I. C. A., 1981, Anthelmintic efficacy of low-dose phenothiazine against strains of sheep nematodes susceptible or resistant to thiabendazole, levamisole and morantel tartrate: efficiency against sequentially administered infections. *Research in Veterinary Science*, **30**, 170–174.
- KITTLE, J. D., CALLE, L. M. and SULLIVAN, P. D., 1981, The effect of substituted phenothiazines on the mutagenicity of benzo[a]pyrene. *Mutation Research*, **80**, 259–264.
- KONTIR, D. M., GLANCE, C. A., COLBY, H. D. and MILES, P. R., 1986, Effects of organic solvent vehicles on benzo[a]pyrene metabolism in rabbit lung microsomes. *Biochemical Pharmacology*, **35**, 2569–2575.
- LUM, P. Y., BURKE, M. D., MAYER, R. T. and IOANNIDES, C., 1986, Alkoxyresorufin O-dealkylases: association with the murine Ah locus. *Cancer Letters*, **32**, 255–262.
- MITCHELL, S. C., 1980, Comparative metabolism of phenothiazine in the rat (*Rattus norvegicus*), mouse (*Mus musculus*), hamster (*Mesocricetus auratus*) and gerbil (*Gerbillus gerbillus*). *Comparative Biochemistry and Physiology*, **67C**, 199–202.
- MITCHELL, S. C., 1982, Mammalian metabolism of orally administered phenothiazine. *Drug Metabolism Reviews*, **13**, 319–343.
- MITCHELL, S. C. and WARING, R. H., 1979, Metabolism of phenothiazine in the guinea pig. *Drug Metabolism and Disposition*, **7**, 399–403.
- MITCHELL, S. C. and WARING, R. H., 1981, A comparison of the *in vivo* metabolism of phenothiazine and promazine in the neonatal guinea pig. *Biology of the Neonate*, **39**, 285–289.
- PARSHIKOV, I. A., FREEMAN, J. P., WILLIAMS, A. J., MOODY, J. D. and SUTHERLAND, J. B., 1999, Biotransformation of N-acetylphenothiazine by fungi. *Applied Microbiology and Biotechnology*, **52**, 553–557.
- RETTIE, A. E., WILLIAMS, F. M., RAWLINS, M. D., MAYER, R. T. and BURKE, M. D., 1986, Major differences between lung, skin and liver in the microsomal metabolism of homologous series of resorufin and coumarin ethers. *Biochemical Pharmacology*, **35**, 3495–3500.
- ROSEBOOM, H. and PERRIN, J. H., 1977, Oxidation kinetics of phenothiazine and 10-methylphenothiazine in acidic medium. *Journal of Pharmaceutical Sciences*, **66**, 1392–1395.
- SCHLENK, D., BEVER, R. J., VERTINO, A. M. and CERNIGLIA, C. E., 1994, P450 catalysed S-oxidation of dibenzothiophene by *Cunninghamella elegans*. *Xenobiotica*, **24**, 1077–1083.
- SOMERS, C. J., DOWNEY, N. E. and O'SHEA, J., 1987, Prophylaxis of trichostrongylid infection afforded by low-dose phenothiazine given in two successive years to first-season calves on a common area of pasture. *Research in Veterinary Science*, **43**, 143–149.
- SUTHERLAND, J. B., EVANS, F. E., FREEMAN, J. P., WILLIAMS, A. J., DECK, J. and CERNIGLIA, C. E., 1994a, Identification of metabolites produced from acridine by *Cunninghamella elegans*. *Mycologia*, **86**, 117–120.
- SUTHERLAND, J. B., FREEMAN, J. P., WILLIAMS, A. J. and CERNIGLIA, C. E., 1994b, N-oxidation of quinoline and isquinoline by *Cunninghamella elegans*. *Experimental Mycology*, **18**, 271–274.
- SUTHERLAND, J. B., RAFII, F., KHAN, A. A. and CERNIGLIA, C. E., 1995, Mechanisms of polycyclic aromatic hydrocarbon degradation. In L. Y. Young and C. E. Cerniglia (eds), *Microbial Transformation and Degradation of Toxic Organic Chemicals* (New York: Wiley-Liss), pp. 269–306.
- TATEISHI, T., NAKURA, H., ASOH, M., WATANABE, M., TANAKA, M., KUMAI, T. and KOBAYASHI, S., 1999, Multiple cytochrome P-450 subfamilies are co-induced with P-glycoprotein by both phenothiazine and 2-acetylaminofluorene in rats. *Cancer Letters*, **138**, 73–79.
- THIMMAIAH, K. N., HORTON, J. K., QIAN, X.-D., BECK, W. T., HOUGHTON, J. A. and HOUGHTON, P. J., 1990, Structural determinants of phenoxazine type compounds required to modulate the accumulation of vinblastine and vincristine in multidrug-resistant cell lines. *Cancer Communications*, **2**, 249–259.
- WADKINS, R. M. and HOUGHTON, P. J., 1993, Kinetics of cellular permeability of phenoxazine and its dependence on P-glycoprotein expression. *FEBS Letters*, **322**, 1–5.
- WANG, R. F., CAO, W. W., KHAN, A. A. and CERNIGLIA, C. E., 2000, Cloning, sequencing, and expression in *Escherichia coli* of a cytochrome P450 gene from *Cunninghamella elegans*. *FEMS Microbiology Letters*, **188**, 55–61.
- WARING, R. H. and MITCHELL, S. C., 1985, The metabolism of phenothiazine in the neonatal calf: identification of drug-polypeptide conjugates from urine. *Xenobiotica*, **15**, 459–468.
- ZHANG, D., FREEMAN, J. P., SUTHERLAND, J. B., WALKER, A. E., YANG, Y. and CERNIGLIA, C. E., 1996a, Biotransformation of chlorpromazine and methdilazine by *Cunninghamella elegans*. *Applied and Environmental Microbiology*, **62**, 798–803.
- ZHANG, D., YANG, Y., LEAKEY, J. E. A. and CERNIGLIA, C. E., 1996b, Phase I and phase II enzymes produced by *Cunninghamella elegans* for the metabolism of xenobiotics. *FEMS Microbiology Letters*, **138**, 221–226.

Pharmacokinetics and absolute bioavailability of sitafloxacin, a new fluoroquinolone antibiotic, in healthy male and female Caucasian subjects

J. O'GRADY^{†*}, A. BRIGGS[‡], S. ATARASHI[‡],
H. KOBAYASHI[‡], R. L. SMITH[§], J. WARD[¶], C. WARD[¶] and
D. MILATOVIC^{||}

[†] Daiichi Pharmaceuticals UK Ltd, 76 Shoe Lane, London EC4A 3JB, UK

[‡] Daiichi Pharmaceutical Co., Ltd, 16-13 Kita Kasai-1-Chome, Edogawa-Ku, Tokyo 134-8630, Japan

[§] Biomedical Sciences Division, Imperial College School of Medicine, South Kensington, London SW7 2AZ, UK

[¶] Covance Clinical Research Unit, Springfield House, Hyde Street, Leeds LS2 9NG, UK

^{||} Eijkman-Winkler Institute, University Medical Center, Utrecht, The Netherlands

Received 28 February 2001

1. The aim was to compare the pharmacokinetics of sitafloxacin from a capsule formulation (dose of 500 mg sitafloxacin) and an intravenous (i.v.) formulation infused over 1 h (dose of 400 mg sitafloxacin) in healthy male and female subjects and to estimate the absolute bioavailability of sitafloxacin from the capsule formulation.

2. Following oral administration, sitafloxacin was rapidly absorbed, with a mean maximum concentration in plasma ($C_{\max}$) of $4.65 \mu\text{g ml}^{-1}$ occurring at a median $t_{\max} = 1.25 \text{ h}$ giving a mean $\text{AUC}(0-\infty) = 28.1 \mu\text{g h ml}^{-1}$. For the i.v. administration, a mean $C_{\max} = 5.53 \mu\text{g ml}^{-1}$ occurred at the end of the 1-h infusion with a mean $\text{AUC}(0-\infty) = 25.4 \mu\text{g h ml}^{-1}$. The mean terminal elimination half-life was 7.0 h (oral) and 6.6 h (i.v.). For the oral and i.v. formulations, the mean total plasma clearance was 296 and 263 ml min^{-1} , respectively and the mean volume of distribution was 180 and 150 litres, respectively.

3. Within 48 h post-dose, ~61% (range 26–86%) of the administered dose was excreted unchanged in urine following capsule administration, compared with ~75% (range 42–101%) following the i.v. formulation. For both formulations, the renal clearance of sitafloxacin (means of 181 and 198 ml min^{-1} for the capsule and i.v. doses, respectively) implies active tubular secretion of the drug.

4. The absolute bioavailability of sitafloxacin from the capsule formulation was high at 89%, with a 95% CI of 84–94%. The intersubject variability (CV%) in the sitafloxacin $\text{AUC}(0-\infty)$ for the capsule was low at 18.6%.

5. Gender differences in the pharmacokinetics of sitafloxacin were small and would not warrant dose adjustment.

6. The findings show that the capsule formulation offers good oral bioavailability and merits further clinical evaluation of sitafloxacin as an orally effective fluoroquinolone antibacterial.

Introduction

Sitafloxacin, (–)-7-[(7*S*)-7-amino-5-azaspiro[2.4]heptan-5-yl]-8-chloro-6-fluoro-1-[(1*R*,2*S*)-2-fluoro-1-cyclopropyl]-1,4-dihydro-4-oxo-3-quinoline-carboxylic acid (figure 1) is a new fluoroquinolone antibacterial agent undergoing clinical development (as the sesquihydrate form) for the treatment of systemic bacterial infections. It exhibits high activity against Gram-positive,

* Author for correspondence: e-mail: ogradyj@daiichi.co.uk

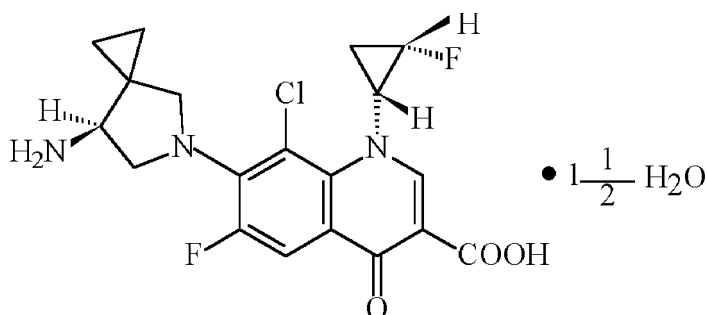


Figure 1. Chemical structure of sitafloxacin (sequihydrate form).

-negative and anaerobic organisms (Milatovic *et al.* 2000) and is effective against resistant strains such as methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant enterococci (VRE) (Shetty and Wilson 2000). Its mechanism of action involves inhibition of bacterial DNA-gyrase (topoisomerase II), an enzyme required for DNA replication.

Several early pilot studies showed that orally administered sitafloxacin, given to healthy subjects (volunteers), was rapidly and extensively absorbed and that elimination of the drug was largely by renal excretion, which could account for some 50–70% of the dose. Metabolism is only a relatively minor pathway of elimination for this drug; urinary and plasma metabolites of sitafloxacin that have been identified include the acyl glucuronic acid conjugate of the parent drug, *N*-acetylsitafloxacin and the two metabolites arising from the oxidative deamination and consequent reduction of the ketone metabolite to the hydroxy form (Daiichi 2000, unpublished data). Although some of these metabolites have antibacterial properties *in vitro* they probably contribute little, if any, to the overall systemic antibacterial properties of sitafloxacin itself.

Sitafloxacin has the properties of an ampholytic electrolyte due to the presence of a carboxyl and an amino group in its molecular structure; it shows a relatively low aqueous solubility ($100 \mu\text{g ml}^{-1}$), but higher solubilities in organic solvents such as chloroform ($3000 \mu\text{g ml}^{-1}$). In view of these solubility characteristics it became of obvious importance to develop and evaluate an oral formulation of sitafloxacin which could provide adequate oral bioavailability and plasma level profiles compatible with providing effective plasma antibacterial concentrations.

This paper describes the pharmacokinetics of intravenously and orally administered (capsule formulation) sitafloxacin given to healthy male and female subjects in an open, randomized, balanced, two-period crossover study.

Materials and methods

Test compounds

Daiichi Pharmaceutical Co. Ltd. (Tokyo, Japan) supplied hard, yellow capsules containing 250 mg sitafloxacin and an intravenous (i.v.) solution of 2 mg ml^{-1} sitafloxacin. The doses were expressed as the equivalents of the anhydrate of sitafloxacin.

Subjects

Twenty-four healthy Caucasian subjects (twelve males, twelve females) participated in this study. Before commencement, ethical approval of the study was obtained from the Covance Clinical Research Unit (Leeds, UK) Independent Review Board and, prior to participation in the study, each subject provided their written informed consent. The subjects were aged between 21 and 52 years. Body weights ranged between 69.4 and 101.3 kg for males and 55.8 and 82.2 kg for females. Subjects were healthy as assessed by physical examination, standard biochemical, haematological and urinalysis screening, vital signs and 12-lead electrocardiography. The subjects had taken no prescribed medications for 14 days before dosing and no over-the-counter medications had been taken by the subjects for 7 days before dosing. Alcohol, caffeine-containing food and beverages, grapefruit and grapefruit juice were prohibited during the study. Treatment randomization to the sequence of oral and i.v. treatments was performed using a computer-generated pseudo-random permutation procedure.

Dosing and sample collection

An oral formulation (capsule) and an i.v. formulation (1 h constant rate infusion) of sitaflloxacin, at dose levels of 500 and 400 mg, respectively, were administered to the subjects in a two-period crossover design. There was an interval of at least 7 days between treatments. Subjects were studied in two groups, each comprising of six males and six females. Each treatment period comprised of day -1 and days 1-3; dosing occurred on the morning of day 1. For each group, in each treatment period, six subjects received the oral formulation and six subjects received the i.v. formulation such that overall each subject received a single oral and a single i.v. dose of sitaflloxacin. Breakfast was not permitted on the day of dosing. Lunch, an afternoon snack and an evening meal were provided at ~4.5, 7.5 and 10.5 h, respectively, after dosing. Water consumption was restricted from 1 h pre-dose up to 2 h post-dose.

Blood samples for the determination of plasma concentrations of sitaflloxacin were collected from each subject in each treatment period pre-dose and at 30, 45, 60, 75, 90 min, 2, 3, 4, 6, 12, 18, 24, 36 and 48 h post-dose following oral dosing and pre-dose, 30, 60 (end of infusion), 65, 70, 80, 90, 105 min, 2, 3, 4, 6, 8, 12, 18, 24, 36 and 48 h after the start of the infusion. The sampling times were selected from previous pharmacokinetic studies with sitaflloxacin to give a reliable measure of the extent of bioavailability. The samples were centrifuged within 1 h of collection, at ~1500 g for 10 min at ~4°C. For each sample, the separated plasma was transferred into two 5 ml polypropylene tubes and stored at approximately -20°C, pending analysis. Urine samples were collected from pre-dose (-12 to 0 h) and 0-2, 2-4, 4-6, 6-8, 8-12, 12-24, 24-36 and 36-48 h after oral dosing or the start of the infusion. Two subsamples (each ~10 ml) were removed into polypropylene tubes and stored at about -20°C, pending analysis.

Quantification of sitaflloxacin in plasma and urine

The concentrations of sitaflloxacin in plasma and urine were determined using a validated method incorporating solid-phase extraction for sample preparation and high-performance liquid chromatography (HPLC) with post-column photolysis

and fluorescence detection. A post-column photolysis procedure was used as it has been found that it produces a marked enhancement of the fluorescent intensity of halogenated quinolones through the elimination of halogen substituents (Aoki *et al.* 1994) and structural rearrangements (Matsumoto *et al.* 1992).

Briefly, plasma and urine study samples, quality control (QC) samples and control matrix were thawed at room temperature and each tube was then vortex mixed and centrifuged at a nominal 4°C for 5 min at 3500 rpm. A 200- μ l aliquot of sample was then transferred into glass tubes, to which 400 μ l 50 μ M potassium dihydrogen orthophosphate was added, followed by 200 μ l (1.25 μ l ml⁻¹ solution for plasma and 10 μ g ml⁻¹ solution for urine) of a structurally related internal standard (7-[(8*S*)-8-amino-6-azaspiro[3,4]octan-6-yl]-8-chloro-6-fluoro-1-[(1*R*,2*S*) 2-fluoro-1-cyclopropyl]-1,4-dihydro-4-oxo-3-quinoline-carboxylic acid). All tubes were then vortex mixed.

Before loading with sample, each solid-phase extraction cartridge (C8 Isolute, Jones Chromatography, Hengoed, UK) was primed with 3 ml methanol followed by 2 ml water and finally 3 ml 50 μ M potassium dihydrogen orthophosphate. The sample solutions (800 μ l) were then individually transferred to the cartridges. Each solid-phase cartridge was washed with 3 ml 50 μ M potassium dihydrogen orthophosphate followed by 2 ml of a solution of tetrahydrofuran (THF)/water (20:80 v/v). Sitafloxacin and the internal standard were then eluted from each solid-phase extraction cartridge into glass tubes with 2 ml of a solution of THF/0.15% orthophosphoric acid (30:70 v/v). The resulting eluate was mixed thoroughly and transferred into amber microvials.

Chromatography was achieved using an Inertsil ODS2-150 (5 μ m) analytical column (15 $\times$ 4.6 mm i.d., Hichrom, Reading, UK) together with an Inertsil ODS2-10C (5 μ m) guard column (10 $\times$ 3.2 mm i.d.; Hichrom) at room temperature. An injection volume of 50–100 μ l was used. The mobile phase was potassium dihydrogen orthophosphate (50 μ M, pH 2.0)/THF/ammonium acetate solution (1 μ M) (3240:760:40 v/v/v) at a flow rate of 1.0 ml min⁻¹. The analytical instrumentation consisted of a PU-980 pump (Jasco UK, Great Dunmow, UK) with a Gilson 231XL autosampler (Anachem, Luton, UK). The post-column photolysis was achieved using a Beam Boost system incorporating a Beam Boost lamp (IC89500) together with a Beam Boost reaction coil (5 m $\times$ 0.3 mm i.d.) supplied by Technicol (Congleton, UK). Sitafloxacin and the internal standard were detected using a FP-920 fluorescence detector (Jasco) with an excitation wavelength of 280 nm and an emission wavelength of 430 nm.

The signal from the fluorescence detector was integrated to produce peak height. Concentrations in calibration standards, QC samples and study samples were obtained by interpolation of their peak height ratios (sitafloxacin/internal standard) from the calibration curves. Chromatography showed the sitafloxacin and internal standard peaks at retention times of $\sim$ 10 and 15 min, respectively, with a run time of 20 min.

The lower limit of quantification of sitafloxacin in plasma was 10 ng ml⁻¹ and the interassay precision of the QC samples analysed throughout the study ranged between 6.2 and 7.1% at concentrations of 30–1500 ng ml⁻¹ (analysed after dilution with control human plasma), with the interassay accuracy between 104.5 and 112.4%. The lower limit of quantification of sitafloxacin in urine was 0.1 μ g ml⁻¹ and the interassay precision of QC samples analysed throughout the study ranged between 3.1 and 7.6% at concentrations of 0.3–15.0 μ g ml⁻¹ (analysed after dilution

with control human urine), with the interassay accuracy between 104.5 and 112.4%.

Pharmacokinetic calculations and statistical analysis

The software used for the pharmacokinetic analyses was SAS v.6.12 (SAS Institute, Inc., Cary, NC, USA). Pharmacokinetic parameters were calculated using non-compartmental procedures from the bioanalytical data. Maximum plasma concentrations ($C_{\max}$) and time to maximum concentration ($t_{\max}$) were determined by examination of the plasma concentration-time profiles. The apparent terminal elimination rate constant, λ_z , was determined by linear regression of the logarithm of plasma concentration-time curve over the terminal linear disposition phase. The start points of the elimination phase for each subject were defined by visual inspection of the profiles of logarithmic plasma concentrations. The terminal elimination half-life ($t_{1/2}$) was calculated from $\ln(2)/\lambda_z$. The areas under the curve for plasma concentrations were calculated using linear trapezoids from pre-dose to $t_{\max}$ and log-linear trapezoids from $t_{\max}$ to 24 h post-dose for AUC(0-24 h) and to the last quantifiable plasma concentration (C_z at time t_z) for AUC(0-tz). Extrapolation to infinity from the last quantifiable plasma concentration (C_z/λ_z) was performed to estimate AUC(0- ∞). Mean residence time (MRT) was calculated for the oral dose as the ratio of the area under the first moment curve to infinity [(AUMC(0- ∞)] to AUC(0- ∞). The intrinsic MRT (MRT_{int}) for the i.v. dose was calculated as for the oral dose, but with a correction of minus half the infusion time. Total plasma clearance of sitafloracin, CL/F for the oral dose and CL for the i.v. dose, were calculated from the dose divided by AUC(0- ∞). During the elimination phase, the volume of distribution, V_z/F and V_z for the oral and i.v. doses, respectively, was calculated from (CL/F)/ λ_z and CL/ λ_z , respectively. The amount of sitafloracin excreted in the urine (A_e) was calculated for each collection period and then combined over 0-48 h post-dose. The percentage of the dose excreted, f_e was calculated as the ratio of A_e to the dose for each collection interval and over 0-48 h post-dose. Renal clearance, CL_R, was calculated over 0-48 h post-dose as the ratio of A_e to AUC. The absolute bioavailability of sitafloracin was calculated as the ratios of AUC(0- ∞) (primary variable) and A_e (0-48 h) (secondary variable), between the oral and i.v. doses, corrected for the dose level administered.

Statistical analyses of the pharmacokinetic data were performed for all subjects with the exception of three females (subjects 12, 19, 23). For Subjects 12 and 19, there were practical problems obtaining some blood samples for the analysis of sitafloracin following the i.v. treatment. For subject 23, data following the i.v. treatment only were obtained as the subject was withdrawn from study after completing treatment period 1.

The pharmacokinetic parameters (including dose and body weight normalized variables (norm)), with the exception of $t_{\max}$, were analysed by analysis of variance. Least squares (LS) means were calculated for the capsule and i.v. formulations of sitafloracin for males, females and both sexes combined (overall). Ratios and 95% CI were calculated for the comparisons of the capsule formulation relative to the i.v. formulation for all subjects and male and female subjects separately for AUC(0- ∞) (norm) and f_e (0-48 h). Ratios and 95% CI were calculated for the comparison of females relative to males for each formulation separately for all

parameters analysed. For t_{max} , median differences and 95% CI for the gender comparisons were performed using non-parametric methods.

Results

Safety and tolerability

Twenty-four subjects (12 males, 12 females) entered the study of which 23 completed as planned. One female was withdrawn due to concerns about rechallenge with sitafloxacin, following an urticarial rash during the i.v. dosing in the first treatment period, which resolved after 2 h 40 min. The urticaria was mild in severity and considered as probably treatment-related. Overall, sitafloxacin was generally well tolerated following administration of the capsule and i.v. formulations. There were no severe, serious or life-threatening adverse events during the study. The incidence of treatment-emergent adverse events was low following the capsule and i.v. formulations with most subjects not reporting any drug-related adverse events. The majority of drug-related adverse events were mild in severity. The most frequently reported adverse event for both oral (capsule) and i.v. dosing was mild diarrhoea, reflecting the results of previous studies with sitafloxacin. There were no clinically significant findings in the vital signs or 12-lead ECG parameters for each formulation, and there were no changes in any of the laboratory safety parameters during the study considered to be of clinical significance. In addition, there were no clinically significant changes in the physical examination or the dermatological examination findings during the study and no trends in body weight.

Pharmacokinetic parameters of sitafloxacin

Following i.v. administration of a single 400 mg dose of sitafloxacin, plasma concentrations of sitafloxacin were maximal at the end of the 1 h infusion (figure 2). Following oral administration of a single 500 mg dose of sitafloxacin as the capsule

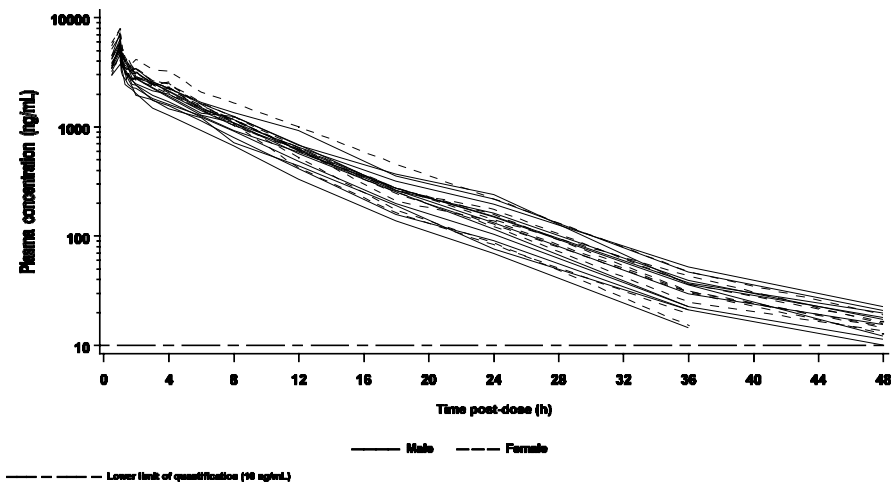


Figure 2. Plasma concentrations of sitafloxacin for all subjects over the 48 h period post-dose following IV formulation administration (400 mg sitafloxacin) (log-linear scale).

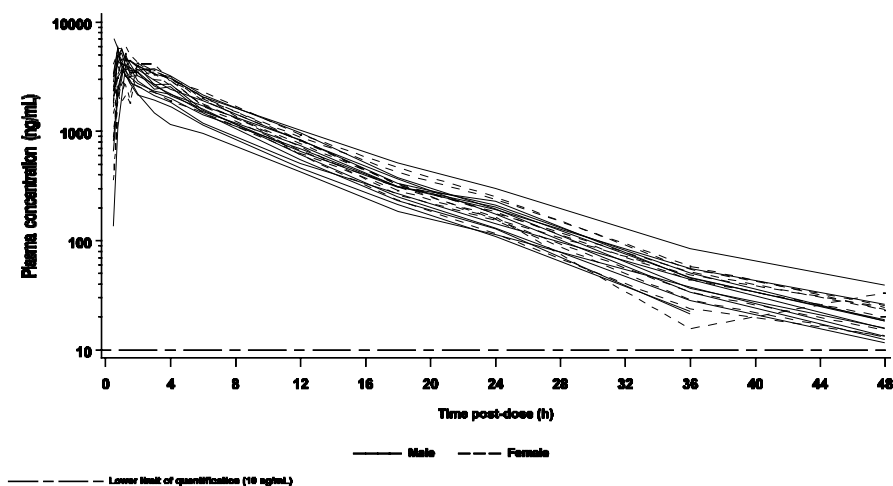


Figure 3. Plasma concentrations of sitafoxacin for all subjects over the 48 h period post-dose following oral capsule formulation administration (500 mg sitafoxacin) (log-linear scale).

formulation, plasma concentrations of sitafoxacin were above the lower limit of quantification (10 ng ml^{-1}) at 30 min post-dose (the first blood sampling time) for all subjects (figure 3). After reaching C_{max} , the semilogarithmic concentration-time curves revealed that the plasma concentrations of sitafoxacin declined in a biphasic manner for both formulations. Sitafoxacin concentrations were quantifiable in plasma for at least 36 h post-dose in all subjects and up to 48 h post-dose in the majority of subjects, following administration of both formulations.

The pharmacokinetic parameters for the i.v. and oral doses of sitafoxacin are given in tables 1 and 2. The mean plasma concentration-time curves showed sitafoxacin was rapidly absorbed from the oral capsule formulation with median t_{max} occurring at 75 min post-dose. Comparison of the pharmacokinetics of sitafoxacin in males and females showed that $\text{AUC}(0-24 \text{ h})$ and $\text{AUC}(0-\infty)$ were $\sim 14\%$ higher in females than in males for the capsule formulation and $\sim 15\%$ higher for females for the i.v. formulation. These differences were essentially accounted for by the differences in body weight between the two genders. Following the i.v. formulation, mean total plasma clearance in females and males was 243 and 278 ml min^{-1} , respectively, with corresponding volumes of distribution during the terminal phase (V_z) of 137 and 160 litres. These small differences between males and females were again reduced following correction for body weight. For both the i.v. and capsule formulation, the terminal elimination half-life of sitafoxacin was similar for both genders, at $\sim 7 \text{ h}$.

The rate of urinary excretion of sitafoxacin, following both the i.v. and capsule formulations was greatest during the 0–2 h period post-dose, with means of 20.7 and 10.6% of the dose being excreted for the two treatments, respectively. The fraction excreted then became progressively less with time. The fraction of the administered dose renally excreted as sitafoxacin over the 48 h post-dose was $\sim 75\%$ for the i.v. formulation and 61% for the capsule. For both formulations, the fraction excreted over 48 h post-dose was lower for females than males (by ~ 14 and 24% for the i.v. and capsule formulations, respectively). The renal clearance (CLR) of sitafoxacin gave means of 198 and 181 ml min^{-1} for the i.v. and capsule formulations, respectively.

Table 1. Pharmacokinetic parameters of sitafloxacin following I.V. formulation administration (400 mg sitafloxacin).

Parameter	Intravenous formulation (400 mg sitafloxacin)			
	Males (n = 12)	Females (n = 9)	Overall (n = 21)	Ratio of females: males LS mean ratio (95% CI)
AUC(0–24 h) (µg.h ml ⁻¹)	22.7 (16.2–29.9)	26.2 (24.1–36.4)	24.2 (16.2–36.4)	1.15 (0.992, 1.34)
AUC(0–∞) (µg.h ml ⁻¹)	23.9 (16.8–31.9)	27.4 (25.4–38.3)	25.4 (16.8–38.3)	1.15 (0.982, 1.34)
C _{max} (ng ml ⁻¹)	5.46 (3.70–7.24)	5.62 (4.18–7.92)	5.53 (3.71–7.92)	1.03 (0.864, 1.23)
t _{max} † (h)	1.00 (1.00–1.00)	1.00 (1.00–1.00)	1.00 (1.00–1.00)	0 (0, 0)
AUC(0–24 h)(norm)	4.25 (3.60–4.90)	4.48 (3.39–5.36)	4.25 (3.39–5.36)	1.05 (0.935, 1.18)
AUC(0–∞)(norm)	4.48 (3.74–5.22)	4.68 (3.48–5.63)	4.57 (3.48–5.63)	1.04 (0.925, 1.18)
C _{max} (norm)	10.2 (6.76–13.9)	9.60 (7.90–11.3)	9.95 (6.76–13.9)	0.940 (0.812, 1.09)
t _{1/2} (h)	6.64 (5.37–7.68)	6.53 (4.80–7.45)	6.59 (4.80–7.68)	0.984 (0.870, 1.11)
MRT _{int} (h)	7.00 (5.76–8.27)	6.66 (5.18–7.43)	6.85 (5.18–8.27)	0.954 (0.861, 1.06)
CL (ml min ⁻¹)	278 (209–398)	243 (174–263)	263 (174–398)	0.873 (0.748, 1.02)
CL (ml min ⁻¹ kg ⁻¹)	3.72 (3.19–4.46)	3.56 (2.96–4.79)	3.65 (2.96–4.79)	0.957 (0.848, 1.08)
V _z (l)	160 (128–204)	137 (97.2–169)	150 (97.2–204)	0.859 (0.720, 1.03)
V _z (l kg ⁻¹)	2.14 (1.86–2.52)	2.01 (1.55–2.44)	2.08 (1.55–2.52)	0.942 (0.809, 1.10)
A _e (0–48 h) (mg)	321 (272–376)	274 (169–404)	300 (169–404)	NP
f _e (0–48 h) (%)	80.2 (67.9–94.1)	68.7 (42.2–101)	75.1 (42.2–101)	0.856 (0.663, 1.11)
CL _R (0–48 h) (ml min ⁻¹)	225 (183–273)	168 (76.0–264)	198 (76.0–273)	0.747 (0.557, 1.00)
CL _R (0–48 h) (ml min ⁻¹ kg ⁻¹)	3.00 (2.63–3.63)	2.45 (1.29–3.79)	2.75 (1.29–3.79)	0.819 (0.619, 1.08)

Geometric mean (min–max) data are presented except where stated otherwise.

† Median (min–max) and median difference (95% CI) presented.

N, number of subjects studied.

norm, Normalized for dose and body weight (mg kg⁻¹).

NP, not performed.

Absolute bioavailability of sitafloxacin from the capsule formulation

The absolute bioavailability [F(AUC)(%)] of sitafloxacin for the capsule formulation, based on a statistical analysis of AUC(0–∞), was 89% (95% CI of 84–94%) for males and females overall, and was very similar in males compared with females (89.4 and 88.3%, respectively). For individual subjects, the absolute bioavailability based on AUC(0–∞) ranged from 70.2 to 106% for males and 74.9 to 103% for females. The absolute bioavailability based on a statistical analysis of f_e(0–48 h), for males and females overall, at 80% (95% CI of 74–87%), was less than that based on AUC(0–∞) and was lower for females (75.6%) compared with males (84.7%). For individual subjects, the absolute bioavailability based on f_e(0–48 h) ranged from 68.9 to 100% for males and 57.7 to 103% for females.

Table 2. Pharmacokinetic parameters of sitafloxacin following oral capsule formulation administration (500 mg sitafloxacin).

Parameter	Capsule formulation (500 mg sitafloxacin)			
	Males (<i>n</i> = 12)	Females (<i>n</i> = 9)	Overall (<i>n</i> = 21)	Ratio of females: males LS mean ratio (95% CI)
AUC(0–24 h) ($\mu\text{g}\cdot\text{h ml}^{-1}$)	25.1 (17.2–34.9)	28.6 (23.0–33.6)	26.5 (17.1–34.9)	1.14 (0.984, 1.33)
AUC(0– ∞) ($\mu\text{g}\cdot\text{h ml}^{-1}$)	26.6 (18.4–38.1)	30.3 (24.8–35.8)	28.1 (18.4–38.1)	1.14 (0.976, 1.33)
<i>F</i> (AUC)(%)	89.0 (70.2–106)	88.4 (74.9–103)	88.7 (70.2–106)	NA
<i>C</i> _{max} ($\mu\text{g ml}^{-1}$)	4.84 (3.55–7.06)	4.42 (3.51–5.94)	4.65 (3.51–7.06)	0.911 (0.764, 1.09)
<i>t</i> _{max} [†] (h)	1.00 (0.500–1.25)	1.50 (0.750–3.00)	1.25 (0.500–3.00)	0.750 (0, 1.25)
AUC(0–24 h)(norm)	3.77 (29.4–46.8)	3.90 (3.02–4.84)	3.83 (2.94–4.84)	1.04 (0.922, 1.17)
AUC(0– ∞)(norm)	4.00 (3.15–5.00)	4.13 (3.13–5.19)	4.06 (3.13–5.19)	1.03 (0.914, 1.17)
<i>C</i> _{max} (norm)	7.28 (5.44–10.3)	6.01 (4.53–7.94)	6.71 (4.53–10.3)	0.826 (0.713, 0.956)
<i>t</i> _{1/2} (h)	6.73 (5.03–8.16)	7.42 (6.64–9.64)	7.02 (5.03–9.64)	1.10 (0.975, 1.25)
MRT (h)	8.07 (6.73–9.36)	8.13 (7.15–9.28)	8.09 (6.73–9.36)	1.01 (0.908, 1.11)
CL/ <i>F</i> (ml min^{-1})	313 (219–454)	275 (233–336)	296 (219–454)	0.878 (0.752, 1.02)
CL/ <i>F</i> ($\text{ml min}^{-1} \text{ kg}^{-1}$)	4.16 (3.33–5.30)	4.03 (3.21–5.32)	4.11 (3.21–5.32)	0.969 (0.858, 1.09)
<i>V</i> _z / <i>F</i> (l)	182 (132–307)	177 (141–242)	180 (132–307)	0.968 (0.811, 1.16)
<i>V</i> _z / <i>F</i> (l kg^{-1})	2.42 (1.84–3.50)	2.59 (1.93–3.33)	2.50 (1.84–3.50)	1.07 (0.918, 1.24)
<i>A</i> _e (0–48 h) (mg)	340 (306–428)	260 (129–431)	303 (129–431)	NP
<i>F</i> (<i>A</i> _e)(%)	84.7 (6.89)	75.7 (57.7–103)	80.7 (57.7–103)	NA
<i>f</i> _e (0–48 h) (%)	68.0 (61.3–85.7)	52.0 (25.9–86.3)	60.6 (25.9–86.3)	0.764 (0.592, 0.986)
CL _R (0–48 h) (ml min^{-1})	214 (176–282)	144 (60.5–242)	181 (60.5–282)	0.672 (0.501, 0.900)
CL _R (0–48 h) ($\text{ml min}^{-1} \text{ kg}^{-1}$)	2.85 (2.27–3.43)	2.11 (1.04–3.50)	2.51 (1.04–3.50)	0.741 (0.561, 0.980)

Geometric mean (min–max) data are presented except where stated otherwise.
†Median (min–max) and median difference (95% CI) presented.
N, number of subjects studied.
norm, Normalized for dose and body weight (mg kg^{-1}).
NP, not performed.
NA, not available.

The between-subject coefficient of variation (CV%) for the bioavailability of sitafloxacin based on the primary variable, AUC(0– ∞), was shown to be low at 18.6% for the capsule, only slightly higher than that for the i.v. infusion (CV% = 17.3%). Based on the secondary parameter, *f*_e(0–48 h), CV% = 34.3 and 25.3% for the capsule and i.v., respectively.

Discussion

This Phase I, open, randomized, balanced, two-period, single-dose crossover study showed sitafloxacin was generally well tolerated in male and female subjects receiving 500 mg sitafloxacin as a solid oral capsule formulation and 400 mg sitafloxacin as a 1 h constant rate i.v. infusion. The most frequently reported drug-related adverse event following both oral (capsule) and i.v. dosing was mild diarrhoea, reflecting the results of previous studies with sitafloxacin. There were no findings in the safety data of clinical significance.

The mean plasma concentration-time curves showed sitafloxacin was rapidly absorbed from the oral capsule formulation with median $t_{\max}$ occurring at 75 min post-dose. Comparison of the pharmacokinetics of sitafloxacin in males and females showed only small differences between genders and these were essentially accounted for by the lower body weight of females compared with males. These small gender differences would not warrant any dose adjustment for sitafloxacin when administered to males and females.

The absolute bioavailability of sitafloxacin from the capsule formulation was high with a mean (95% CI) of 89% (84–94%) when based on $AUC(0-\infty)$ data. This high bioavailability was associated with a low between-subject variability in $AUC(0-\infty)$, with the between-subject coefficient of variation (CV%) being 18.6% for the capsule, only slightly higher than that for the i.v. infusion (CV% = 17.3%). Based on $f_e(0-48\text{ h})$, the absolute bioavailability determined for the capsule formulation was slightly less at 80%. The reason for the differences in absolute bioavailability between the $AUC(0-\infty)$ and $f_e(0-48\text{ h})$ may demonstrate a small dose-route related difference in the urinary excretion of sitafloxacin. Based on the secondary parameter, $f_e(0-48\text{ h})$, the CV% were higher than those based on $AUC(0-\infty)$, but showed a similar trend for the two formulations (CV% = 34.3 and 25.3% for capsule and i.v., respectively).

The fraction of the administered dose renally excreted as sitafloxacin over the 48 h post-dose was ~75% for the i.v. formulation and 61% for the capsule. The renal clearance of sitafloxacin was higher than the glomerular filtration rate, implying active tubular secretion of sitafloxacin. The high urinary concentrations of sitafloxacin may be beneficial in the treatment of urinary tract infections.

Sitafloxacin has a high *in vitro* activity against Gram-positive and -negative bacteria and anaerobes (Milatovic *et al.* 2000). Integration of pharmacokinetics and antibacterial activity *in vitro* or *in vivo* provides important information about the pharmacodynamic properties of an antibacterial agent. For the fluoroquinolones the $AUC(0-24\text{ h})$ minimum inhibitory concentration (MIC) ratio has been identified as the pharmacokinetic/pharmacodynamic parameter best correlating with the microbiological and clinical efficacy (Craig 1998).

In the case of sitafloxacin (based on a mean $AUC(0-24\text{ h}) = 25\text{ }\mu\text{g}\cdot\text{h ml}^{-1}$), such AUC/MIC ratios would be achieved by $MIC \leq 0.25\text{ }\mu\text{g ml}^{-1}$ for Gram-negative and $\leq 0.5\text{ }\mu\text{g ml}^{-1}$ for Gram-positive bacteria. Thus, combining the pharmacokinetic results of the present study with the MIC data from a recently published large European surveillance study (Milatovic *et al.* 2000) and applying the above-mentioned pharmacodynamic breakpoints, sitafloxacin appears to be active against 100% of the three major respiratory tract pathogens (*S. pneumoniae*, *H. influenzae*, *M. catharrhalis*), 96% of staphylococci including methicillin-resistant strains, 90% of the enterobacterial strains, 70% of the enterococci including vancomycin-resistant strains and 63% of *Pseudomonas* strains (table 3).

Table 3. Percentage of strains with sitaflloxacin MICs of $\leq 0.25 \mu\text{g ml}^{-1}$ for Gram-negative bacteria and $\leq 0.5 \mu\text{g ml}^{-1}$ for Gram-positive species (adopted from Milatovic 2000) producing AUC/MIC breakpoints for sitaflloxacin of ≥ 100 and ≥ 30 , respectively.

		Percentage strains inhibited at indicated sitaflloxacin concentration ($\mu\text{g ml}^{-1}$)	
	No. of strains	0.25	0.5
Gram-negative bacteria			
<i>E. coli</i>	411	86.6	
<i>Klebsiella pneumoniae</i>	445	94.4	
<i>Klebsiella oxytoca</i>	241	97.5	
<i>Proteus mirabilis</i>	319	84.6	
<i>Citrobacter freundii</i>	109	89.0	
<i>Enterobacter cloacae</i>	378	90.2	
<i>Morganella morganii</i>	138	91.3	
<i>Serratia marcescens</i>	211	87.7	
<i>Pseudomonas aeruginosa</i>	615	63.4	
<i>Haemophilus influenzae</i>	224	100	
<i>Moraxella catarrhalis</i>	213	100	
Gram-positive bacteria			
<i>Staphylococcus aureus</i>	891		95.1
<i>Staphylococcus epidermidis</i>	650		97.5
Beta-haemolytic streptococci	454		100
Viridans streptococci	97		100
<i>Streptococcus pneumoniae</i>	427		100
<i>Enterococcus faecalis</i>	257		75.3
<i>Enterococcus faecium</i>	175		61.9

Clinical studies evaluating the pharmacodynamics of sitaflloxacin would have to prove this efficacy assumption, although Phase II clinical studies in Europe and South Africa with sitaflloxacin (400 mg i.v., once daily) have shown bacteriological efficacy. In randomized studies, satisfactory bacteriological responses were obtained in all 24 (100%) of patients with intra-abdominal infections treated with sitaflloxacin (Daiichi 1999, unpublished data); and in patients with pneumonia (Feldman *et al.* 2001), 19 of the 20 (95%) bacteriologically evaluable patients in the sitaflloxacin treatment group had a satisfactory bacteriological response 21–35 days post-treatment. In an open study with resistant organisms, six of nine (67%) patients with vancomycin-resistant enterococci and four of 11 (36%) patients with methicillin-resistant *Staphylococcus aureus* treated with sitaflloxacin achieved a satisfactory bacteriological assessment at the first evaluation (2–5 days post-treatment) (Shetty and Wilson 2000).

In conclusion, this study has shown the capsule formulation of sitaflloxacin to offer excellent bioavailability with plasma levels compatible with providing effective antibacterial concentrations with no marked gender differences, a low variability between subjects and a high renal excretion of unchanged drug. These characteristics merit further clinical evaluation of this drug as an orally effective quinolone antibacterial.

References

- AOKI, H., OHSHIMA, Y., TANAKA, M., OKAZAKI, O. and HAKUSUI, H., 1994, High-performance liquid chromatographic determination of the new quinolone antibacterial agent DU-6859a in human serum and urine using solid-phase extraction with phoyolysis-fluorescence detection. *Journal of Chromatography B*, **660**, 365–374.

- CRAIG, W. A., 1998, Pharmacokinetic/pharmacodynamic parameters: rationale for antibacterial dosing of mice and men. *Clinical Infectious Diseases*, **26**, 1–12.
- FELDMAN, C., WHITE H., O'GRADY, J., BRIGGS, A. and RICHARDS, G., 2001, An open, randomised, multi-centre study comparing the safety and efficacy of sitaflloxacin and imipenem/cilastatin in the intravenous treatment of hospitalised patients with pneumonia. *Journal of Antimicrobial Agents*, **17**, 177–188.
- MATSUMOTO, M., KOJIMA, K., NAGANO, H., MATSUBARA, S and YOKOTA, T., 1992, Photostability and biological activity of fluoroquinolones substituted at 8 position after UV irradiation. *Antimicrobial Agents and Chemotherapy*, **36**, 1715–1719.
- MILATOVIC, D., SCHMITZ, F.-J., BRISSE, S. VERHOEF, J and FLUIT, A. C., 2000, In vitro activities of sitaflloxacin (DU-6859a) and six other fluoroquinolones against 8,796 clinical bacterial isolates. *Antimicrobial Agents and Chemotherapy*, **44**, 1102–1107.
- SHETTY, N and WILSON, A. P. R., 2000, Sitaflloxacin in the treatment of patients with infections caused by vancomycin-resistant enterococci (VRE) and methicillin-resistant *Staphylococcus aureus*. *Journal of Antimicrobial Chemotherapy*, **46**, 633–638.



Evaluation of the absorption, excretion and metabolism of [^{14}C] etoperidone in man

G. W. CALDWELL^{†*}, W. N. WU[‡] and J. A. MASUCCI[†]

[†] Division of Drug Discovery and [‡] Division of Preclinical Development, R. W. Johnson Pharmaceutical Research Institute, Spring House, PA 19477, USA

Received 24 July 2001

1. The absorption, excretion and metabolism of 2-{3-[4-(3-chlorophenyl)-1-piperazinyl]propyl}-4,5-diethyl-2,4-dihydro-3H-1,2,4-triazole-3-one hydrochloride (etoperidone HCl) was investigated in six healthy men. Subjects were fasted overnight before receiving a single oral dose of a 100 mg solution [^{14}C] etoperidone HCl.

2. Plasma (0–48 h), urine (0–120 h) and faecal (0–120 h) samples were collected. The terminal half-life of the total radioactivity from plasma was 21.7 ± 2.8 h with an apparent clearance of 1.01 ± 0.08 ml min⁻¹. Recoveries of total radioactivity in urine and faeces were $78.8 \pm 3.6\%$ and $9.6 \pm 4.1\%$ of the dose, respectively.

3. Etoperidone and 21 metabolites were isolated and identified in the plasma, urine and faecal extracts. Unchanged etoperidone accounted for <0.01% of the dose in all excreta samples. Nine metabolites were identified in the plasma extracts and 21 urinary metabolites were identified. Seven faecal metabolites were identified.

4. Five proposed pathways were used to describe the formation of the metabolites: alkyl oxidation, piperazinyl oxidation, *N*-dealkylation, phenyl hydroxylation and conjugation. Alkyl oxidation of etoperidone resulted in the formation of 2-{3-[4-(3-chlorophenyl)-1-piperazinyl]propyl}-4-ethyl-2,4-dihydro-5-(1-hydroxyethyl)-3H-1,2,4-triazole-3-one. Piperazinyl oxidation of this metabolite leads to the formation of its *N*-oxide. *N*-dealkylation of the piperazinyl group led to the formation of 1-(3-chlorophenyl) piperazine and triazole propionic acid. Phenyl hydroxylation led to three important metabolites in the urine and faeces.

Introduction

1,2,4-Triazol-3-one heterocyclic carbon drugs such as trazodone HCl [2-{3-[4-(3-chlorophenyl)-1-piperazinyl]propyl}-1,2,4-triazole-[4,3- α]-pyridin-3-(2H)-one hydrochloride] (Brogden *et al.* 1981), nefazodone HCl [2-{3-[4-(3-chlorophenyl)-1-piperazinyl]propyl}-5-ethyl-2,4-dihydro-4-(2-phenoxyethyl)-3H-1,2,4-triazole-3-one hydrochloride] (Davis *et al.* 1997), and etoperidone HCl [2-{3-[4-(3-chlorophenyl)-1-piperazinyl]propyl}-4,5-diethyl-2,4-dihydro-3H-1,2,4-triazole-3-one hydrochloride] (Ramacci *et al.* 1979) are known for their therapeutic use in treating depression. These drugs selectively block post-synaptic serotonin 5-hydroxytryptamine (5-HT) receptors and moderately inhibit serotonin and nor-adrenaline (nor-epinephrine) re-uptake (Raffa *et al.* 1992, Bolden-Watson *et al.* 1993). After oral administration, these drugs are well absorbed; however, they are extensively metabolized in the liver primarily by hydroxylation, *N*-dealkylation and *N*-oxidation processes (Wu *et al.* 1991, Nilsen and Dale 1992, Greene and Barbhuiya 1997). Of special interest is the metabolite 1-(3-chlorophenyl) piperazine (mCPP), which is formed by all three drugs by *N*-dealkylation of the piperazinyl

* Author for correspondence; e-mail: gcaldwel.prius.jnj.com

nitrogen. Although the plasma levels of mCPP account for only a small fraction of the total drug concentration, repeated administration of these drugs may result in the formation of biologically significant amounts, which could contribute to the antidepressant effects of the parent drug (Maes *et al.* 1997). The main route of elimination for these drugs is via metabolism with approximately 75% of a 25–100 mg oral dose of [^{14}C] trazodone HCl being recovered in urine as metabolites within the first 72 h (Brogden *et al.* 1981). Approximately 45% of a 50–200 mg oral dose of [^{14}C] nefazodone HCl was recovered in urine within the first 24 h (Mayol *et al.* 1994). Approximately 79% of a 100 mg oral dose of [^{14}C] etoperidone HCl was recovered in urine within the first 120 h (Wu *et al.* 1991). Preliminary absorption, excretion and metabolism data of etoperidone in man have been presented (Wu *et al.* 1991). However, no structures of the metabolites formed *in vivo* have been reported.

The present work describes the absorption, excretion and metabolism of [^{14}C] etoperidone following oral administration of a single 100 mg solution dose to six healthy men. We report the isolation, characterization and identification of unchanged etoperidone and its 21 metabolites in human plasma, urine and faeces.

Materials and methods

Chemicals

[^{14}C] etoperidone (RWJ-26872-002, M1) was synthesized at the R. W. Johnson Pharmaceutical Research Institute (Spring House, PA, USA) and radiochemical purity was shown to be >98% by thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC) (figure 1). Appropriate dilutions of [^{14}C] etoperidone were made with unlabelled etoperidone to obtain the desired specific activity ($1.01 \mu\text{Ci mg}^{-1}$) for the various studies. 5-(1-Hydroxyethyl) etoperidone (RWJ-36766, M2), 5-(1-oxoethyl) etoperidone (RWJ-36771, M3), methyl ester of triazole propionic acid (RWJ-41678, M13) and mCPP (RWJ-24315, M22) were synthesized at the R. W. Johnson Pharmaceutical Research Institute. All synthesized compounds had purities >95% by TLC and HPLC. The structures of these isolated metabolites (figure 1) were used as reference compounds for HPLC, TLC, nuclear magnetic resonance (NMR) and mass spectrometry (MS) analyses (Wu *et al.* 1995).

Glusulase[®], a mixture of arylsulphatase and β -glucuronidase (1:4 v/v) from *Helix pomatia*, was obtained from Endo Laboratories, Inc. (Wilmington, DE, USA). Diazald[®] (diazomethane reagent) was purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). Formula-989[®], used as a scintillation solution, was obtained from New England Nuclear (Boston, MA, USA). HPLC-grade solvents were obtained from the Fisher Scientific Co. (Fair Lawn, NJ, USA) and glass-distilled solvents were purchased from Burdick and Jackson Laboratories, Inc. (Muskegon, MI, USA). Other reagent chemicals were obtained from commercial sources and used without further purification.

Human study

The study was an open design with six subjects enrolled to receive treatment. Only adult male subjects in healthy condition were included, as determined by a detailed medical history, a physical examination, determination of clinical laboratory values (e.g. hematology, serum chemistries and urinalysis evaluations) and an electrocardiogram. The subject's ages ranged from 23 to 29 years and their body weight ranged from 64 to 83 kg (table 1). The subjects each received a single solution dose of 100 mg [^{14}C] etoperidone HCl at approximately 8 a.m. on day 1. The dosing solution was prepared by adding 100 ml distilled water to 100 mg [^{14}C] etoperidone HCl (dry power) in a 4-ounce bottle and gently swirling until the powder dissolved. Each subject consumed the entire contents of the bottle and two 60-ml rinses of the bottle. The subjects were cloistered from the evening of the day prior to dosing until 5 days (120 h) after dosing. The subjects underwent a supervised fast from 12 h predose until 4 h after dosing when a standard lunch was provided. Dinner was provided 6 h after lunch. Water intake was prohibited for 2 h predose and 2 h post-dose except for the 220 ml water administered with the study treatment.

Blood samples (~15 ml) were collected in heparinized green top Venoject[®] tubes using disposable needles (Becton-Dickinson & Co., Franklin Lakes, NJ, USA) at preselected times after dosing. The

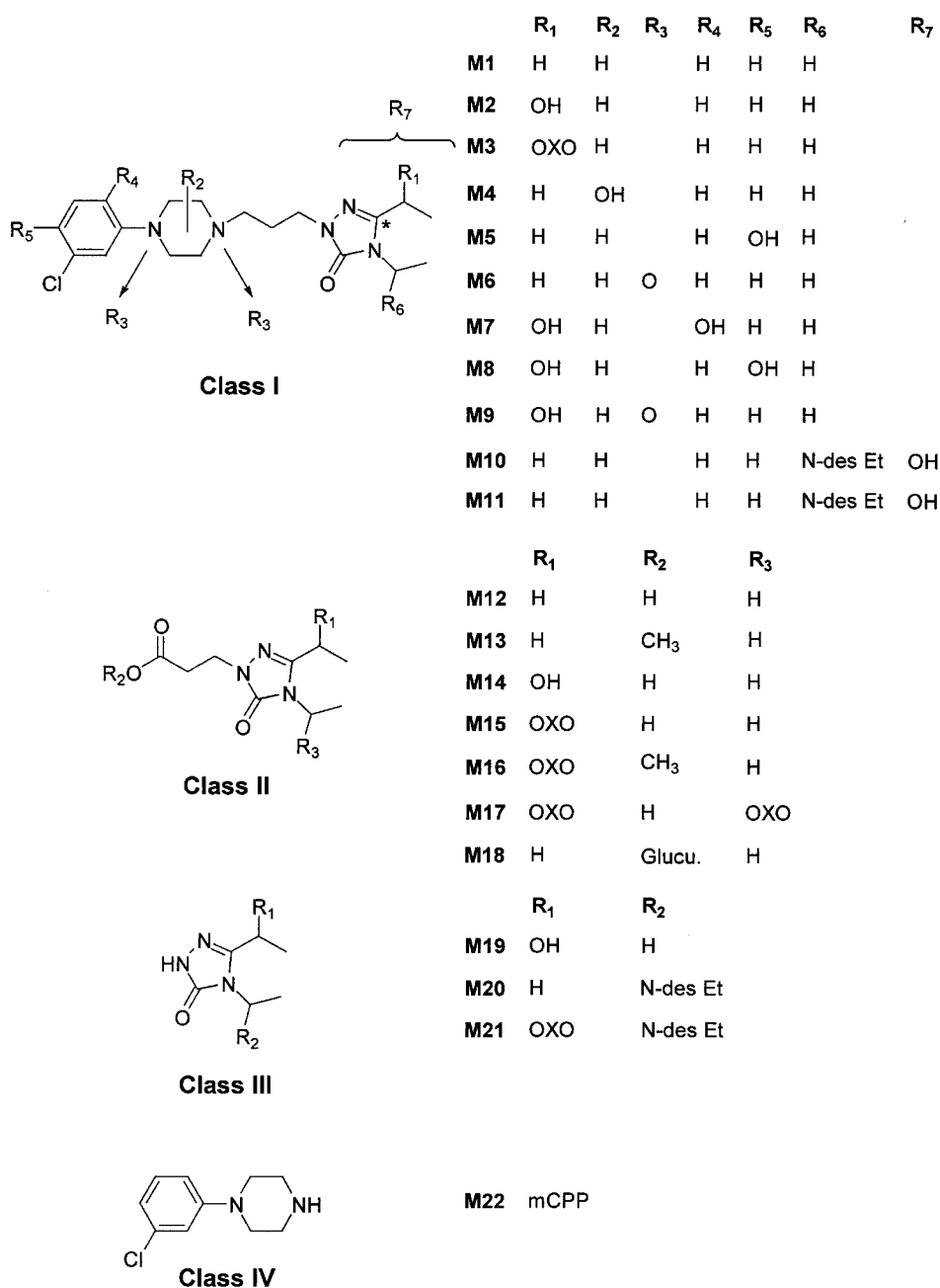


Figure 1. Structures of etoperidone (M1) and its metabolites (M2–21) identified in humans.

* Position of ¹⁴C label.

blood samples were centrifuged for 20min at 2000rpm to obtain plasma. Plasma was stored in polypropylene screw-cap tubes at -20°C before and after assay. Urine and faecal samples were quantitatively collected at specified intervals for 5 days following dose administration. Urine was stored in polypropylene screw-cap bottles and faeces were stored in polypropylene containers at -20°C before and after assay.

Table 1. Summary of the plasma levels and pharmacokinetic parameters of total radioactivity (ng eq. ml⁻¹) in man after a single oral solution dose of [¹⁴C] etoperidone (100 mg).

	Subject number						
Number:	1	2	3	4	5	6	
Age (years):	23	29	26	28	25	26	
Weight (kg):	76	65	82	83	64	64	Mean ± SD
Time (h)							
0	0	0	0	0	0	0	
0.25	509	1771	2021	705	2025	1967	1500 ± 700
0.50	2476	2623	2217	1980	2493	2507	2383 ± 238
0.75	2119	2676	2284	1909	2239	2284	2252 ± 252
1.0	1958	1846	1628	1673	1994	2056	1859 ± 176
1.5	1641	1722	1405	1450	1708	1592	1586 ± 132
2.0	1512	1476	1240	1258	1481	1405	1395 ± 119
3.0	1227	1352	1079	1062	1204	1195	1187 ± 106
4.0	1008	963	814	825	959	990	943 ± 66
6.0	758	749	602	602	620	629	660 ± 73
8.0	642	616	455	491	504	513	537 ± 75
10.0	535	544	424	473	482	459	486 ± 46
12.0	472	426	302	392	446	427	411 ± 59
16.0	331	378	334	378	334	338	351 ± 22
24.0	285	284	237	259	265	247	263 ± 19
30.0	230	232	192	226	226	227	222 ± 15
48.0	127	142	105	160	127	117	130 ± 19
Peak concentration (ng eq. ml ⁻¹)	2476	2676	2284	1980	2493	2507	2403 ± 242
Peak time (h)	0.50	0.75	0.75	0.50	0.50	0.50	0.6 ± 0.1
AUC (0–48 h)	19 874	20 638	17 175	18 285	19 308	19 007	19 048 ± 1214
Cl/F (ml min ⁻¹)	0.96	0.94	1.17	0.98	0.98	1.04	1.01 ± 0.08
T _{1/2} (h)	20.4	22.6	19.0	26.8	21.0	20.1	21.7 ± 2.8

Analytical equipment

Thin-layer radiochemical analyses were performed using 5 × 20 cm silica gel GF plates (250 µm gel thickness; Analtech, Inc., Newark, DE, USA). TLC plates were analysed using a Radiochromatogram Imaging System (BID100) (Bioscan, Inc., Washington, DC, USA). The HPLC used was a CRT (cathode ray tube)-based gradient liquid chromatograph (Beckman Instruments, Fullerton, CA, USA) connected to a radioactivity flow detector (IN/US Service Corp., Fairfield, NJ, USA). LiChrosorb RP-18 MPLC guard and analytical columns (10 µm, 130 × 4.6 mm i.d.) were used for sample analysis (Brownlee Labs., Inc., Santa Clara, CA, USA). Electron ionization (EI) mass spectra were obtained on a VG7070E (VG Micromass, Manchester, UK) where the operating conditions were source temperature at 200 °C; ionization potential at 70 eV; multiplier voltage at 1.8 KeV; electrometer setting at 10⁻⁷ A/V; mass ranges 18–1000 daltons. Chemical ionization (CI) mass spectra were obtained on a Finnigan 3300 (San Jose, CA, USA) where the operating conditions were source temperature at 200 °C; ionization potential at 200 eV; multiplier voltage at 2.0 KeV; reagent gas ammonia; mass ranges 100–800 daltons. A VG 7070E mass spectrometer was used to obtain fast-atom bombardment (FAB) mass spectra with 3-mercaptopropane-1,2-diol as the matrix and argon as the gas for the FAB gun. The acceleration voltage was 5 kV with a mass range of 50–1000 daltons. Proton (¹H) NMR spectra were obtained on a Bruker AM-360 Fourier transform spectrometer equipped with an Aspect 3000 data system. Typical ¹H-NMR spectra were obtained using a pulse width of 3 µs (30°); a 1 s recycling delay; 100 transients processed with a 0.1 Hz exponential weighting function and a digital resolution of 0.5 Hz per point. The compounds were dissolved in deuteroacetonitrile in 5 mm tubes. The chemical shifts (δ) measured in parts-per-million (ppm) were referenced internally to tetramethylsilane.

Radioactivity determination

Duplicate aliquots (0.1–1.0 ml) of plasma, urine and faecal extracts were assayed for total radioactivity in 10 ml Formula-989 by liquid scintillation spectrometry. Faeces were homogenized in 2 vols methanol and vacuum filtered resulting in methanolic faecal extracts and faecal residues. Faecal residues were air-dried before combustion analysis. Triplicate aliquots (100–300 mg) of the faecal residues were analysed using a Packard Model 306 Tricarb[®] oxidizer and a Beckman 3801 Liquid Scintillation Counting System purchased from Beckman Instruments (Fullerton, CA, USA).

Chromatography

Selected samples were pooled and analysed by TLC and HPLC for biotransformation profiling. Prior to chromatographic analysis, it was necessary to purify most of the samples. The plasma samples at 0.5 and 4 h were freeze-dried and extracted with methanol. The radioactivity profiles of the plasma samples were comparable before and after purification. The percentage of radioactivity recovered following purification was >83%. The urine samples (0–120 h) were purified by two different procedures. Urine samples were freeze-dried and extracted with methanol. The percentage of radioactivity recovered following purification was 95%. Urine samples were also hydrolysed with 1% Glusulase[®] in 1 M acetate buffer at 38°C overnight, freeze-dried and extracted with methanol. The percentage of radioactivity recovered following purification was 98%. Faecal extract samples (0–120 h) were evaporated to dryness and reconstituted with methanol. The percentage of radioactivity recovered following purification was 100%.

TLC analysis of the samples was performed using the following systems: an acidic solvent system, methylene chloride/methanol/glacial acetic acid (80/15/5 v/v/v) and a basic solvent system, methylene chloride/methanol/ammonium hydroxide (90/10/1 v/v/v). Each TLC plate was developed to a height of 14 cm above the spot or band where the sample was originally applied. Each sample was co-chromatographed with etoperidone, 5-(1-hydroxyethyl)etoperidone (RWJ-36766, M2) and 5-(1-oxyethyl)etoperidone (RWJ-36771, M3), the methyl ester of triazole propionic acid (RWJ-41678, M13) and mCPP (RWJ-24315, M22) as reference compounds, which were visualized with shortwave ultraviolet (UV) light after each plate was air-dried. Each plate was also analysed utilizing a RTLC scanner radiochromatogram imaging system (Radiomatic Instrument and Chemical Co., Tampa, FL, USA).

HPLC analysis of each sample pool was performed using a gradient elution method (Holland and Heebner 1991). Solvent A was water (4 litres) containing 0.1 M sodium acetate (0.6 ml), amylamine (2.24 ml), heptane sulphonic acid (2.0 g) and glacial acetic acid (10 ml), while solvent B was methanol/acetonitrile (1/3 v/v, 4 litres) containing the same amounts of reagents for solvent A. The gradient programme was carried out from 0% B to 60% B in 15 min, 60% B to 100% B in 15 min and 100% B for 2 min at a flow rate of 1 ml min⁻¹. All UV tracings were obtained using a Beckman 165 UV absorbance detector at 254 nm. All [¹⁴C] tracings were obtained using an on-line radioactivity flow detector.

Metabolite isolation and identification

Following sample purification, individual metabolites were isolated using alumina column chromatography and HPLC peak collection methods. Unresolved column fractionation residues or HPLC metabolite peaks were further separated using TLC zone scraping methods. The TLC and HPLC procedures were identical to those outlined above. All metabolites were characterized by desorption ammonia chemical ionization (DCI-NH₃) MS. Mass spectra of the isolated metabolites and their derivatives were compared with those of authentic samples, if available, for further structure confirmation. Proton (¹H) NMR spectra were obtained for some of the metabolites and compared with those of authentic samples. Overall, each metabolite was identified using a combination of three or more characteristics obtained from TLC, HPLC, NMR and MS (CI, EI, FAB) analysis. Some of the metabolites were derivatized using ethereal diazomethane to form methyl ethers or esters. These derivatives were further analysed using NMR and MS.

Results

The total radioactivity from each of the six subjects was measured for each plasma sample obtained from the study and, therefore, represents unchanged drug plus circulating plasma metabolites (table 1). The mean peak concentration was 2403 ± 242 ng eq. ml⁻¹ (%CV 10.1%), which occurred at 0.6 ± 0.1 h after dose administration. The area under the total radioactivity plasma concentration versus time curve (AUC_{0–48 h}) for each subject was calculated using the trapezoidal approximation (Gibaldi and Perrier 1982). The mean AUC_{0–48 h} of total radioactivity in plasma was 19048 ± 1214 ng eq. h ml⁻¹ (%CV 6.4%). Plasma elimination

Table 2. Per cent of total radioactivity (ng eq. ml⁻¹) recovered in urine and faeces in man after a single oral solution dose of [¹⁴C] etoperidone (100mg).

Time (h)	Subject number Per cent of administered dose						Mean ± SD
	1	2	3	4	5	6	
Urine							
0-2	28.9	29.4	28.8	28.8	n.s.	22.1	27.6 ± 3.1
0-4	44.8	45.7	45.6	45.4	56.6	43.5	46.9 ± 4.8
0-6	54.1	55.3	54.5	53.8	64.8	53.1	55.9 ± 4.4
0-8	60.3	60.9	60.3	58.9	n.s	59.2	59.9 ± 0.8
0-10	64.0	64.7	64.4	62.2	69.0	62.3	64.4 ± 2.5
0-12	67.5	67.7	67.8	64.6	71.5	65.1	67.4 ± 2.5
0-24	76.1	74.0	73.4	68.7	76.2	71.2	73.3 ± 2.9
0-48	82.0	77.3	76.8	72.6	79.4	73.5	76.9 ± 3.5
0-72	82.9	78.3	79.4	73.6	80.4	74.0	78.1 ± 3.7
0-96	83.1	78.6	79.9	74.3	81.0	74.2	78.5 ± 3.6
0-120	83.3	79.0	80.1	74.6	81.1	74.4	78.8 ± 3.6
Faeces							
0-24	0.0	0.0	n.s.	2.1	4.9	9.0	3.2 ± 3.8
0-48	0.1	6.2	0.4	n.s.	11.1	14.7	6.5 ± 6.5
0-72	2.2	7.5	5.5	6.8	13.2	14.9	8.4 ± 4.8
0-96	4.3	8.0	6.8	7.9	13.4	15.1	9.3 ± 4.1
0-120	4.4	8.2	7.3	8.7	13.5	15.3	9.6 ± 4.1
Total recovery	87.7	87.2	87.4	83.3	94.6	89.7	88.4 ± 3.7

n.s., No sample.

half-life was calculated by linear regression of the terminal phase of the plasma concentration versus time curve and the oral clearance was calculated from the plasma AUC data (Gibaldi and Perrier 1982). The terminal half-life of the total radioactivity from plasma was calculated to be 21.7 ± 2.8 h with a clearance of 1.01 ± 0.08 ml min⁻¹. The recovery of total radioactivity in urine (0-120 h) and faeces (0-120 h) was determined for mass balance considerations (table 2). The cumulative results were 78.8 ± 3.6% and 9.6 ± 4.1% of the dose excreted in the urine and the faeces, respectively.

Unchanged etoperidone and a total of 21 metabolites were isolated and identified. Their structures are presented in figure 1, while their TLC *R_f*, HPLC *R_t*, proton (¹H) NMR chemical shifts (ppm) and MS fragments (*m/z*) are presented in table 3. The per cent of administered dose represented by each metabolite in plasma, urine and faecal extract pools are summarized in table 4. For plasma pools, the numbers are expressed as per cent of total radioactivity in the sample, since the per cent of doses cannot be calculated. The per cents were obtained by peak area integration of radiochromatograms. In general, these values had a coefficient of variation and accuracy within 15% if the values were >5% of the sample. Therefore, metabolites accounting for <5% of the radioactivity in a sample are represented by single plus or double plus symbols instead of the estimated value to indicate that they are present but cannot be accurately quantified. The single plus represented samples that were <5% while the double plus represented samples that were between 5 and 20% but could not be accurately quantified. The total per cent of radioactivity in each sample pool represented by identified metabolites are also listed at the bottom of the table 4. More than 80% of the

Table 3. TLC R_f (basic solvent system), HPLC R_t , proton (^1H) NMR chemical shifts and MS fragments of etoperidone and its metabolites.

Metabolite	TLC R_f (min)	HPLC R_t (min)	^1H -NMR ^b chemical shifts (δ) (ppm)	MS (DCI-NH ₃) m/z (% relative intensity)
M1	0.72–0.82	23.2–24.5	6.75–7.40 (m, 4H), 3.80 (t, 2H), 3.65 (q, 2H), 3.22 (t, 4H), 2.63 (t, 4H), 2.60 (t, 2H), 2.52 (q, 2H), 2.00 (q, 2H), 1.23 (t, 3H) and 1.25 (t, 3H)	378 (MH ⁺ , 100%), 237 (5%), 223 (2%), 211 (38%), 195 (5%), 182 (10%), 168 (3%), 154 (2%), 142 (10%) and 139 (5%)
M2	0.57–0.67	20.2–22.5	6.74–7.25 (m, 4H), 3.81 (t, 2H), 3.50 (q, 2H), 3.20 (t, 4H), 2.55 (t, 4H), 2.42 (t, 2H), 4.72 (q, 2H), 1.90 (q, 2H), 1.25 (t, 3H) and 1.55 (d, 3H)	394 (MH ⁺ , 100%), 227 (50%), 209 (20%) and 139 (20%); 393 (M ⁺ , 100%, EI), 378 (M ⁺ -CH ₃ , 100%, EI), 277 (90%, EI) and 209 (45%, EI)
M3	0.90–0.98	24.5–25.5	6.75–7.15 (m, 4H), 4.02 (t, 2H), 3.85 (q, 2H), 3.17 (t, 4H), 2.52 (t, 4H), 2.45 (t, 2H), 1.95 (q, 2H), 1.22 (t, 3H) and 2.45 (s, 3H)	392 (MH ⁺ , 100%), 225 (60%), 209 (40%), 196 (20%) and 139 (20%)
M4	0.25–0.35	18.0–20.0		394 (MH ⁺ , 100%), 213 (60%), 198 (100%) and 195 (20%)
M5	0.30–0.40	18.2–20.1	6.75–6.97 (m, 3H), 3.65 (t, 2H), 3.90 (q, 2H), 3.85 (s, 3H), 3.50 (t, 4H), 2.50 (t, 4H), 2.42 (t, 2H), 2.10 (q, 2H), 2.20 (q, 2H), 1.25 (t, 3H) and 1.30 (d, 3H)	408 (MH ⁺ , 100%), 239 (5%), 225 (5%), 211 (15%) and 142 (10%); 407 (M ⁺ , 20%, EI), 392 (M ⁺ -CH ₃ , 15%, EI), 239 (20%, EI), and 211 (100%, EI)
M6	0.10–0.20	13.0–15.0		378 (100%), 237 (10%), 213 (15%), 211 (10%), 197 (80%), 182 (100%) and 142 (30%); 394 (MH ⁺ , 100%, FAB) and 378 (MH ⁺ -O, 15%, FAB)
M7	0.15–0.25	14.4–15.5	6.74–7.55 (m, 3H), 3.70 (t, 2H), 3.60 (q, 2H), 3.10 (t, 4H), 2.42 (t, 4H), 2.42 (t, 2H), 4.70 (q, H), 1.90 (q, 2H), 1.25 (t, 3H) and 1.50 (d, 3H)	410 (MH ⁺ , 100%), 392 (20%), 227 (29%), 209 (40%), 196 (20%) and 139 (20%)
M8	0.15–0.25	16.1–18.1	6.75–6.93 (m, 3H), 3.60 (t, 2H), 3.80 (q, 2H), 3.40 (t, 4H), 2.53 (t, 4H), 2.42 (t, 2H), 4.73 (q, H), 2.20 (q, 2H), 1.20 (t, 3H) and 1.35 (d, 3H)	410 (MH ⁺ , 100%), 321 (20%), 227 (10%), 198 (12%), 184 (5%) and 158 (5%)

(continued)

Table 3—(continued)

Metabolite	TLC <i>R</i> _f (min)	HPLC <i>R</i> _t (min)	¹ H-NMR ^b chemical shifts (δ) (ppm)	MS (DCI-NH ₃) <i>m/z</i> (% relative intensity)
M9	0.10–0.20	13.1–15.0		394 (10%), 213 (55%) and 198 (100%); 410 (MH ⁺ , 100%, FAB), and 394 (MH ⁺ -O, 15%, FAB)
M10	0.02–0.07	n.d. ^a		380 (MH ⁺ , 100%), 213 (20%) and 209 (25%)
M11	0.02–0.07	n.d. ^a		380 (MH ⁺ , 100%) and 213 (30%)
M12	0.02–0.07	11.4–14.3	3.63 (q, 2H), 1.31 (t, 3H), 4.10 (t, 2H), 2.78 (t, 2H), 2.50 (q, 2H), 1.25 (t, 3H) and 3.70 (s, 3H)	214 (MH ⁺ , 10%), 143 (100%) and 123 (30%). Methyl Ester: 227 (M ⁺ , 50%, EI), 196 (25%, EI), 168 (40%, EI), 154 (100%, EI), and 141 (30%, EI)
M13	0.81–0.91	28.0–30.0	3.63 (q, 2H), 1.31 (t, 3H), 4.10 (t, 2H), 2.78 (t, 2H), 2.50 (q, 2H), 1.25 (t, 3H) and 3.70 (s, 3H)	227(M ⁺ , 50%, EI), 196 (25%, EI), 168 (40%, EI), 154 (100%, EI), and 141 (30%, EI)
M14	0.02–0.05	n.d. ^a		244 (MH ⁺ , 100%); 243 (M ⁺ , 30%, EI), 212 (15%, EI), 184 (10%, EI), 170 (20%, EI) and 157 (10%, EI)
M15	0.02–0.10	12.0–14.3		242 (MH ⁺ , 100%); 241 (M ⁺ , 15%, EI), 210 (10%, EI), 182 (10%, EI), 168 (15%, EI) and 155 (10%, EI)
M16	0.91–0.99	28.0–30.0		242(MH ⁺ , 100%); 241(M ⁺ , 15%, EI), 210 (10%, EI), 182 (10%, EI), 168 (15%, EI), and 155 (10%, EI)
M17	0.05–0.10	28.0–30.0		256 (MH ⁺ , 100%)
M18	0	10.0–11.0	Hydrolysed to M12 after Glusulase [®] treatment	Hydrolysed to M12 after Glusulase [®] treatment
M19	0.20–0.30	8.4–11.6		158 (MH ⁺ , 100%)
M20	0.05–0.10	8.4–11.6	2.35 (q, 2H) and 1.20 (t, 3H)	114 (MH ⁺ , 100%); 113 (M ⁺ , 100%, EI)
M21	0.10–0.20	8.4–11.6		128 (MH ⁺ , 100%); 127 (M ⁺ , 100%, EI)
M22	0.40–0.46	20.5–21.5	6.75–7.20 (m, 4H), 3.20 (t, 4H), and 2.00 (t, 4H)	197 (MH ⁺ , 100%)

^a n.d., Not detected.
^b S, singlet; d, doublet; t, triplet; q, quartet; m, multiplet.

Table 4. Per cent of total ^{14}C and per cent of dose represented by etoperidone and its metabolites in each sample.^a

Pooled sample	Plasma (0.5 h)	Plasma (4 h)	Urine (0–48 h)	Faecal extract (0–72 h)
Sample content	2383 (ng eq. mg ⁻¹)	943 (ng eq. mg ⁻¹)	77% dose	6% dose
M1	23	4	+	+
M2	30	30	8	+
M3	+	+	+	+
M4	–	–	+	–
M5	+	+	8	16
M6	7	++	+	–
M7	–	–	++	+
M8	–	–	++	30
M9	7	++	+	–
M10	+	+	+	–
M11	+	+	+	–
M12	15	5	34	++
M13	–	–	+	–
M14	–	–	+	–
M15	–	–	+	–
M16	–	–	+	–
M17	–	–	+	–
M18	–	–	++	–
M19	–	–	+	–
M20	–	–	++	–
M21	–	–	+	–
M22 ^c	+	+	+	–
Total identified	~90	~80	~87	~60

^a All values are the means from the six male subjects. Per cents estimated by radio-TLC method were higher than those of the HPLC method.

^b +, <5% of total ^{14}C in sample; ++, 5–20% of total ^{14}C in sample.

^c Metabolite was identified as a non- ^{14}C component.

radioactivity in plasma and urine and 60% of the radioactivity in faecal extracts has been accounted for by identified metabolities.

Etoperidone metabolites can be categorized into five classes (figure 1). Class I (M1–11) are etoperidone analogues, class II (M12–18) are triazole propionic acid (TPA) analogues, class III (M19–21) are triazole analogues, class IV (M22) are mCPP analogues and conjugates. Unchanged etoperidone (M1) was isolated in plasma and urine and detected in faeces by TLC. It was identified on the basis of TLC, HPLC, NMR and MS data (table 3) in comparison with those data obtained from authentic etoperidone (Wu *et al.* 1995). The DCI-NH₃ MS analysis of unchanged etoperidone provided an intense protonated molecular cation at m/z 378 (100% relative intensity) with a characteristic chlorine isotope pattern. The CI spectra also contained informative fragment cations at m/z 237 (5%), 223 (2%), 211 (38%), 195 (5%), 182 (10%), 168 (3%), 154 (2%), 142 (10%) and 139 (5%). These fragments are interpreted in figure 2. The ^1H -NMR data showed characteristic chemical shifts (ppm) at 6.75–7.40 (m, 4H) (3-chlorophenyl protons); 3.65 (q, 2H) and 1.25 (t, 3H) (triazole *N*-ethyl protons); 3.22 (t, 4H) and 2.63 (t, 4H) (piperazinyl protons); 3.80 (t, 2H), 2.60 (t, 2H) and 2.00 (q, 2H) (propyl protons); 2.52 (q, 2H) and 1.23 (t, 3H) (triazole C-ethyl

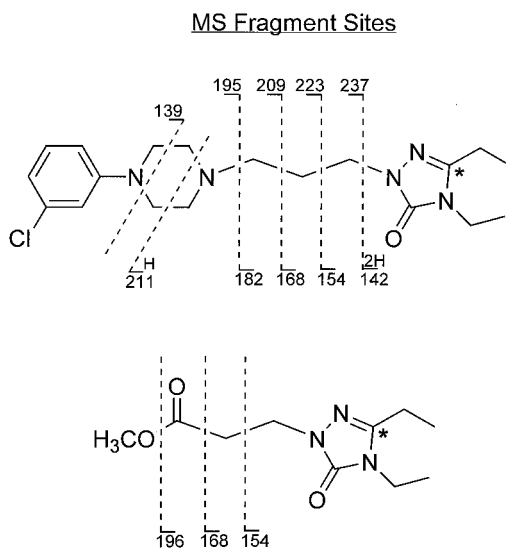


Figure 2. Sites of MS fragment ions in etoperidone and triazole propionic acid. *Position of ¹⁴C label.

protons). Etoperidone was observed in significant quantities in plasma (23% at 0.5 h; table 2).

M2 was isolated from plasma and urine samples and detected in trace amounts by TLC in faeces. The DCI-NH₃ spectrum contained an intense protonated molecular cation at m/z 394 (i.e. 16 amu greater than etoperidone) with a characteristic chlorine isotope pattern. Prominent fragments at m/z 227 (i.e. 211 + 16), 209 and 139 were consistent with a hydroxyl group attached to the triazole moiety (figure 2). The ¹H-NMR spectrum exhibited a quartet resonance with an integration of one methine proton at δ 4.72 ppm and indicated a secondary alcohol located at C1 of the triazole C-ethyl group. Direct comparison of the TLC, HPLC, ¹H-NMR and MS (CI) data of the isolated metabolite with those of an authentic sample (RWJ-36766) indicated its identity. M2 was found in significant quantities in plasma (30% at 0.5 h) and urine (8% of the sample). It further formed as a glucuronide conjugated in minor quantities (1%) in urine.

M3 was observed as a trace metabolite in plasma, urine and faeces. The DCI-NH₃ spectrum of the isolated material from plasma contained an intense protonated molecular cation at m/z 392 (i.e. 14 amu greater than etoperidone) with a characteristic chlorine isotope pattern. Prominent fragments at m/z 225 (i.e. 211 + 14), 209 and 139 indicated an oxo group attached at the triazole moiety (figure 2). Direct comparison of the TLC, HPLC, ¹H-NMR and MS (CI) data of the isolated metabolite with those of an authentic sample (RWJ-36771) identified M3 as oxo-etoperidone.

M4 was isolated by column chromatography from urine samples. The structure of M4 was tentatively elucidated on the basis of MS data only. The DCI-NH₃ spectrum of the isolated M4 showed an apparent protonated molecular cation at m/z 394 (i.e. 16 amu greater than etoperidone) with a characteristic chlorine isotope pattern along with important cations at m/z 213, 198 and 195. Since the m/z 231 (i.e. 195 + 16 + 2H) cation contained a chlorine isotope pattern (figure 2), the MS data suggested a hydroxyl group attaching to either the phenyl or the

piperazinyl ring. Diazomethane treatment of M4 did not derivatize the compound based on TLC and CI MS data. Therefore, M4 was tentatively assigned to have a hydroxyl substitution at the piperazinyl ring. M4 was present in minor amounts in urine only.

M5 was isolated from both urine and faeces and was converted to the methyl ether by diazomethane treatment after isolation from faecal extract. The DCI-NH₃ analysis of M5 methyl ether exhibits an intense apparent protonated molecular cation at m/z 408 (i.e. 30 amu greater than etoperidone) with a characteristic chlorine isotope pattern along with significant cations at m/z 239 (i.e. 209 + 30), 225 (i.e. 195 + 30), 211 and 142 (figure 2). These MS data and the results from M4 suggested a methoxyl group attached to the phenyl ring. The ¹H-NMR spectrum of the methylated product showed an *O*-methyl resonance signal as a singlet with an integration of three protons at δ 3.85 ppm. The three phenyl proton resonances appeared as an ABX pattern between δ 6.97–6.75 ppm (Becker 1969). These NMR data indicated the *O*-methyl group was substituted at the fourth position on the aromatic ring; that is, the *O*-methyl group was *para* relative to the piperazinyl ring (figure 2).

M6 was isolated from urine and plasma samples. DCI-NH₃ spectrum of the isolated metabolite did not contain a protonated molecular cation of etoperidone plus an oxygen atom. However, a protonated molecular cation of etoperidone was exhibited at m/z 378 along with prominent cations at m/z 213, 211, 197, 182 and 142. FAB MS data provided an apparent protonated molecular cation at m/z 394 and 378. Diazomethane treatment of M6 did not derivatize the metabolite based on MS data. M6 was tentatively assigned as an etoperidone N-oxide based on these observations and was found in significant quantities in plasma and in minor quantities in urine.

M7 was observed in small amounts in urine and faecal samples. It was isolated from these samples and analysed using MS and NMR. DCI-NH₃ of the isolated material contained an intense adduct molecular cation at m/z 410 (i.e. 32 amu higher than etoperidone) along with important cations at m/z 392 and 227. DCI-NH₃ analysis of the diazomethane treatment of M7 led to the formation of an intense molecular cation at m/z 424, which was interpreted to be the methyl phenyl ether derivative. The ¹H-NMR spectrum of M7 revealed a quartet signal for a methine proton at the C1 position of the C-ethyl group observed at δ 4.70 ppm and three protons forming an ABX between δ 6.78 and 7.46 ppm (Becker 1969). These NMR data indicated the OH group was substituted at the sixth position on the aromatic ring; that is, the OH group was *para* relative to the chlorine atom in the phenyl ring (figure 2). The MS and NMR data tentatively indicated M7 to be a dihydroxylated etoperidone with a hydroxyl group at the triazole moiety and another at the phenyl ring. M7 was tentatively elucidated to be 2-hydroxyphenyl-1-hydroxyethyl-etoperidone based on these spectroscopic data.

M8 was isolated from both urine and faeces. DCI-NH₃ analysis of this isolated metabolite indicated an intense protonated molecular cation at m/z 410 (i.e. 32 amu greater than etoperidone) with a characteristic chlorine isotope pattern along with significant cations at m/z 227 and 198. DCI-NH₃ analysis of the diazomethane treatment of M8 led to the formation of an intense molecular cation at m/z 424 (i.e. 14 amu increase after derivatization), which was interpreted to be the methyl phenyl ether derivative. Combining these data with the results of M7 suggested M8 to be OH-phenyl-OH-ethyl etoperidone. The ¹H-NMR spectrum

revealed quartet signals for a C1 methine proton of a C-ethyl group observed at δ 4.73 ppm and an ABX pattern for three phenyl proton δ 6.93–6.75. These NMR data indicated the OH group was substituted at the fourth position on the aromatic ring; that is, the OH group was *para* relative to the piperazinyl ring (figure 2). These spectroscopic data suggested M8 to be 4-hydroxyphenyl-1-hydroxyethyl etoperidone (figure 1). M8 was observed in significant amounts in urine and faeces. It was also present as a glucuronide conjugate in small quantities in urine.

M9 was isolated from plasma and DCI-NH₃ analysis of the isolated metabolite did not show an apparent protonated molecular cation. However, three prominent cations at m/z 394, 213 and 198 were different from those fragments associated with the MS analysis of M2. Based on M6 data, these fragments indicated an N-oxide located at the piperidine ring. Further analysis by FAB MS revealed an intense protonated molecular cation at m/z 410 accompanied by 394. M9 was tentatively assigned to be 1-OH-ethyl etoperidone *N*-oxide (M2 *N*-oxide) based on MS data. Treatment of M9 with ethereal diazomethane did not derivatize the metabolite. The position of the N-oxide of M9 is still uncertain. M9 was observed in significant amounts in plasma.

M10 and M11 were found in minor amounts in plasma. Both metabolites were polar and water soluble. They were isolated separately as two methyl derivatives after diazomethane treatment. The mass spectral analysis of the methyl derivatives of M10 and M11 both showed an intense protonated molecular cation at m/z 380 with a characteristic chlorine isotope pattern. Both metabolites also exhibited similar DCI-NH₃ fragment cations; however, the metabolites showed slight differences in their TLC behaviour. From these preliminary MS data M10 and M11 were tentatively assigned as two regioisomeric hydroxyl metabolites. Further structural confirmation of the metabolites was terminated because of insufficient material for NMR analysis.

M12 was isolated from urine and was analysed using DCI-NH₃ and ¹H-NMR. The MS data showed a protonated molecular cation of m/z 214 together with a fragment at m/z 143. Methylation of M12 with diazomethane led to the production of a methyl ester. The DCI-NH₃ spectrum of the methyl ester of M12 showed an apparent molecular cation at m/z 228 and several important fragments (table 3 and figure 2). The ¹H-NMR data of the methyl ester of M12 showed characteristic chemical shifts (ppm) at 3.63 (q, 2H) and 1.31 (t, 3H) (triazole *N*-ethyl protons); 4.10 (t, 2H) and 2.78 (t, 2H) (propyl protons); 2.50 (q, 2H) and 1.25 (t, 3H) (triazole C-ethyl protons); 3.70 (s, 3H) (*O*-methyl). M12 was identified to be triazole propionic acid (TPA) on the basis of TLC, NMR and MS data (table 3) in comparison with those data obtained from an authentic sample of a methyl ester of TPA (RWJ-41678). M12 was found in major amounts in urine (26% of the dose), plasma (15% of the sample at 0.5h) and significant amount in faeces. M12 also formed a polar glucuronide conjugate (M18) present in minor quantities in urine. The polar conjugate was hydrolysed to M12 after Glusulase[®] treatment.

M13 was isolated in small amounts from urine samples. The identity of M13 was confirmed to be the methyl ester of TPA by comparison of TLC, MS and NMR data of the isolated metabolite with those of an authentic sample of the methyl ester of TPA (RWJ-41678).

M14 was isolated in small amounts from urine and further derivatized to a methyl ester using excess ethereal diazomethane. The MS analysis of the meta-

bolite methyl ester showed an intense protonated molecular cation at m/z 244 (i.e. 16 amu greater than M13). Several diagnostic cations at m/z 212, 184, 170 and 157 were observed in the EI MS spectrum. The MS data tentatively proposed the structure of M14 to be 1-OH ethyl derivative of M12. The hydroxyl position of M14 was based on the OH position of M2. M14 was observed in minor quantities in urine.

M15 was isolated in small quantities from urine and converted to a methyl ester derivative with diazomethane. The MS data of the methyl ester showed an intense protonated molecular cation at m/z 242 (i.e. 14 amu greater than M12 and 2 amu less than M14) in DCI-NH₃ along with significant fragment cations at m/z 210, 182, 168 and 155 in EI MS. The MS data tentatively assigned the structure of M15 as the oxo analog of M12. The carbonyl position was tentatively postulated on the basis of that of M3. M15 was present in small amounts in urine.

M16 was only found in trace amounts in urine. The isolated metabolite gave an intense protonated molecular cation at 242. The EI fragments were identical to those of M15 methyl ester. The characteristics of both M16 and M15 methyl ester were identical. Therefore, M16 is the methyl ester of M15.

M17 was isolated from urine and derivatized to a methyl ester using diazomethane. The DCI-NH₃ spectrum showed an intense protonated molecular cation at m/z 256 (i.e. 28 amu greater than M13). The structure of M17 was tentatively proposed as a dioxo-TPA analogue. The structure is still uncertain and due to insufficient amounts of material, further confirmation of the structure of M17 was stopped at this stage. M17 was only observed in trace amounts in urine.

M19 was present in trace amounts in urine. DCI-NH₃ of the isolated sample yielded an intense protonated molecular cation at m/z 158, which was tentatively assigned to a hydroxy triazole analog. Owing to limited amounts of the isolated material, the structure is still uncertain.

M20 was isolated only from urine samples and DCI-NH₃ data of the isolated sample revealed an intense protonated molecular cation at m/z 114. The ¹H-NMR spectrum revealed a triplet resonance signal at δ 1.20 ppm and a quartet resonance signal at δ 2.35 ppm for a C-ethyl protons. The structure of M20 was identified to be a *N*-desethyl triazole analogue from a combination of MS and NMR data. M20 was present in significant quantities only in the urine.

M21 was isolated from urine and DCI-NH₃ spectrum of the isolated metabolite showed the apparent molecular cation at m/z 128 (i.e. 14 amu greater than M20). The MS data tentatively assigned the structure of M21 as the oxo analogue of M20. The carbonyl position was tentatively postulated on the basis of that of M19. Owing to the limited amounts of the isolated material, the structure is still uncertain. M21 was present in trace amounts in urine.

M22 was isolated as a non-radioactive component from plasma. DCI-NH₃ spectrum showed an intense protonated molecular cation at m/z 197 with a characteristic chlorine isotope pattern. Both MS and NMR data of the isolated metabolite were identical to those of authentic mCPP (RWJ-24315). MCPP was present in minor quantities in both plasma and urine.

Discussion

The terminal half-life of the total radioactivity from plasma was calculated to be 21.7 ± 2.8 h with a clearance of $1.01 \pm 0.08 \text{ ml min}^{-1}$ (table 1). A total of $88.4 \pm 3.7\%$ of the administered radioactive dose was recovered in excreta in the 5 days and accounted for $78.8 \pm 3.6\%$ and $9.6 \pm 4.1\%$ of the radioactive dose in urine and faeces, respectively, following a single 100 mg dose of [^{14}C] etoperidone to healthy men (table 2). Unchanged etoperidone accounted for $<0.01\%$ of the dose in excreta samples. These data indicate that etoperidone was well absorbed and extensively metabolized by man, and renal excretion was the major route of elimination of etoperidone metabolites.

Unchanged etoperidone and 21 metabolites were isolated, characterized and identified in humans (tables 3 and 4, figures 1 and 2). Five major metabolic pathways including alkyl oxidation, piperazinyl oxidation, *N*-dealkylation, phenyl hydroxylation and conjugation described formation of these metabolites (figure 3). The proposed metabolic pathways for the formation of metabolites are depicted in figure 4. Alkyl oxidation of etoperidone at *Site A* resulted in the formation of the major metabolite 2-{3-[4-(3-chlorophenyl)-1-piperazinyl] propyl}-4-ethyl-2,4-dihydro-5-(1-hydroxyethyl)-3H-1,2,4-triazole-3-one (M2). This metabolite was found in large quantities in plasma and urine. Further oxidation of M2 led to the production of the trace level M3 and the major plasma circulating *N*-oxide metabolite-nine (M9). Oxidative *N*-dealkylation of the piperazinyl group *Site B* led to the formation of 1-(3-chlorophenyl) piperazine (M22) a minor plasma and urinary metabolite and triazole proprionic acid (M12) a major urinary metabolite. M12 was further conjugated as an acyl glucuronide (M18) to a small extent in the urine. *N*-dealkylation at *Site B* in conjunction with oxidation at *Sites A* and *A'* led to the generation of three less quantitatively significant metabolites, M14, M15, M17 and M18. Formation of two minor ester metabolites, M13 and M16, could be artefact products due to the presence of methanol in the sample workup process. Oxidative *N*-dealkylation at *Site B'* and/or *Site B''* in conjunction with *Site A* generated three minor urinary metabolites, M19, M20 and M21. Piperazinyl ring oxidation at *Site C* produced M4 and M6 in plasma and

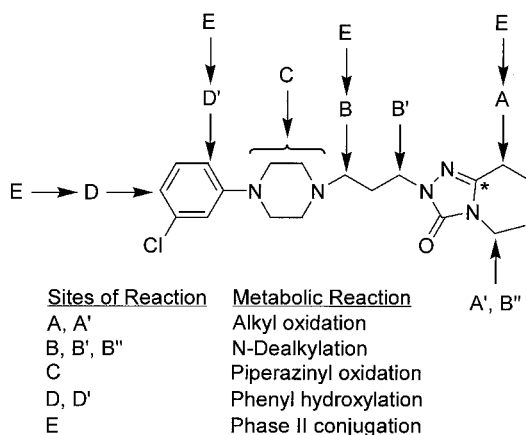


Figure 3. Sites of oxidation and corresponding metabolic reactions involving etoperidone in humans.

* Position of ^{14}C label.

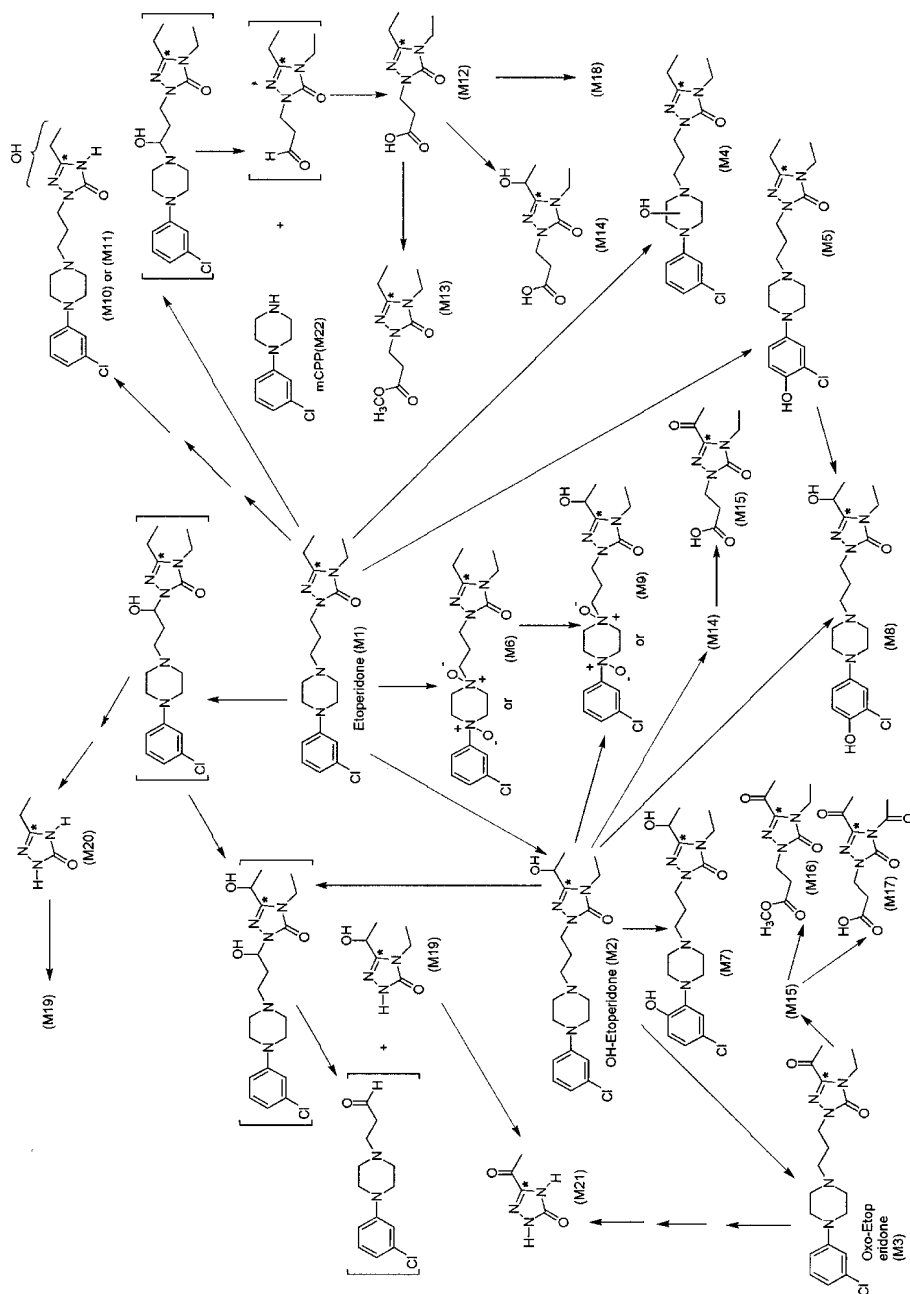


Figure 4. Proposed metabolic pathway for etoposide in human. Brackets indicate intermediates that have not been detected.

urine. M6 may be further oxidized at *Site A* to produce M9. Phenyl hydroxylation also appeared to be an important metabolic process. Hydroxylation at *Site D* produced M5, which was found in significant amounts in the urine and faeces. Phenyl hydroxylation in conjunction with oxidation at *Site A* resulted in the formation of two dihydroxylated metabolites, M7 and M8, excreted in both urine and faeces in significant amounts. M2, M5, M7, M8 and M12 were further conjugated as glucuronides; however, these metabolites were only found in low concentrations in urine.

The human plasma metabolite pattern of etoperidone (table 4) was somewhat similar to those of dogs (Wu *et al.* 1995). Wu *et al.* identified four plasma metabolites of etoperidone in dogs. 5(Hydroxyethyl)-etoperidone (M2) was found as a major metabolite along with a minor amount of M3. These results were similar to the human results. However, 1-(3-chlorophenyl) piperazine (mCPP, M22), an active metabolite that was produced in major quantities in dogs, appeared as a minor plasma metabolite in humans. Oxidation of mCPP at the piperazine ring produced a hydroxy mCPP plasma metabolite in dogs. This oxidative metabolite of mCPP was not observed in humans. In conclusion, etoperidone is well absorbed, rapidly excreted and extensively metabolized in humans. Renal excretion is the major route of elimination of the metabolites of this drug.

Acknowledgements

The authors thank Dr L. E. Weaner for synthesizing [^{14}C] etoperidone, Mr M. S. Mutter for providing NMR spectra and Dr J. D. Rodgers for synthesizing etoperidone metabolites.

References

- BECKER, E. D., 1969, *High Resolution NMR* (New York: Academic Press).
- BOLDEN-WATSON, C. and RICHELSON, E., 1993, Blockade by newly-developed antidepressants of biogenic amine uptake into rat brain synaptosomes. *Life Sciences*, **52**, 1023.
- BROGDEN, R. N., HEEL, R. C., SPEIGHT, T. M. and AVERY, G. S., 1981, Trazodone: A review of its pharmacological properties and therapeutic use in depression and anxiety. *Drugs*, **21**, 401.
- DAVIS, R., WHITTINGTON, R. and BRYSON, H. M., 1997, Nefazodone: a review of its pharmacology and clinical efficacy in the management of major depression. *Drugs*, **53**, 608.
- GIBALDI, M. and PERRIER, D., 1982, *Pharmacokinetics* (New York: Marcel Dekker).
- GREENE, D. S. and BARBHAIYA, R. H., 1997, Clinical pharmacokinetics of nefazodone. *Clinical Pharmacokinetics*, **33**, 260.
- HOLLAND, M. L. and HEEBNER, E. T., 1991, High-performance liquid chromatographic assay with ultraviolet detection for the determine of etoperidone and two active metabolites, 5-(1-hydroxyethyl)etoperidone and 1-(3-chlorophenyl)piperazine, in plasma, *Journal of Chromatography*, **567**, 433.
- MAES, M., WESTENBERG, H., VANDOOALAECH, E., DEMEDTS, P., WAUTERS, A., NEELS, H. and MELTZER, H. Y., 1997, Effects of trazodone and fluoxetine in the treatment of major depression: therapeutic pharmacokinetic and pharmacodynamic interactions through formation of meta-chlorophenylpiperazine. *Journal of Clinical Psychopharmacology*, **17**, 358.
- MAYOL, R. F., COLE, C. A., LUKE, G. M., COLSON, K. L. and KERNS, E. H., 1994, Characterization of the metabolites of the antidepressant drug nefazodone in human urine and plasma. *Drug Metabolism and Disposition*, **22**, 304.
- NILSEN, O. G. and DALE, O., 1992, Single dose pharmacokinetics of trazodone in healthy subjects. *Pharmacology and Toxicology (Copenhagen)*, **71**, 150.
- RAFFA, R. B., SHANK, R. P. and VAUGHT, J. L., 1992, Etoperidone, trazodone and MCP: *in vitro* and *in vivo* identification of serotonin 5-HT $_{1A}$ (antagonistic) activity. *Psychopharmacology (Berlin)*, **108**, 320.

- RAMACCI, M. T., GHIRARDI, O., MACCARI, F., PACIFICI, L. and SALE, P., 1979, Pharmacological evaluation of Δ^2 , 1,2,4-triazolin-5-one [1,3(4-m-chlorophenyl-1-piperazinyl)-propyl]-3,4-diethyl-hydrochloride (etoperidone), a new potential antidepressant drug. *Arzneim.-Forsch. Drug Research*, **29**, 294.
- WU, W. N., CALDWELL, G. W. and UETZ, J. A., 1991, Biotransformation of etoperidone in man. *Pharmacologist*, **33**, 208.
- WU, W. N., MASUCCI, J. A., MCKOWN, L. A. and RODGERS, J. D., 1995, Identification of four plasma metabolites of Etoperidone in dogs. *Journal of Pharmacology and Biomedical Analysis*, **13**, 177.

Evaluation of the marmoset as a model species for drug glucuronidation

M. G. SOARS[†], R. J. RILEY^{‡*} and B. BURCHELL[†]

[†] Department of Molecular and Cellular Pathology, Ninewells Hospital and Medical School, Dundee DD1 9SY, UK

[‡] Department of Physical and Metabolic Science, AstraZeneca Charnwood, Bakewell Road, Loughborough LE11 5RH, UK

Received 19 March 2001

1. The *in vitro* glucuronidation of a wide range of compounds has been studied in microsomes prepared from marmoset liver and kidney. These studies have been undertaken to evaluate the marmoset as a model species for drug glucuronidation and for comparison with conjugation by other species.

2. The compounds studied were glucuronidated by marmoset liver microsomes to varying extents (e.g. naproxen CL_{int} $0.4 \mu\text{l min}^{-1} \text{mg}^{-1}$, 1-naphthol CL_{int} $43 \mu\text{l min}^{-1} \text{mg}^{-1}$). Both marmoset and rat liver microsomes glucuronidated morphine at the 3-position (marmoset CL_{int} $19 \mu\text{l min}^{-1} \text{mg}^{-1}$, rat CL_{int} $6.3 \mu\text{l min}^{-1} \text{mg}^{-1}$) but glucuronidation at the 6-position was below the level of radiodetection in both species.

3. Interestingly, marmoset liver microsomes were able to catalyse the glucuronidation of the tertiary amine imipramine to a significant extent ($0.05 \text{ nmol min}^{-1} \text{mg}^{-1}$). However, no glucuronidation was detected by rat liver microsomes.

4. Conjugation of a range of substrates was detectable by marmoset kidney microsomes in contrast to rat kidney microsomes, which only catalysed the glucuronidation of bilirubin and 1-naphthol (CL_{int} $17 \mu\text{l min}^{-1} \text{mg}^{-1}$ and $18 \mu\text{l min}^{-1} \text{mg}^{-1}$, respectively).

5. This report and previous work in dog and human tissue microsomes suggest that the marmoset may be an alternative animal model for human drug glucuronidation, especially when the pathway of drug glucuronidation is known to differ between lower laboratory species and man.

Introduction

Uridine diphosphate glucuronosyltransferases (UGT) are a large family of membrane-bound enzymes responsible for the detoxification of a wide range of xenobiotics and endogenous compounds (Dutton 1980, Mackenzie *et al.* 1997). The large number of drugs glucuronidated, whether directly or following Phase I functionalization necessitates a thorough understanding of glucuronidation for efficient drug development. Historically, pharmaceutical companies have used rodents such as the rat as animal models for pharmacokinetic studies. Whilst a considerable understanding of glucuronidation by the rat has been obtained from the cloning and expression in recombinant cell lines containing the major rat UGT isoforms (Bock *et al.* 1993, Prichard *et al.* 1994, Coffman *et al.* 1995, Green *et al.* 1995), less information is available on drug glucuronidation from microsomal studies (Koster *et al.* 1986, El Mouelhi and Bock 1991).

* Author for correspondence; e-mail: rob.riley@astrazeneca.com

The dog has commonly been used as a second animal model in the development of new chemical entities, however substantial data on drug glucuronidation by the dog has only recently become available. Soars *et al.* (2001) suggested that a wide range of drugs are more extensively glucuronidated by dog liver microsomes compared with human hepatic microsomes. Qualitative differences in drug glucuronidation were observed between dog and humans with extrahepatic glucuronidation also markedly reduced in the dog.

Late preclinical safety assessment studies often require a third species to be used when metabolic pathways in rat and dog differ greatly from human. Primates have often been the species chosen for this task and have been commonly used in further studies to test opioid agonists and antagonists (France and Woods 1989, Bertalmio *et al.* 1992, Winger *et al.* 1992). Information concerning UGT in primates has been limited compared with rat or humans. However, the cloning and expression of several UGT isoforms from the cynomolgus monkey (Green *et al.* 1997, Barbier *et al.* 1999, Belanger *et al.* 1999) has suggested that the conjugation of certain substrates (e.g. steroid glucuronidation) may be conserved between monkey and human. More recently, there has been a trend towards using the marmoset as a simian model for drug development due to the advantageous nature of its smaller size when compared with other primates. An understanding of drug glucuronidation by the marmoset would therefore provide novel information of value to industry and academia alike.

Traditionally, *in vitro* pharmacokinetic studies have focused on the liver fractions and cells as the major metabolic organ (Dutton 1980). This practice has afforded an accurate assessment of clearance by Phase I enzymes (with the possible exception of drugs metabolized by CYP3A4; Houston 1994). However, an underestimate in the metabolism of drugs primarily cleared by Phase II process of glucuronidation, which may be expressed significantly in extrahepatic tissues, may become apparent when using this method. Previous work in this lab by Sutherland *et al.* (1993) has shown the expression of the major drug-metabolizing UGT isoforms to be present in human kidney. The importance of this finding was illustrated with the anaesthetic propofol, which was glucuronidated more rapidly by human kidney than liver (Soars *et al.* 2001). More recently, Strassburg *et al.* (2000) demonstrated the expression of many UGT isoforms in human intestine. However, to date, only limited information on functional drug glucuronidation by this tissue is available.

The aim of the present paper was to investigate drug glucuronidation in marmoset tissue microsomes to assess the suitability of using the marmoset as a model for glucuronidation in humans. The *in vitro* kinetic parameters $V_{\max}$ and K_m were determined in microsomes prepared from both marmoset liver and kidney for a range of chemically diverse drugs known to be predominantly glucuronidated in humans (Bertz and Granneman 1997). Similar studies were also performed using rat liver and kidney microsomes. Using the data generated in this report and results obtained previously in human and dog (Soars *et al.* 2001), the marmoset was evaluated as a model for drug glucuronidation in man.

Material and methods

Materials

Substrates, UDP-glucuronic acid and other reagents used in the assays were purchased from Sigma (Gillingham, UK), Aldrich (Gillingham, UK) or BDH (Poole, UK) and were of the highest grade

available. [^{14}C] UDPGA (293.6 mCi mmol $^{-1}$, 99.7% purity) and [^3H] imipramine hydrochloride (48.7 Ci mmol $^{-1}$, 98.5% purity) were purchased from NEN Dupont (Stevenage, UK).

Microsomal preparation from liver and kidney

Rat liver and kidney samples were removed from four healthy male Sprague-Dawley rats (250–350 g), immediately frozen in liquid nitrogen and stored at -80°C . Marmoset liver and kidney samples were removed from six healthy marmosets (male/female ratio 2:1, age 2–3 years, 300–400 g) and frozen in the same way as rat tissues. All microsomes were prepared using a method adapted from that of Coughtrie *et al.* (1987). Briefly, livers or kidneys were minced and homogenized in 4 vols ice-cold 0.25 M sucrose, 5 mM HEPES, pH 7.4. The resultant homogenates were then centrifuged for 15 min at 10 000g and the supernatants were centrifuged at 100 000g for 60 min. Microsomal pellets were resuspended in 1 vol. (ml) ice-cold 0.25 M sucrose, 5 mM HEPES, pH 7.4, equal to the wet weight (g) of tissue. Protein in microsomal samples was determined using the method of Lowry *et al.* (1951) using bovine serum albumin as a standard. Aliquots were stored at -80°C .

For each microsomal preparation, tissue was sonicated for 1, 2, 3, 4 and 5 $\times$ 5-s bursts (Microson Ultrasonic Cell Disruptor, Heat Technologies, Farmingdale, NY, USA) with 1 min on ice between bursts. The resultant microsomal sonicates were used in UGT assays to determine the optimum level of sonication (4 $\times$ 5-s bursts) required for maximal glucuronidation using 1-naphthol as the substrate.

Pooled human liver microsomes (figure 1D) were prepared by pooling microsomes from six human livers (male/female ratio 1:2, age range 27–62 years, one smoker) obtained from Keystone Skin Bank (Exton, PA, USA). Human kidney samples (figure 2D) were ethically obtained from pathological specimens from patients undergoing a nephrectomy. Non-pathological segments from three specimens were immediately frozen in liquid nitrogen and stored at -80°C .

Dog liver microsomes (figure 1C) were prepared from a single male beagle dog liver. Dog kidney microsomes (figure 2C) were prepared from the kidneys of two healthy male beagle dogs (2–5 years old, 10–15 kg).

UGT assays

UGT assays (except bilirubin and imipramine) were performed as described by Ethell *et al.* (1998). Briefly, 100 mM Tris/maleate buffer, pH 7.4, containing 5 mM MgCl_2 , typically 500 μM substrate, 250–350 μg microsomal or cellular sonicate, 2 mM UDPGA (0.1 μCi [^{14}C] UDPGA/assay) were combined in a total volume of 100 μl . The β -glucuronidase inhibitor, saccharic acid 1,4-lactone (10 mM), was present in all incubations so that any difference in levels of UGT activities was not a consequence of differential hydrolysis of glucuronides. Incubations were conducted at 37°C for 60 min (the reaction was shown to be linear over this time) and then terminated by the addition of 100 μl methanol prechilled to -20°C . The mixture was centrifuged for 10 min at 1000g. The resulting supernatant was then transferred to an HPLC vial with 150 μl of this volume being directly injected onto a gradient HPLC system. [^{14}C] UDPGA and glucuronide were detected using a Reeve 9701 Radioactivity Monitor fitted with 200 μl solid scintillant flowcell.

Substrate solutions were prepared and diluted on the day of assay. Typically, the range of substrate concentrations used were 10, 50, 100, 250, 500 and 1000 μM . Kinetic parameters (V_{max} , K_{m} , CL_{int}) were calculated by fitting the experimental data (duplicate incubations at six substrate concentrations) to the Michaelis-Menten equation by a non-linear least-squares regression method (Prism v.2, Graphpad Software, San Diego, CA, USA). All data have been quoted to an appropriate level of accuracy.

A colorimetric assay was used to measure bilirubin glucuronidation (Heirwegh *et al.* 1972). Imipramine glucuronidation was determined using the organic phase extraction method using [^3H] Imipramine described by Coughtrie and Sharp (1991).

Results

Glucuronidation by the liver

Sixteen compounds covering a wide range of chemical diversity and therapeutic areas were studied in pooled hepatic microsomes from marmoset and rat. V_{max} and K_{m} were determined and the results are shown in tables 1 and 2. Intrinsic clearance (CL_{int}), an indication of the inherent metabolic stability of the compounds studied, was calculated from $V_{\text{max}}/K_{\text{m}}$.

Marmoset hepatic microsomes were capable of catalysing the glucuronidation of all the compounds studied to varying extents (table 1). Interestingly, marmoset liver could glucuronidate the tertiary amine imipramine to form a quaternary

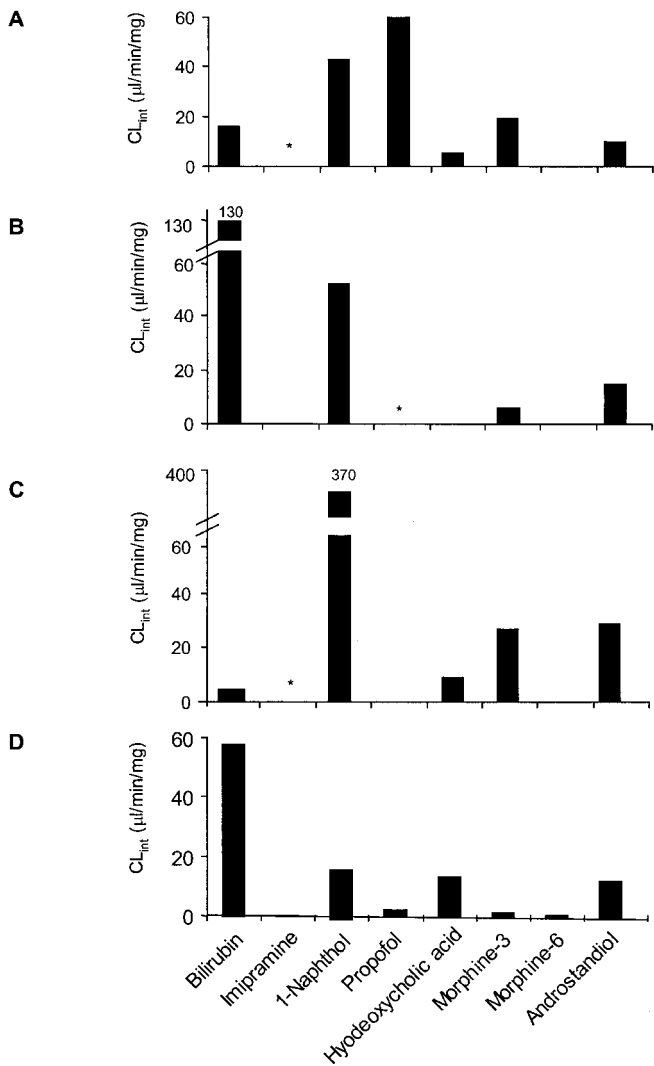


Figure 1. Comparison of CL_{int} for seven probe substrates obtained in hepatic microsomes prepared from (A) marmoset, (B) rat, (C) dog and (D) human. Marmoset and rat CL_{int}'s were determined three times, dog and human CL_{int}'s were determined on either two or three occasions. (C, D) Compiled from Soars *et al.* (2001). *Activity detectable.

ammonium glucuronide (0.05 nmol min⁻¹ mg⁻¹). However, the sensitivity of this assay did not permit accurate kinetics to be determined. In contrast, this pathway of metabolism was not detectable in rat hepatic microsomes under the assay conditions employed. Rat liver microsomes were also unable to glucuronidate codeine, ethinylestradiol, furosemide and the bile acid hydoxycholic acid (table 2), all of which were glucuronidated significantly by marmoset liver microsomes.

When glucuronidation of several substrates diagnostic for the major human UGT isoforms was compared in the livers of the common animal species (rat, dog, marmoset) and human (figure 1), it was clear that several key interspecies differences were apparent. Differences were apparent in both the rate and the route of glucuronidation.

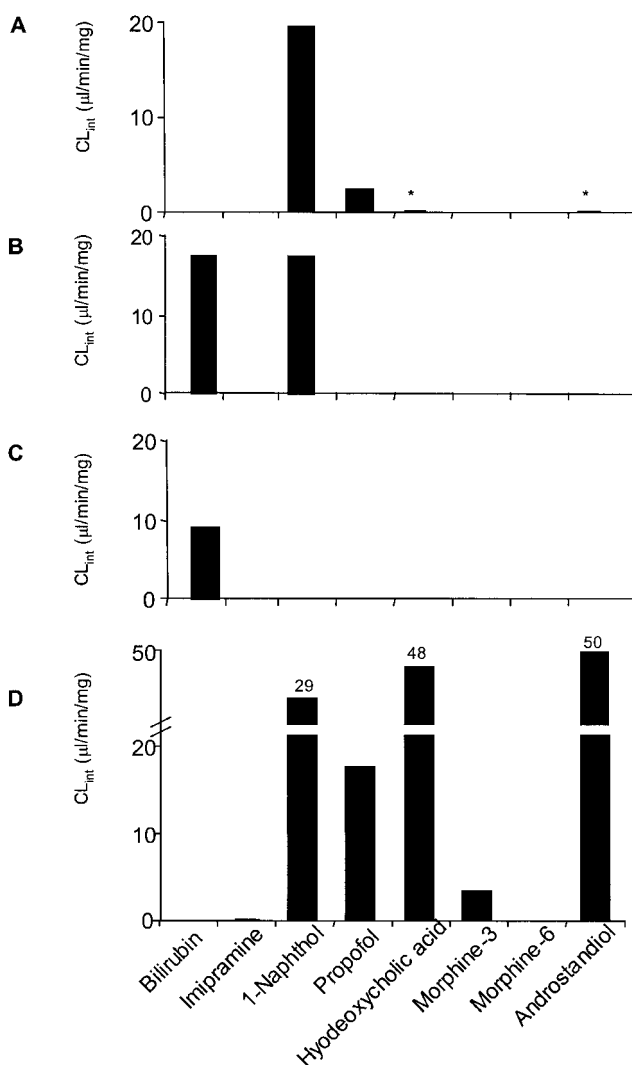


Figure 2. Comparison of CL_{int} for seven probe substrates obtained in renal microsomes prepared from (A) marmoset, (B) rat, (C) dog and (D) human. Marmoset and rat CL_{int} 's were determined three times, dog and human CL_{int} 's were determined on either two or three occasions. (C, D) Compiled from Soars *et al.* (2001). *Activity detectable.

Glucuronidation of propofol was detectable by rat liver microsomes ($0.18 \text{ nmol min}^{-1} \text{ mg}^{-1}$). Propofol glucuronidation was catalysed efficiently by human liver microsomes ($CL_{int} 2 \mu\text{L min}^{-1} \text{ mg}^{-1}$) and even more extensively by marmoset liver microsomes ($CL_{int} 60 \mu\text{L min}^{-1} \text{ mg}^{-1}$). No direct conjugation of this substrate was detectable in dog liver microsomes. Further major interspecies differences were illustrated by the comparison of morphine glucuronidation. Human hepatic microsomes catalysed the formation of both the 3- and 6-glucuronides. However, only glucuronidation at the three position was significant in microsomes prepared from the livers of rat, dog and marmoset. Trace amounts of the 6-glucuronide could be detected in the latter species by more sensitive

Table 1. Kinetic parameters for the glucuronidation of 16 compounds by marmoset liver and kidney microsomes.

Compound	Liver				Kidney			
	$V_{\max}$ (nmol min ⁻¹ mg ⁻¹)	K_m (μM)	CL _{int} (μl min ⁻¹ mg ⁻¹)		$V_{\max}$ (nmol min ⁻¹ mg ⁻¹)	K_m (μM)	CL _{int} (μl min ⁻¹ mg ⁻¹)	
Androstenediol	1.5 ± 0.22	150 ± 25	10 ± 3		*0.10 ± 0.05			
Bilirubin	0.34 ± 0.05	21 ± 0.4	16 ± 3		ND	ND		ND
Codaine	0.28 ± 0.05	450 ± 90	0.6 ± 0.07		ND	ND		ND
Ethinylestradiol	0.53 ± 0.02	130 ± 15	4.2 ± 0.4		ND	ND		ND
Furosemide	0.53 ± 0.05	240 ± 13	2.2 ± 0.3		ND	ND		ND
Gemfibrozil	1.5 ± 0.07	100 ± 5	14 ± 1		*0.09 ± 0.02			
Hydromorphone	12 ± 0.98	620 ± 130	20 ± 3		ND	ND		ND
Hydroxychohic acid	0.70 ± 0.21	140 ± 41	5.6 ± 3.6		*0.12 ± 0.06			
Imipramine	*0.05 ± 0.03				ND			
Ketoprofen	0.51 ± 0.05	740 ± 80	0.7 ± 0		ND	ND		ND
Morphine (3 gluc)	9.2 ± 0.67	510 ± 170	19 ± 6		ND	ND		ND
Morphine (6 gluc)	ND	ND	ND		ND	ND		ND
Naloxone	2.3 ± 0.03	680 ± 100	3.5 ± 0.6		*0.16 ± 0.01			
1-Naphthol	1.6 ± 0.92	380 ± 38	43 ± 5		5.7 ± 0.64	290 ± 15		20 ± 3
Naproxen	0.63 ± 0.06	1700 ± 410	0.4 ± 0.1		ND	ND		ND
Propofol	27 ± 4.3	450 ± 49	60 ± 10		2.1 ± 0.15	1000 ± 200		2.1 ± 0.3
Valproic acid	1.9 ± 0.20	5700 ± 470	0.3 ± 0.04		*0.19 ± 0.04			

Data are mean ± SD of three determinations.
Marmoset liver microsomes were prepared from the pooled livers of five marmosets.
Marmoset kidney microsomes were prepared from the pooled kidneys of five marmosets.
ND, below the level of detection (8 pmol min⁻¹mg⁻¹).
* Data are mean ± SD of three activities.

analysis using mass spectrometry (verified by authentic standards; data not shown).

Rat liver microsomes displayed a very high affinity for the endobiotic bilirubin resulting in extensive glucuronidation ($CL_{int} = 130 \mu\text{l min}^{-1} \text{mg}^{-1}$). Bilirubin was also significantly glucuronidated by both marmoset ($CL_{int} = 16 \mu\text{l min}^{-1} \text{mg}^{-1}$) and human hepatic microsomes ($CL_{int} = 58 \mu\text{l min}^{-1} \text{mg}^{-1}$). The glucuronidation of the endobiotic steroid, androstanediol, also displayed similar results between microsomes prepared from both human and marmoset livers ($CL_{int} = 13$ and $10 \mu\text{l min}^{-1} \text{mg}^{-1}$ respectively).

Glucuronidation by the kidney

The glucuronidation of the compounds was also studied in marmoset and rat kidney microsomes, and the results summarized in tables 1 and 2. The glucuronidation of several UGT substrates by the kidneys of the common animal species (rat, dog, marmoset) and humans (figure 2) was also compared. Table 2 clearly shows the limited drug glucuronidation capacity of rat kidney microsomes where only the glucuronidation of 1-naphthol and bilirubin was detected. This agreed with data obtained using dog kidney microsomes where bilirubin was the only compound glucuronidated (Soars *et al.* 2001). By contrast, the glucuronidation of a range of compounds was detectable with marmoset (table 1) and human kidney microsomes (figure 2). 1-Naphthol was extensively glucuronidated by microsomes prepared from marmoset kidney ($CL_{int} = 20 \mu\text{l min}^{-1} \text{mg}^{-1}$) and propofol was glucuronidated to a significant level ($CL_{int} = 2 \mu\text{l min}^{-1} \text{mg}^{-1}$). Androstanediol, gemfibrozil, hyodeoxycholic acid, naloxone and valproic acid were also glucuronidated, albeit to a limited extent, in marmoset renal microsomes (figure 2).

Discussion

Glucuronidation is a major Phase II process responsible for the detoxification of a wide range of xenobiotics and endogenous compounds. Non-primate species such as rat and dog are commonly used as animal models for early preclinical pharmacokinetic studies. Primates are often used in metabolism and safety assessment studies when metabolic pathways differ markedly between the lower laboratory animal and human. Traditionally, cynomolgus and rhesus monkeys have been used for pharmacokinetic and pharmacodynamic studies. However, there has been a recent trend towards using the marmoset as a simian model. Therefore, a knowledge of glucuronidation in the marmoset would aid the development of new drugs. The data in this study have shown that marmoset liver microsomes can glucuronidate a wide range of compounds known to be glucuronidated by human liver microsomes (Soars *et al.* 2001). The majority of the drugs studied exhibited a greater *in vitro* CL_{int} in marmoset hepatic microsomes than those determined previously in human liver microsomes (Soars *et al.* 2001). This quantitative difference agrees with the preliminary findings of Sharer *et al.* (1995), who reported paracetamol glucuronidation to be six to eight times greater in cynomolgus and rhesus monkey compared with human.

Although there exists this general quantitative difference in the rate of hepatic drug glucuronidation between marmoset and human liver microsomes there were many similarities observed in the glucuronidation of specific compounds. The

Table 2. Kinetic parameters for the glucuronidation of 16 compounds by rat liver and kidney microsomes.

Compound	Liver			Kidney		
	$V_{\max}$ (nmol min ⁻¹ mg ⁻¹)	K_m (μM)	CL _{int} (μl min ⁻¹ mg ⁻¹)	$V_{\max}$ (nmol min ⁻¹ mg ⁻¹)	K_m (μM)	CL _{int} (μl min ⁻¹ mg ⁻¹)
Androstenediol	1.8 ± 0.08	120 ± 21	15 ± 2.3	ND	ND	ND
Bilirubin	1.1 ± 0.08	8.8 ± 2.4	130 ± 26	0.29 ± 0.04	17 ± 1.0	17 ± 3.4
Codeine	ND	ND	ND	ND	ND	ND
Ethinylestradiol	ND	ND	ND	ND	ND	ND
Furosemide	ND	ND	ND	ND	ND	ND
Gemfibrozil	2.0 ± 0.55	91 ± 28	24 ± 11	ND	ND	ND
Hydromorphone	0.82 ± 0.09	560 ± 39	1.5 ± 0.1	ND	ND	ND
Hydroxycholeic acid	ND	ND	ND	ND	ND	ND
Imipramine	ND	ND	ND	ND	ND	ND
Ketoprofen	0.50 ± 0.06	650 ± 190	0.8 ± 0.2	ND	ND	ND
Morphine (3 gluc)	2.1 ± 0.19	340 ± 37	6.3 ± 0.3	ND	ND	ND
Morphine (6 gluc)	ND	ND	ND	ND	ND	ND
Naloxone	1.4 ± 0.22	500 ± 76	2.8 ± 0.01	ND	ND	ND
1-Naphthol	29 ± 3.4	630 ± 280	52 ± 21	0.74 ± 0.11	44 ± 16	18 ± 3.4
Naproxen	0.59 ± 0.13	760 ± 57	0.8 ± 0.2	ND	ND	ND
Propofol	*0.18 ± 0.03			ND	ND	ND
Valproic acid	2.1 ± 0.10	490 ± 35	4.2 ± 0.1	ND	ND	ND

Data are mean ± SD of three determinations.
Rat liver microsomes were prepared from the pooled livers of four Sprague-Dawley rats.
Rat kidney microsomes were prepared from the pooled kidneys of four Sprague-Dawley rats.
ND, below the level of detection (8 pmol min⁻¹ mg⁻¹).
* Data are mean ± SD of three activities.

glucuronidation of androstenediol by marmoset hepatic microsomes was similar to that by human liver microsomes ($CL_{int} = 10$ and $13 \mu\text{l min}^{-1} \text{mg}^{-1}$ respectively). Interestingly the K_m for this reaction in marmoset and human liver microsomes was also comparable ($150 \mu\text{M}$ in marmoset, $175 \mu\text{M}$ in human; Soars *et al.* 2001) suggesting a similar affinity in both species for this endobiotic steroid. These *in vitro* results concur with previous studies by Guillemette *et al.* (1996), who reported that cynomolgus monkey and humans are unique in their high levels of circulating androstenediol glucuronide. The cloning of simian UGT2B20 (Barbier *et al.* 1999), believed to be the orthologue of human UGT2B15, further suggested a conserved pathway for steroid metabolism between the monkey and humans.

The ability of marmoset liver microsomes to glucuronidate the tertiary amine, imipramine to form a quaternary ammonium glucuronide confirmed the findings of Tsai and Gorrod (1999), who described the formation of nicotine glucuronide using marmoset hepatic microsomes. The ability to glucuronidate tertiary amines enhances the use of the marmoset (as an animal model for glucuronidation in humans) as an alternative to most laboratory animals which have been shown to be unable to catalyse this route of metabolism (Hucker *et al.* 1978), confirmed here using rat liver microsomes (table 2).

Another compound glucuronidated significantly in microsomes prepared from both marmoset and human liver (figure 1) was the anaesthetic propofol. Interestingly propofol, known to be metabolized in human by UGT1A9 (Ebner *et al.* 1993), was not directly glucuronidated by dog liver microsomes (figure 1) and glucuronidated at a lower rate by rat liver microsomes (table 2). Although the major UGT isoforms present in the rat have been extensively studied, to date no rat UGT1A9 orthologue has been isolated suggesting that glucuronidation of propofol by the rat may be catalysed by an alternative isoform. The species differences in propofol glucuronidation seen between the lower laboratory animals and humans therefore suggest that they may not be good models for drugs known to be metabolized by human UGT1A9.

In contrast to the similarities in glucuronidation observed above between marmoset and human hepatic microsomes, the glucuronidation of the opioid, analgesic morphine represented a clear interspecies difference. Marmoset liver microsomes were only capable of catalysing the glucuronidation of morphine at the 3-position to any significant degree (table 1), whereas human liver microsomes could form glucuronides at both the 3- and 6-positions (figure 1). Green *et al.* (1997) showed the cynomolgus monkey capable of glucuronidating morphine at both the 3- and 6-positions, a process catalysed by monkey UGT2B9. Rat hepatic microsomes were also shown to glucuronidate morphine almost exclusively at the 3-position (table 2), which agrees with Oguri *et al.* (1990). Morphine glucuronidation was shown to be catalysed in rat by UGT2B1 (Pritchard *et al.* 1994).

Although the liver is widely regarded as the main metabolic organ in man, the kidney has been established as an extrahepatic tissue with the capacity to glucuronidate a wide range of drugs (McGurk *et al.* 1998, Soars *et al.* 2001). Rat kidney microsomes have been shown only to glucuronidate bilirubin and 1-naphthol and therefore lack the drug glucuronidation potential of human kidney microsomes (figure 2). A similar situation was seen in dog kidney microsomes where no drug glucuronidation was evident (figure 2).

In contrast to rat and dog, marmoset kidney microsomes catalyse the glucuronidation of a range of substrates. Although only 1-naphthol and propofol were

glucuronidated extensively enough to obtain CL_{int} estimates activity towards substrates such as hydoxycholeic acid and androstanediol was also detectable. This suggested that the marmoset kidney had the highest glucuronidation capacity compared with the other animal species studied. Interestingly, marmoset renal microsomes did not catalyse the glucuronidation of bilirubin, which agrees with the lack of activity seen towards bilirubin in human kidney microsomes (figure 2). Sutherland *et al.* (1993) showed that the lack of bilirubin activity in human kidney microsomes was due to the lack of UGT1A1 expression in this organ. The lack of bilirubin activity in marmoset kidney may also be due to the lack of expression of a UGT responsible for bilirubin glucuronidation. However, further work must be done for this to be confirmed. It is tempting to speculate that dietary or other evolutionary pressures have eliminated the need for glucuronidation of such endobiotics by the kidney in these species, as postulated for the CYP family of enzymes (Nelson and Strobel 1987).

The present paper has compared and contrasted hepatic and renal glucuronidation in the marmoset and man. When evaluating the relevance of drug glucuronidation in marmoset, other commonly used laboratory species have also been considered. Although a significant amount of work has been previously performed on individual recombinant UGT isoforms in rat, this paper has extensively studied drug glucuronidation in microsomes from both the rat liver and kidney. *In vitro* data suggest that the marmoset may be a useful alternative animal model for human drug glucuronidation, especially when the rate and pathway of drug glucuronidation are known to differ significantly between lower laboratory species and humans. The significant extrahepatic glucuronidation observed in the marmoset in comparison with other animals further merits its use for preclinical pharmacokinetic and safety evaluation studies. Although *in vitro* data play a crucial role in the development of novel chemical entities, further work should be performed to confirm that these observations accurately represent the situation *in vivo*.

Acknowledgements

The authors thank Drs Bridget Sutton and Cathy Temple (Roche Discovery Welwyn) for kindly providing the marmoset tissue in this study. This work was funded by AstraZeneca, Roche and The Wellcome Trust.

References

- BARBIER, O., BELANGER, A. and HUM, D. W., 1999, Cloning and characterization of a simian UDP-glucuronosyltransferase enzyme UGT2B20, a novel C₁₉ steroid-conjugating protein. *Biochemical Journal*, **337**, 567–574.
- BELANGER, G., BARBIER, O., HUM, D. W. and BELANGER, A., 1999, Molecular cloning, expression and characterization of a monkey steroid UDP-glucuronosyltransferase, UGT2B19, that conjugates testosterone. *European Journal of Biochemistry*, **260**, 701–708.
- BERTALMO, A. J., MEDZIHRADESKY, F., WINGER, G. and WOODS, J. H., 1992, Differential influence of N-dealkylation on the stimulus properties of some opioid agonists. *Journal of Pharmacology and Experimental Therapeutics*, **261**, 278–284.
- BERTZ, R. J. and GRANNEMAN, G. R., 1997, Use of *in vitro* and *in vivo* data to estimate the likelihood of metabolic pharmacokinetic interactions. *Clinical Pharmacokinetics*, **32**, 210–258.
- BOCK, K. W., FORSTER, A., GSCHLIDMEIER, H., BRUCK, M., MUNZEL, P., SCHARECK, W., FOURNEL GIGLEUX, S. and BURCHELL, B., 1993, Paracetamol glucuronidation by recombinant rat and human phenol UDP-glucuronosyltransferases. *Biochemical Pharmacology*, **45**, 1809–1814.

- COFFMAN, B. L., GREEN, M. D., KING, C. D. and TEPHLY, T. R., 1995, Cloning and stable expression of a cDNA encoding a rat liver UDP-glucuronosyltransferase (UDP-glucuronosyltransferase 1. 1) that catalyses the glucuronidation of opioids and bilirubin. *Molecular Pharmacology*, **47**, 1101–1105.
- COUGHTRIE, M. W. H., BURCHELL, B. and BEND, J. R., 1987, Purification and properties of rat kidney UDP-glucuronosyltransferase. *Biochemical Pharmacology*, **36**, 245–251.
- COUGHTRIE, M. W. H. and SHARP, S., 1991, Glucuronidation of imipramine in rabbit and human liver microsomes: assay conditions and interaction with other tertiary amine drugs. *Biochemical Pharmacology*, **42**, 1497–1501.
- DUTTON, G. J., 1980, *Glucuronidation of Drugs and Other Compounds* (Boca Raton: CRC Press).
- EBNER, T., REMMEL, R. P. and BURCHELL, B., 1993, Human bilirubin UDP-glucuronosyltransferase catalyses the glucuronidation of ethinylestradiol. *Molecular Pharmacology*, **43**, 649–654.
- EL MOUELHI, M. and BOCK, K. W., 1991, Stereoselective (S)- and (R)-naproxen glucuronosyltransferases of rat liver. *Drug Metabolism and Disposition*, **19**, 304–308.
- ETHELL, B. T., ANDERSON, G., BEAUMONT, K., RANCE, D. J. and BURCHELL, B., 1998, A universal radiochemical high performance liquid chromatographic assay for the determination of UDP-glucuronosyltransferase activity. *Analytical Biochemistry*, **225**, 142–147.
- FRANCE, C. P. and WOODS, J. H., 1989, Discriminative stimulus effects of naltrexone in morphine-treated rhesus monkeys. *Journal of Pharmacology and Experimental Therapeutics*, **250**, 937–943.
- GREEN, M. D., BELANGER, G., HUM, D. W., BELANGER, A. and TEPHLY, T., 1997, Glucuronidation of opioids, carboxylic acid-containing drugs, and hydroxylated xenobiotics catalysed by expressed monkey UDP-glucuronosyltransferase 2B9 protein. *Drug Metabolism and Disposition*, **25**, 1389–1394.
- GREEN, M. D., CLARKE, D. J., OTURU, E. M., STYCZYNSKI, P. B., JACKSON, M. R., BURCHELL, B. and TEPHLY, T. R., 1995, Cloning and expression of a rat liver phenobarbital-inducible UDP-glucuronosyltransferase (2B12) with specificity for monoterpenoid alcohols. *Archives of Biochemistry and Biophysics*, **322**, 460–468.
- GUILLEMETTE, C., HUM, D. W. and BELANGER, A., 1996, Levels of plasma C₁₉ steroids and 5 α -reduced C₁₉ steroid glucuronides in primates, rodents, and domestic animals. *American Journal of Physiology*, **271**, E348–353.
- HEIRWEGH, K. P., VAN DE VIJVER, M. and FEVERY, J., 1972, Assay and properties of digitonin-activated bilirubin uridine diphosphate glucuronyltransferase from rat liver. *Biochemical Journal*, **129**, 605–618.
- HOUSTON, J. B., 1994, Utility of *in vitro* drug metabolism data in predicting *in vivo* metabolic clearance. *Biochemical Pharmacology*, **47**, 1469–1479.
- HUCKER, H. B., STAUFFER, S. C., BALLETO, A. J., WHITE, S. D., ZACCHEI, A. G. and ARISON, B. H., 1978, Physiological disposition and metabolism of cyclobenzaprine in the rat, dog, rhesus monkey, and man. *Drug Metabolism and Disposition*, **6**, 659–672.
- KOSTER, A. S., SCHIRMER, G. and BOCK, K. W., 1986, Immunochemical and functional characterization of UDP-glucuronosyltransferases from rat liver, intestine and kidney. *Biochemical Pharmacology*, **35**, 3971–3975.
- LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. and RANDALL, R. J., 1951, Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry*, **193**, 265–275.
- MACKENZIE, P. I., OWENS, I. S., BURCHELL, B., BOCK, K. W., BAIROCH, A., BELANGER, A., FOURNEL GIGLEUX, S., GREEN, M., HUM, D. W., IYANAGI, T., LANCET, D., LOUISOT, P., MAGDALOU, J., CHOWDHURY, J. R., RITTER, J. K., SCHACHTER, H., TEPHLY, T. R., TIPTON, K. F. and NEBERT, D. W., 1997, The UDP glycosyltransferase gene superfamily: recommended nomenclature based on evolutionary divergence. *Pharmacogenetics*, **7**, 255–269.
- MCGURK, K. A., BRIERLEY, C. H. and BURCHELL, B., 1998, Drug glucuronidation by human renal UDP-glucuronosyltransferases. *Biochemical Pharmacology*, **55**, 1005–1012.
- NELSON, D. R. and STROBEL, H. W., 1987, Evolution of cytochrome P-450 proteins. *Molecular Biology and Evolution*, **4**, 572–593.
- OGURI, K., HANIOKA, N. and YOSHIMURA, H., 1990, Species differences in metabolism of codeine: urinary excretion of codeine glucuronide, morphine-3-glucuronide and morphine-6-glucuronide in mice, rats, guinea pigs and rabbits. *Xenobiotica*, **20**, 683–688.
- PRITCHARD, M., FOURNELGIGLEUX, S., SIEST, G., MACKENZIE, P. and MAGDALOU, J., 1994, A recombinant phenobarbital-inducible rat liver UDP-glucuronosyltransferase (UDP-glucuronosyltransferase 2B1) stably expressed in V79 cells catalyses the glucuronidation of morphine, phenols, and carboxylic acids. *Molecular Pharmacology*, **45**, 42–50.
- SHARER, J. E., SHIPLEY, L. A., VANDENBRANDEN, M. R., BINKLEY, S. N. and WRIGHTON, S. A., 1995, Comparisons of phase I and phase II *in vitro* hepatic enzyme activities of human, dog, rhesus monkey, and cynomolgus monkey. *Drug Metabolism and Disposition*, **23**, 1231–1241.
- SOARS, M. G., RILEY, R. J., FINDLAY, K. A. B., COFFEY, M. J. and BURCHELL, B., 2001, Evidence for significant differences in microsomal drug glucuronidation by canine and human liver and kidney. *Drug Metabolism and Disposition*, **29**, 121–126.

- STRASSBURG, C. P., KNEIP, S., TOPP, J., OBERMAYERSTRAUB, P., BARUT, A., TUKEY, R. H. and MANNS, M. P., 2000, Polymorphic gene expression and interindividual variation of UDP-glucuronosyltransferase activity in human small intestine. *Journal of Biological Chemistry*, **275**, 36164–36171.
- SUTHERLAND, L., EBNER, T. and BURCHELL, B., 1993, The Expression of UDP-glucuronosyltransferases of the UGT1 family in human liver and kidney in response to drugs. *Biochemical Pharmacology*, **45**, 295–301.
- TSAI, M. C. and GORROD, J. W., 1999, Evidence for the biosynthesis of a glucuronide conjugate of (*S*)-(–)-nicotine but not (*S*)-(–)-cotinine or ($\pm$)-*trans*-3'-hydroxycotinine by marmoset hepatic microsomes. *Drug Metabolism and Drug Interactions*, **15**, 223–237.
- WINGER, G., SKJOLDAGER, P. and WOODS, J. H., 1992, Effects of buprenorphine and other opioid agonists and antagonists on alfentanil- and cocaine-reinforced responding in rhesus monkeys. *Journal of Pharmacology and Experimental Therapeutics*, **261**, 311–317.

Evaluation of 7-benzyloxy-4-trifluoromethylcoumarin, some other 7-hydroxy-4-trifluoromethylcoumarin derivatives and 7-benzyloxyquinoline as fluorescent substrates for rat hepatic cytochrome P450 enzymes

A. B. RENWICK†, G. LAVIGNETTE‡, P. D. WORBOYS§, B. WILLIAMS§, D. SURRY§, D. F. V. LEWIS¶, R. J. PRICE†, B. G. LAKE†* and D. C. EVANS||

†TNO BIBRA International Ltd, Woodmansterne Road, Carshalton SM5 4DS, UK

‡ENS BANA, Université de Bourgogne, Campus Universitaire, 1 Esplanade Erasme, F-21100 Dijon, France

§Merck Sharp and Dohme Ltd, Neuroscience Research Centre, Terlings Park, Eastwick Road, Harlow CM20 2QR, UK

¶School of Biomedical and Life Sciences, University of Surrey, Guildford GU2 5XH, UK

||Drug Metabolism, Merck & Co., 126 East Lincoln Avenue, Rahway, NJ 07065, USA

Received 11 April 2001

1. The aim of this study was to evaluate a number of derivatives of 7-hydroxy-4-trifluoromethylcoumarin (HFC) and 7-benzyloxyquinoline (7BQ) as novel fluorescent substrates for monitoring rat hepatic cytochrome P450 (CYP) enzyme specificity in a 96-well plate format. The HFC derivatives examined comprised 7-benzyloxy-4-trifluoromethylcoumarin (BFC), 2,5-bis(trifluoromethyl)-7-benzyloxy-4-trifluoromethylcoumarin (BFBFC), 3,5-bis(trifluoromethyl)-7-benzyloxy-4-trifluoromethylcoumarin (BTBFC), 2-(trifluoromethyl)-7-benzyloxy-4-trifluoromethylcoumarin (2TFBFC), 3-(trifluoromethyl)-7-benzyloxy-4-trifluoromethylcoumarin (3TFBFC) and 3-(trifluoromethoxy)-7-benzyloxy-4-trifluoromethylcoumarin (3TFMeOBFC).

2. The CYP specificity of the fluorescent probe substrates was examined using characterized liver microsomes from male Sprague–Dawley rats treated with β -naphthoflavone (BNF), sodium phenobarbitone (NaPB), isoniazid, pregnenolone-16 α -carbonitrile (PCN), dexamethasone (DEX) and methylclofenapate to induce CYP1A, CYP2B, CYP2E, CYP3A, CYP3A and CYP4A forms, respectively. Studies were also performed with microsomes from baculovirus-infected insect cells containing rat cDNA-expressed CYP1A1, CYP1A2, CYP2B1, CYP3A1 and CYP3A2.

3. BFC metabolism was most markedly induced by BNF and NaPB, whereas BFBFC metabolism was most markedly induced by PCN and DEX and BTBFC was not metabolized by rat liver microsomes. BFC was a high-affinity substrate for cDNA-expressed CYP1A1 and CYP2B1, whereas BFBFC exhibited a high affinity for CYP3A1 and CYP3A2.

4. The metabolism of 2TFBFC and 3TFBFC was induced by NaPB, PCN and DEX. 3TFBFC was a relatively specific substrate for cDNA-expressed CYP2B1, whereas 2TFBFC could be metabolized by CYP2B1, CYP3A1 and CYP3A2.

5. 3TFMeOBFC metabolism was markedly induced by BNF treatment and 3TFMeOBFC was extensively metabolized by cDNA-expressed CYP1A1.

6. The metabolism of 7BQ to 7-hydroxyquinoline was induced by treatment with PCN and DEX. 7BQ was a substrate for cDNA-expressed CYP3A2 and to a lesser extent for CYP3A1.

* Author for correspondence; e-mail: blake@tnobibra.co.uk

7. In summary, some of the HFC derivatives studied and 7BQ are useful fluorescent probe substrates for rat CYP enzymes. BFC appears to be a probe for CYP1A and CYP2B, 2TFBFC for CYP2B and CYP3A and 3TFBFC for CYP2B. While 3TFMeOBFC appears to be a relatively specific probe for CYP1A1, both BFBFC and 7BQ are good probes for the induction of CYP3A.

Introduction

Cytochrome P450 (CYP) forms comprise a superfamily of haemoproteins and the mammalian enzymes have a major role in the oxidative metabolism of both xenobiotics and certain endogenous compounds (Conney 1986, Nelson *et al.* 1996, Parkinson 1996). In recent years, much progress has been made in the development of *in vitro* tools that can be used by the pharmaceutical industry in the development of new therapeutic agents. For example, human liver microsomes, hepatocytes, liver slices and cDNA-expressed CYP forms can be employed to elucidate the pathways of metabolism of new drugs and to help to predict drug-drug interactions (Wrighton *et al.* 1993, Crespi *et al.* 1997, 1998, Lake 1997, Clarke 1998, Rodrigues 1999). Rapid screening for CYP inhibition can be performed in a 96-well plate format using either liver microsomes or cDNA-expressed CYP forms and substrates that are metabolized to highly fluorescent products (Crespi *et al.* 1997, 1998, Chauret *et al.* 1999). *In vitro* techniques can also be employed to ascertain whether a new therapeutic agent has the potential to induce CYP enzymes. For example, rodent and human hepatocytes can be cultured in a 96-well plate format and induction of CYP forms monitored with substrates that are metabolized to fluorescent products (Donato *et al.* 1993, Gómez-Lechón *et al.* 1997, Price *et al.* 2000). While the induction of CYP forms in humans can lead to alterations in the pharmacological effects of administered therapeutic agents (Parkinson 1996, Lin and Lu 1998, Pelkonen *et al.* 1998, Tanaka 1998), CYP induction can also be important in preclinical studies. For example, several non-genotoxic CYP2B, CYP3A and CYP4A inducers have been shown to produce liver tumours in rodents (Nims *et al.* 1987, Grasso and Hinton 1991, Lake 1995, Whysner *et al.* 1996).

One requirement for conducting rapid CYP inhibition and induction studies is the availability of suitable probe substrates for the CYP enzymes of interest. In this respect, substrates metabolized to highly fluorescent products are more suitable for rapid screens than substrates which require chromatographic separation of metabolites before quantification by spectroscopic, radiometric or mass spectrometry techniques. With respect to rat liver, the resorufin derivatives 7-ethoxyresorufin (7ER) and 7-methoxyresorufin may be used as fluorescent probes for induction of CYP1A forms, whereas 7-benzoyloxyresorufin (7BR) and 7-pentoxeresorufin are useful probes for the induction of CYP2B forms (Burke *et al.* 1985, 1994, Lubet *et al.* 1990, Nerurkar *et al.* 1993, Nims and Lubet 1996). Other compounds that have been employed as fluorescent probe substrates for rat CYP forms include 7-alkoxycoumarins, 7-alkoxyquinolines, 3-cyano-7-ethoxycoumarin and derivatives of 7-hydroxy-4-trifluoromethylcoumarin (HFC) such as 7-benzoyloxy-4-trifluoromethylcoumarin (BFC) and 7-ethoxy-4-trifluoromethylcoumarin (DeLuca *et al.* 1988, White 1988, Mayer *et al.* 1990, Buters *et al.* 1993, Price *et al.* 2000). However, while the specificity of certain resorufin derivatives for CYP1A and CYP2B forms is well established, no compounds have

been reported to be specific fluorescent probe substrates for induction of rat hepatic CYP3A forms.

The aim of the present study was to evaluate a number of HFC derivatives as novel fluorescent substrates for rat hepatic CYP forms employing a 96-well plate format. The HFC derivatives examined comprised BFC, 2,5-bis(trifluoromethyl)-7-benzyloxy-4-trifluoromethylcoumarin (BFBFC), 3,5-bis(trifluoromethyl)-7-benzyloxy-4-trifluoromethylcoumarin (BTBFC), 2-(trifluoromethyl)-7-benzyloxy-4-trifluoromethylcoumarin (2TFBFC), 3-(trifluoromethyl)-7-benzyloxy-4-trifluoromethylcoumarin (3TFBFC), 2-(trifluoromethoxy)-7-benzyloxy-4-trifluoromethylcoumarin (2TFMeOBFC) and 3-(trifluoromethoxy)-7-benzyloxy-4-trifluoromethylcoumarin (3TFMeOBFC). In addition, studies were also conducted with 7-benzyloxyquinoline (7BQ), which is known to be metabolized to 7-hydroxyquinoline (7HQ) by rat and human liver microsomes (Mayer *et al.* 1990, Renwick *et al.* 2001). The rat CYP enzyme specificity of the fluorescent probes studied (for structures, see figure 1) was determined by studies with characterized liver microsomal preparations from rats treated with some known inducers of CYP1A, CYP2B, CYP2E, CYP3A and CYP4A forms and with cDNA-expressed rat CYP1A, CYP2B and CYP3A forms.

Materials and methods

Materials

Sodium phenobarbitone (NaPB) was purchased from BDH Chemicals (Poole, UK), methylclofenapate (MCP) from Lancaster Synthesis (Morecambe, UK) and β -naphthoflavone (BNF), dexamethasone (DEX), isoniazid (ISN), pregnenolone-16 α -carbonitrile (PCN), palmitoyl-CoA, 4-nitrophenol, testosterone, resorufin, 7BR, 7ER, Tris and enzyme cofactors from Sigma-Aldrich Co. Ltd (Poole, UK). BFC, HFC, 7BQ and 7HQ were obtained from Salford Ultrafine Chemicals and Research Ltd (Manchester, UK), whereas BFBFC (L-871,408), BTBFC (L-871,420), 2TFBFC (L-871,397), 3TFBFC (L-871,399), 2TFMeOBFC (L-855,098) and 3TFMeOBFC (L-871,407) were kindly provided by MSD Research Laboratories (Harlow, UK). Microsomes from baculovirus-infected insect (BTI-TN-5B1-4) cells containing cDNA-expressed rat CYP forms (GENTEST Corp., Woburn, MA, USA) were obtained from Cambridge Bioscience (Cambridge, UK) and stored at -80°C . The samples of baculovirus-infected insect cell microsomes comprised CYP1A1 + rat NADPH-cytochrome P-450 reductase (OR), CYP1A2 + rat OR, CYP2B1 + human OR + human cytochrome b5 (cyt-b5), CYP3A1 + rat OR + human cyt-b5 and CYP3A2 + rat OR + human cyt-b5.

Animals and treatment

Male Sprague-Dawley rats were obtained from Harlan Olac (Bicester, UK) and allowed free access to R&M No. 1 laboratory animal diet (Special Diets Services, Witham, UK) and water. The animals were housed in groups of two to four in mesh-floored cages in accommodation maintained at $22 \pm 3^{\circ}\text{C}$ with relative humidity of 40–70% and allowed to acclimatize to these conditions for at least 1 week before the study began. Rats (210–225 g body weight) were treated with $100 \text{ mg kg}^{-1} \text{ day}^{-1}$ of either NaPB, BNF, PCN or ISN, $75 \text{ mg kg}^{-1} \text{ day}^{-1}$ MCP and $50 \text{ mg kg}^{-1} \text{ day}^{-1}$ DEX by daily oral gavage for 4 days. Control animals received corresponding quantities (5 ml kg^{-1} body weight) of the 0.56% (w/v) gum tragacanth or corn oil vehicles. Gum tragacanth was used as the vehicle for NaPB, PCN, DEX and ISN and corn oil for BNF and MCP. Animals were killed by exposure to carbon dioxide gas followed by cervical dislocation and the livers immediately excized for biochemical studies.

Biochemical investigations

Whole liver homogenates ($0.25 \text{ g fresh tissue ml}^{-1}$) were prepared in ice-cold 0.154 M KCl containing 50 mM Tris-HCl, pH 7.4, using a Potter-type, Teflon-glass, motor-driven homogenizer (A. H. Thomas, Philadelphia, PA, USA). Liver-washed microsomal fractions were prepared by differential centrifugation and assayed for total CYP content (Lake 1987). Whole homogenate and microsomal protein content was determined by the method of Lowry *et al.* (1951), employing bovine serum albumin as standard. Aliquots of whole homogenate and washed microsomes were stored at -80°C . Liver whole homogenate palmitoyl-CoA oxidation activity was determined as described by Gray *et al.* (1983), but with the

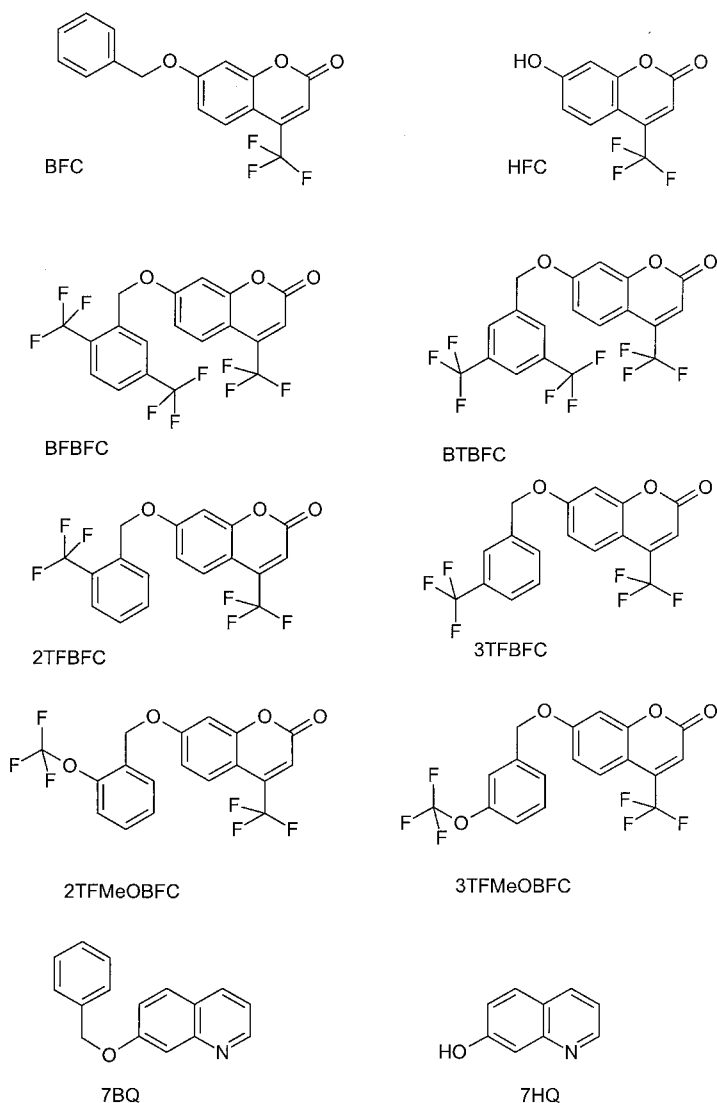


Figure 1. Structures of BFC, HFC, HFC derivatives, 7BQ and 7HQ.

addition of 10 μM FAD to each cuvette. Liver microsomes were assayed for 7-ethoxyresorufin *O*-deethylase, 7-benzyloxyresorufin *O*-debenzylase and 4-nitrophenol hydroxylase activities employing substrate concentrations of 2, 10 and 300 μM , respectively, as described (Japenga *et al.* 1993, Lake 1987). Microsomal testosterone metabolism to 7 α -, 6 β - and 16 β -hydroxytestosterones and androstenedione was determined in incubation mixtures containing 250 μM [4- ^{14}C]testosterone (0.5 $\mu\text{Ci}/\text{tube}$), 1 mM NADPH, 0.5 mg microsomal protein and 50 mM Tris-HCl buffer, pH 7.4, in a final volume of 1 ml. [4- ^{14}C]-testosterone metabolites were extracted and separated by high-performance liquid chromatography as described by Sonderfan *et al.* (1987).

Metabolism of BFC and other HFC derivatives by liver microsomes

Incubations were performed in Nunc MicrowellTM (Life Technologies Ltd, Paisley, UK) polystyrene plates with conical wells. The washed microsomal fractions were diluted with 0.154 M KCl containing 50 mM Tris-HCl buffer, pH 7.4, to a protein concentration of 0.017–1.0 mg ml⁻¹. Stock 0.25, 1.0, 2.5 and 25 mM solutions of BFC were prepared in DMSO and were diluted 100 $\times$ with 100 mM phosphate buffer, pH 7.4, so that the final DMSO concentration in the incubations was 0.1% (v/v).

Incubation mixtures contained either 0.25, 1.0, 2.5 or 25 μM BFC (i.e. 10 μl of the appropriate diluted BFC substrate solution), 30 μl diluted microsomal preparation (0.5–30 μg protein) and 50 μl 100 mM phosphate buffer, pH 7.4. For each BFC substrate concentration, microsomal protein concentration and incubation time, four wells were incubated with NADPH (tests) and two wells without NADPH (blanks). After mixing the plates on a gyratory shaker, the plates were pre-incubated for 5 min in a 37°C incubator. The reaction was then initiated by the addition of 1 mM (final concentration) NADPH. After mixing the plates on a gyratory shaker, they were incubated at 37°C for 5–40 min and were terminated by the addition of 100 μl ice-cold 0.25 M Tris-base in 60% (v/v) acetonitrile. After the addition of NADPH to the blank wells, the plates were centrifuged at 260g for 10 min at 4°C. A 150- μl aliquot was removed from each well into a white Porvair 96-well plate (Perkin Elmer Ltd, Beaconsfield, UK) and the fluorescence was determined with a Perkin Elmer model LS-50B luminescence spectrophotometer equipped with a Perkin Elmer model LS-50 plate reader. Fluorescence measurements were made with excitation and emission wavelengths of 410 and 510 nm, respectively, employing 3-nm slits. Standard curves were constructed with 0–250 pmol HFC per well, the standard being added to phosphate buffer and taken through the procedure described above. For the six other HFC derivatives, namely BFBFC, BTBFC, 2TFBFC, 3TFBFC, 2TFMeOBFC and 3TFMeOBFC, the enzyme assay procedures were the same as for BFC, except that substrate concentrations of 0.25, 2.5 and 25 μM were employed.

Metabolism of 7BQ by liver microsomes

The metabolism of 7BQ was performed as described above for BFC employing substrate concentrations of 0.25, 1.0, 2.5 and 25 μM . Incubations were terminated with 100 μl ice-cold 0.1 M Tris-HCl buffer, pH 7.8, in 60% (v/v) acetonitrile. The plates were processed as described above for BFC. Fluorescence measurements were made with excitation and emission wavelengths of 410 and 510 nm respectively, employing 7-nm slits. Standard curves were constructed with 0–500 pmol 7HQ per well.

Metabolism of BFC and other HFC derivatives by cDNA-expressed rat CYP forms

Incubations were performed in Nunc Micro WellTM polystyrene 96-well plates with conical wells. For each of the cDNA-expressed CYP preparations (i.e. CYP1A1, CYP1A2, CYP2B1, CYP3A1 and CYP3A2), each substrate concentration and each incubation time, four test wells and one blank well (without microsomal protein) were incubated. Incubation mixtures containing 1, 2.5 and 25 μM BFC (final DMSO concentration 0.1% v/v), 1 mM NADPH and 100 mM phosphate buffer, pH 7.4, were pre-incubated for 5 min at 37°C. The reaction was initiated with 1–4 pmol of each of the cDNA-expressed CYP preparations (30 μl prepared in 100 mM phosphate buffer) and incubations terminated after 2.5–15 min and processed as described above for BFC. For the four other HFC derivatives studied, namely BFBFC, 2TFBFC, 3TFBFC and 3TFMeOBFC, the enzyme assay procedures were the same as for BFC, except that substrate concentrations of 0.25, 2.5 and 25 μM were employed.

Metabolism of 7BQ by cDNA-expressed rat CYP forms

The metabolism of 7BQ was performed as described above for BFC employing substrate concentrations of 1, 2.5 and 25 μM . Incubations were terminated with 100 μl of ice-cold 0.1 M Tris-HCl buffer, pH 7.8, in 60% (v/v) acetonitrile. The plates were processed as described above for the liver microsome 7BQ metabolism studies.

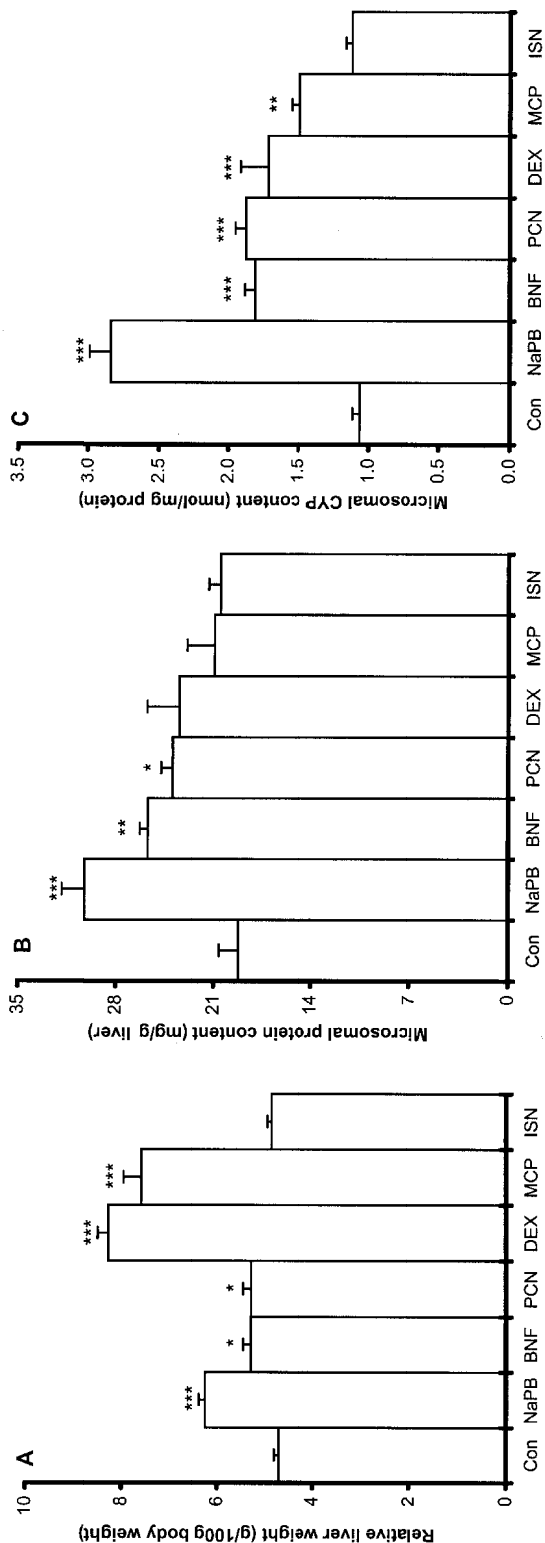
Statistical analysis

Statistical evaluation of data was performed by one-way analysis of variance. Comparisons between means were made using the least significant difference test.

Results

Characterization of liver microsomes from rats treated with selected CYP inducers

Initial studies were performed to characterize liver microsomes from rats treated with selected CYP inducers, namely NaPB, BNF, PCN, DEX, MCP and ISN. The dose levels of the six inducers studied were taken from Lake *et al.* (1998). As no significant differences were observed between gum tragacanth- and corn oil-treated rats, these groups were combined to provide a single control group for these studies (data not shown). Only DEX treatment produced a decrease in the body weight of male Sprague–Dawley rats (data not shown). With the exception of ISN, all the inducers produced a significant increase (figure 2A) in



Treatment

Figure 2. Effect of administration of CYP enzyme inducers to rats for 4 days on relative liver weight (A), microsomal protein content (B) and total CYP content (C). Results are expressed as mean $\pm$ SEM of four animals. Values significantly different from controls (Con) are: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

relative liver weight (i.e. liver weight per 100 g body weight). The largest increases to 175 and 160% of control were observed in rats treated with DEX and MCP respectively. Treatment with NaPB, BNF and PCN produced a significant increase in microsomal protein content (figure 2B), whereas with the exception of ISN all the inducers significantly increased microsomal CYP content (figure 2C). NaPB produced the most marked effect on both parameters inducing microsomal protein and CYP content to 155 and 270% of control, respectively.

The induction of CYP forms by the different compounds was assessed by measuring some selected enzyme activities. Whole homogenate cyanide-insensitive palmitoyl-CoA oxidation was employed as a marker for induction of CYP4A forms (Lake 1995, Lake and Lewis 1996), whereas microsomal 7ER, 7BR and 4-nitrophenol metabolism were employed as markers of CYP1A, CYP2B and CYP2E forms, respectively (Burke *et al.* 1985, Reinke *et al.* 1985, Japenga *et al.* 1993, Nerurkar *et al.* 1993, Parkinson 1996, Lake *et al.* 1998). In addition, microsomal testosterone metabolism to 7 α -, 16 β - and 6 β -hydroxytestosterones was determined as markers of induction of CYP2A, CYP2B and CYP3A subfamily forms, respectively (Parkinson 1996).

Only MCP treatment produced a significant increase in whole homogenate palmitoyl-CoA oxidation activity to 1440% of control (figure 3A). Treatment with DEX resulted in a significant decrease in enzyme activity to 70% of control. Microsomal 4-nitrophenol hydroxylase activity was significantly induced by NaPB and ISN treatment to 190 and 420% of control, respectively (figure 3B). Treatment with BNF produced a marked induction of microsomal 7ER *O*-deethylase activity to 30 000% of control with much smaller increases being observed after treatment with NaPB, PCN, DEX and ISN (figure 3C). In contrast, microsomal 7BR *O*-debenzylase activity was markedly induced to 17 700% of control by NaPB treatment with much smaller increases being observed after treatment with the other compounds studied (figure 3D). Microsomal testosterone metabolism to 6 β -hydroxytestosterone was significantly induced by treatment with NaPB, PCN and DEX (figure 4A). Treatment with NaPB and BNF produced small increases in 7 α -hydroxytestosterone formation, whereas a significant decrease was observed after treatment with PCN, DEX and ISN (figure 4B). Only treatment with NaPB produced a marked induction of testosterone metabolism to 16 β -hydroxytestosterone (figure 4C). Finally, treatment with NaPB, PCN and DEX significantly induced testosterone metabolism to androstenedione, whereas a significant decrease was observed after treatment with BNF and MCP (figure 4D).

Metabolism of HFC derivatives and 7BQ in rat liver microsomes

In initial studies, the metabolism of the HFC derivatives and 7BQ was investigated with pooled liver microsomes from control and CYP form inducer treated rats. These studies were performed to establish assay conditions for each fluorescent probe substrate where product formation (i.e. HFC or 7HQ) was linear with respect to incubation time and microsomal protein concentration (data not shown). To obtain information on the affinity of each fluorescent probe for different CYP forms, three or four different substrate concentrations were employed. With liver microsomes from control rats, BFC metabolism could be quantified at substrate concentrations of 1, 2.5 and 25 μ M and BFBFC, 2TFBFC, 3TFBFC and 3TFMeOBFC at substrate concentrations of 0.25, 2.5 and 25 μ M

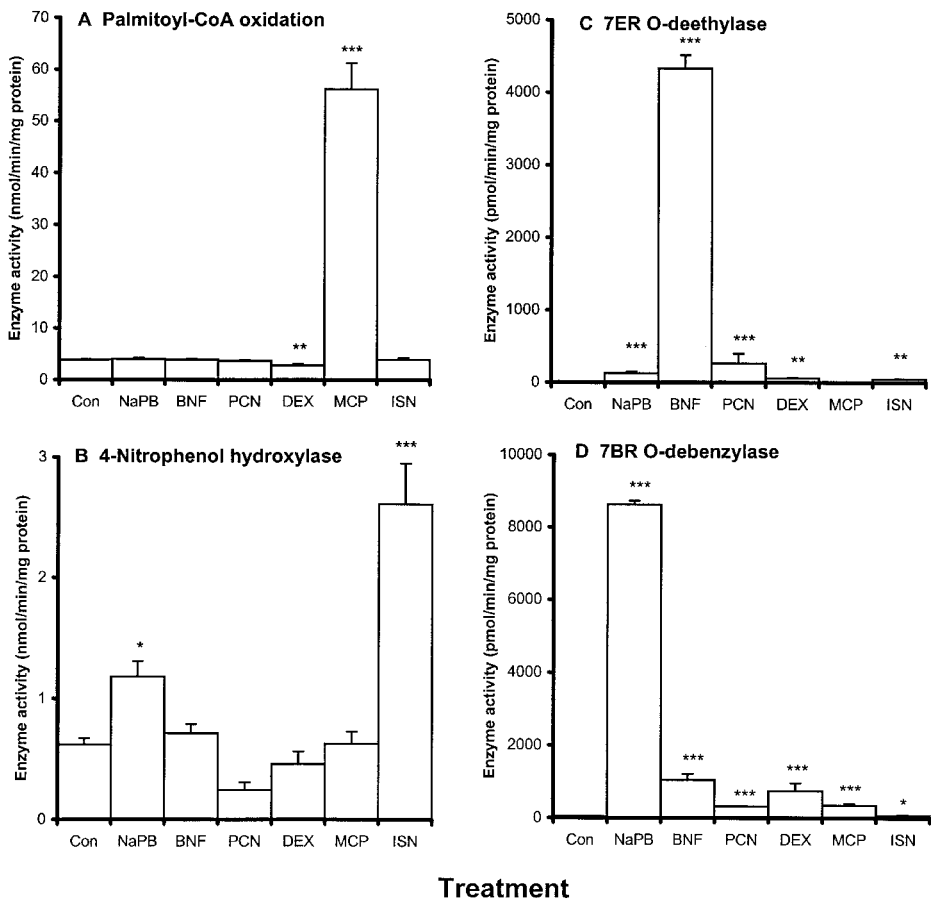


Figure 3. Effect of administration of CYP enzyme inducers to rats for 4 days on hepatic whole homogenate palmitoyl-CoA oxidation (A) and microsomal 4-nitrophenol hydroxylase (B), 7-ethoxyresorufin (7ER) *O*-deethylase (C) and 7-benzoyloxyresorufin (7BR) *O*-debenzylase (D) activities. Results are expressed as mean $\pm$ SEM of four animals. Values significantly different from controls (Con) are: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

(table 1). Because only a small amount of 2TFMeOBFC was available, preliminary studies were only performed with liver microsomes from control-, BNF- and DEX-treated rats (data not shown). Unlike the HFC derivatives shown in table 1, 7BQ metabolism could only be reliably quantified in control liver microsomes at substrate concentrations of 2.5 and 25 μM . In contrast to all the other substrates studied, no significant metabolism of BTBFC was detected in liver microsomes from either control animals or from rats treated with the CYP inducers (data not shown). At the highest substrate concentration examined, namely 25 μM , 7BQ exhibited the highest rate of metabolism in control rat liver microsomes, followed by 2TFBFC and BFC and then by 3TFMeOBFC, BFBFC and 3TFBFC (table 1).

The results of the studies on the metabolism of the HFC derivatives and 7BQ by rat liver microsomes are shown in figure 5. BFC metabolism was markedly induced by BNF and NaPB treatment at all three substrate concentrations (1, 2.5 and 25 μM) examined (figure 5A). BFC metabolism was also induced by DEX, with

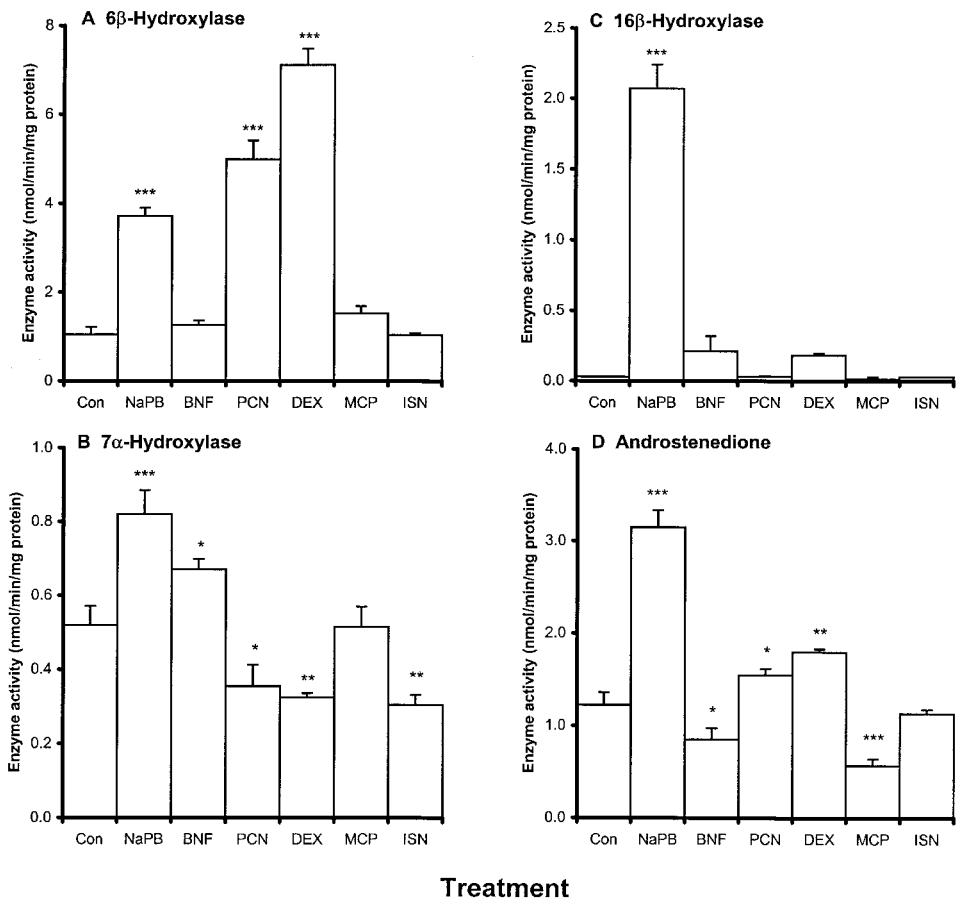


Figure 4. Effect of administration of CYP enzyme inducers to rats for 4 days on hepatic microsomal testosterone 6β- (A), 7α- (B) and 16β- (C) hydroxylase activities and androstenedione formation (D). Results are expressed as mean $\pm$ SEM of four animals. Values significantly different from controls (Con) are: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Table 1. Metabolism of HFC derivatives and 7BQ by liver microsomes from the male Sprague–Dawley rat.

Substrate concentration (μM)	Enzyme activity ($\text{pmol min}^{-1} \text{mg protein}^{-1}$) ^a					
	BFC	BFBFC	2TFBFC	3TFBFC	3TFMeOBFC	7BQ
0.25	n.d. ^b	2 ± 0.2	3 ± 1	15 ± 1	10 ± 1	n.d.
1	46 ± 2	— ^c	—	—	—	n.d.
2.5	68 ± 2	21 ± 3	111 ± 8	20 ± 2	25 ± 1	109 ± 15
25	364 ± 37	43 ± 5	389 ± 28	34 ± 2	57 ± 2	969 ± 65

^a Results are expressed as mean $\pm$ SEM of four animals (two gum tragacanth and two corn oil-treated rats).

^b n.d., Enzyme activity not detectable.

^c Not assayed.

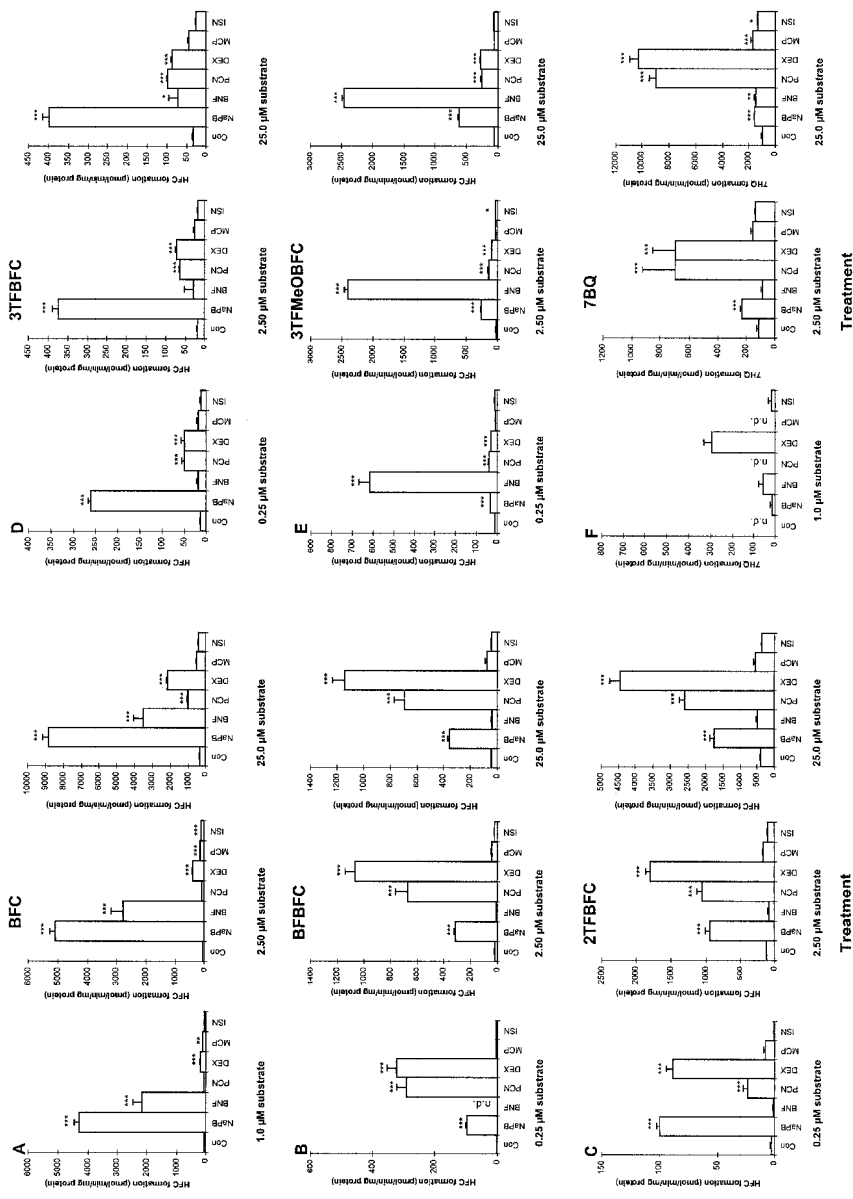


Figure 5. Effect of administration of CYP enzyme inducers to rats for 4 days on hepatic microsomal BFC (A), BFBFC (B), 2TFBFC (C), 3TFBFC (D), 3TFMeOBFC (E) and 7BQ (F) metabolism. Results are expressed as mean $\pm$ SEM of four animals. The absence of any detectable enzyme activity is indicated as n.d. Values significantly different from controls (Con) are: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

smaller effects being observed after treatment with PCN, MCP and ISN. The metabolism of BFBFC was markedly induced by PCN and DEX treatment at all three substrate concentrations (0.25, 2.5 and 25 μM) examined (figure 5B). Treatment with NaPB also resulted in a smaller induction of BFBFC metabolism (figure 5B). 2TFBFC metabolism was induced by treatment with NaPB, PCN and DEX, with the greatest effect being produced by DEX at substrate concentrations of 2.5 and 25 μM (figure 5C). In contrast, NaPB produced the greatest induction of 3TFBFC metabolism, with much smaller effects being produced by PCN and DEX (figure 5D). The metabolism of 3TFMeOBFC was markedly induced by BNF treatment at all three substrate concentrations examined (figure 5E). Much smaller increases in 3TFMeOBFC metabolism were observed after treatment with NaPB, PCN and DEX. In contrast to 3TFMeOBFC, preliminary studies on the metabolism of 2TFMeOBFC demonstrated that metabolism of this substrate was markedly induced by DEX and not by BNF (data not shown).

At a substrate concentration of 1 μM 7BQ metabolism could be quantified in liver microsomal preparations from DEX treated rats and to a lesser extent with some of the other CYP inducers studied (figure 5F). Treatment with both PCN and DEX markedly induced 7BQ metabolism at substrate concentrations of 2.5 and 25 μM . In addition, smaller increases in 7BQ metabolism were observed with NaPB, BNF, MCP and ISN at 7BQ substrate concentrations of either 2.5 and/or 25 μM (figure 5B).

Metabolism of HFC derivatives and 7BQ by cDNA-expressed CYP forms

To provide additional information on the CYP specificity of the fluorescent substrates, metabolism studies were performed with baculovirus-infected insect cell microsomes containing cDNA-expressed rat CYP1A1, CYP1A2, CYP2B1, CYP3A1 or CYP3A2 (figure 6). The linearity of metabolism of BFC, BFBFC, 2TFBFC, 3TFBFC, 3TFMeOBFC and 7BQ by all five cDNA-expressed CYP forms and at all substrate concentrations used was established in preliminary experiments (data not shown).

BFC was metabolized to HFC by the CYP1A1 and CYP2B1 preparations at substrate concentrations of 1, 2.5 and 25 μM (figure 6A). Lower rates of BFC metabolism were observed with the CYP1A2, CYP3A1 and CYP3A2 preparations. The highest rates of metabolism of 1, 2.5 and 25 μM BFBFC were observed with the CYP3A1 and CYP3A2 preparations (figure 6B). 2TFBFC was most extensively metabolized by the CYP2B1, CYP3A1 and CYP3A2 preparations at all three substrate concentrations examined (figure 6C). In contrast, only low rates of 2TFBFC metabolism were observed with the CYP1A2 preparation and metabolism could not be detected with the CYP1A1 preparation. 3TFBFC was most extensively metabolized by the CYP2B1 preparation at all three substrate concentrations examined (figure 6D). Lower rates of 3TFBFC metabolism were observed at some substrate concentrations with the CYP1A1, CYP1A2, CYP3A1 and CYP3A2 preparations. 3TFMeOBFC was extensively metabolized by the CYP1A1 preparation at all three substrate concentrations studied (figure 6E). In contrast, only very low rates of metabolism were observed with the CYP1A2, CYP2B1, CYP3A1 and CYP3A2 preparations.

The metabolism of 7BQ was studied with substrate concentrations of 1, 2.5 and 25 μM (figure 6F). At the lowest substrate concentration of 1 μM , 7BQ metabolism

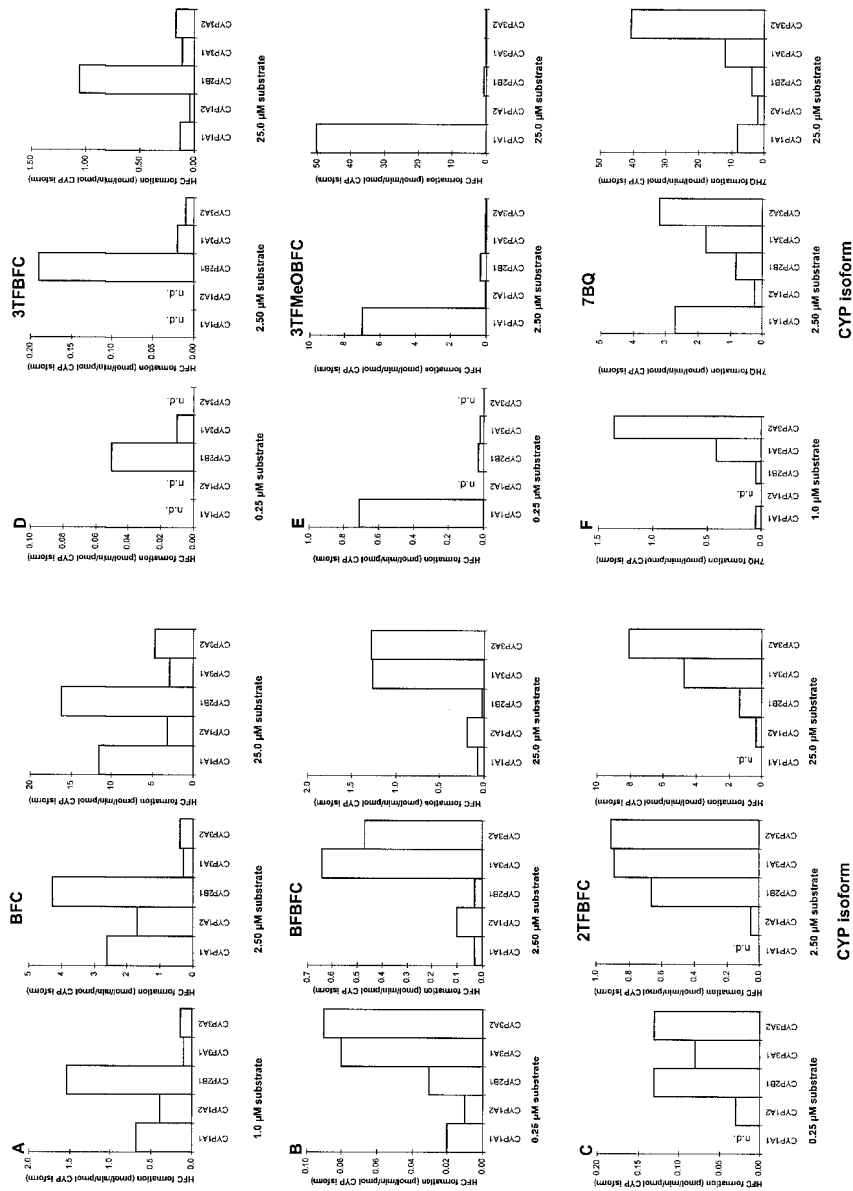


Figure 6. Metabolism of BFC (A), BFBFC (B), 2TFBFC (C), 3TFBFC (D), 3TFMeOBFC (E) and 7BQ (F) by baculovirus-infected insect cell microsomes containing cDNA-expressed rat CYP1A1, CYP1A2, CYP2B1, CYP3A1 and CYP3A2. The results of a single experiment are shown. Each value is the mean of four wells. The absence of any detectable enzyme activity is indicated as n.d.

was greatest with the CYP3A1 and CYP3A2 preparations. At substrate concentrations of 2.5 and 25 μM the highest rates of 7BQ metabolism were observed with the CYP3A2 preparation, with lower rates of metabolism being observed with the other cDNA-expressed CYP forms examined.

Discussion

The development of rapid screens for investigating the effects of new chemical entities on CYP enzymes would help both reduce the cost of drug development and the usage of experimental animals. When developing a new therapeutic agent, it is necessary to identify which CYP forms will be involved in the metabolism of the compound and whether there will be any potential for drug interactions due to either the inhibition or induction of CYP forms (Wrighton *et al.* 1993, Parkinson 1996, Clarke 1998, Lin and Lu 1998, Pelkonen *et al.* 1998, Tanaka 1998, Rodrigues 1999). CYP induction is also of importance in preclinical studies, because CYP inducers may produce liver tumours in rodents (Nims *et al.* 1987, Grasso and Hinton 1991, Lake 1995, Whysner *et al.* 1996).

The objective of this study was to evaluate the usefulness of a number of fluorescent substrates as probes for monitoring selected CYP enzymes in rat liver. While some resorufin derivatives are available as probes for rat hepatic CYP1A and CYP2B forms (Burke *et al.* 1985, 1994, Nerurkar *et al.* 1993, Nims and Lubet 1996), no fluorescent probes are available for CYP3A forms in rat liver. In these studies the usefulness of some HFC derivatives and 7BQ as probes for rat hepatic CYP forms was evaluated using characterized liver microsomal preparations from rats treated with known CYP inducers and with some cDNA-expressed rat CYP forms.

Liver microsomes were prepared and characterized from rats treated with NaPB, BNF, PCN, DEX, MCP and ISN. BNF, ISN and MCP were selected as inducers of CYP1A, CYP2E and CYP4A subfamily forms, respectively (Okey 1990, Nelson *et al.* 1996, Lake *et al.* 1998). NaPB is known to induce CYP2B forms and also CYP2A and CYP3A forms, whereas PCN and DEX are inducers of CYP3A and to a lesser extent of CYP2B forms (Maurel 1996, Nelson *et al.* 1996, Nims and Lubet 1996, Parkinson 1996, Lake *et al.* 1998). The induction of CYP forms was monitored using 7ER, 7BR, 4-nitrophenol and testosterone as probe substrates (Burke *et al.* 1985, 1994, Reinke *et al.* 1985, Okey 1990, Japenga *et al.* 1993, Nerurkar *et al.* 1993, Parkinson 1996, Lake *et al.* 1998). In addition, whole homogenate palmitoyl-CoA oxidation was determined as a marker for CYP4A induction as other studies have demonstrated good correlations between the induction of peroxisomal (palmitoyl-CoA oxidation) and microsomal (CYP4A-dependent) fatty acid oxidizing enzymes in rodent liver (Lake 1995, Lake and Lewis 1996).

Treatment with BNF, NaPB, PCN, DEX, MCP and ISN resulted in the expected CYP induction profiles. Relative liver weight and total CYP content were induced by all the compounds studied except ISN. 7ER metabolism was markedly induced by the CYP1A inducer BNF, whereas 7BR metabolism was most markedly induced by the CYP2B inducer NaPB. Treatment with NaPB also resulted in an induction of testosterone metabolism to 7 α -, 16 β - and 6 β -hydroxy-testosterones which are known to be catalysed by CYP2A, CYP2B and CYP3A forms, respectively (Parkinson 1996). 4-Nitrophenol hydroxylase activity was

induced by the CYP2E inducer ISN and to a lesser extent by NaPB. Like NaPB, the CYP3A inducers PCN and DEX significantly increased testosterone 6 β -hydroxylase activity. Finally, the peroxisome proliferator and CYP4A inducer MCP produced a marked induction of whole homogenate palmitoyl-CoA oxidation.

Overall, the pattern of CYP form induction in this study with male Sprague-Dawley rats are in agreement with those of a previous study where female Sprague-Dawley CD rats were used (Lake *et al.* 1998). One apparent sex difference in response was the greater effect of MCP on palmitoyl-CoA oxidation. Peroxisome proliferators are known to produce more marked effects in the livers of male than in female rats (Lake 1995). In the previous study, Western immunoblotting of liver microsomes was also performed. While NaPB induced levels of CYP2B1/2 apoproteins, CYP3A forms were also induced; whereas DEX induced levels of CYP2B1/2 in addition to levels of CYP3A apoproteins (Lake *et al.* 1998).

The CYP form specificity of BFC, the other HFC derivatives and 7BQ (see figure 1 for the structures) was determined by studies with the characterized liver microsomal preparations and with cDNA-expressed CYP1A1, CYP1A2, CYP2B1, CYP3A1 and CYP3A2. All of these studies were performed using 96-well plates and a fluorescence plate reader. To obtain information on the affinity of each substrate for particular CYP forms, incubations were performed with three substrate concentrations namely 0.25 or 1 μ M together with 2.5 and 25 μ M.

The metabolism of BFC was induced by treatment with NaPB and BNF and to a lesser extent with PCN and DEX. Studies with the cDNA-expressed CYP forms demonstrated that BFC was a substrate for CYP1A1 and CYP2B1 and to a lesser extent for CYP1A2, CYP3A1 and CYP3A2. BFC may therefore be considered a probe for induction of CYP1A and CYP2B forms in rat liver. As such these results are in agreement with a previous study where BFC metabolism was induced in liver microsomes from female Sprague-Dawley CD rats by treatment with NaPB, BNF and DEX and was primarily metabolized by cDNA-expressed CYP1A1, CYP1A2 and CYP2B1 at a substrate concentration of 2.5 μ M (Price *et al.* 2000). These investigations also demonstrated that BFC metabolism in cultured hepatocytes from male Sprague-Dawley rats could be induced by treatment with NaPB and BNF, but not with DEX (Price *et al.* 2000).

In contrast to BFC, BFBFC appears to be a probe for CYP3A forms in rat liver. BFBFC metabolism was more markedly induced by the CYP3A inducers PCN and DEX than by NaPB. In addition, the studies with the cDNA-expressed CYP forms showed a much higher affinity for CYP3A1 and CYP3A2 than for CYP1A1, CYP1A2 and CYP2B1 at all three substrate concentrations examined. Unlike BFBFC, the BTBFC isomer was not metabolized by rat liver microsomes. In other studies, BFBFC, but not BTBFC, has been shown to be metabolized to HFC by human liver microsomes (Renwick *et al.* 2001).

The metabolism of the isomers 2TFBFC and 3TFBFC were induced by treatment with NaPB, PCN and DEX. However, while 3TFBFC metabolism was induced to a greater extent by NaPB treatment at all three substrate concentrations studied, DEX produced the greatest stimulation of 2TFBFC metabolism at substrate concentrations of 2.5 and 25 μ M. The studies with the cDNA-expressed forms demonstrated that 3TFBFC was a relatively specific

substrate for CYP2B1, whereas 2TFBFC can be metabolized by CYP2B1, CYP3A1 and CYP3A2.

Studies were also conducted with the 2TFMeOBFC and 3TFMeOBFC isomers. Because only a small quantity of 2TFMeOBFC was available, only limited studies could be conducted. These preliminary experiments suggested that 2TFMeOBFC metabolism was induced by DEX treatment, with BNF treatment having no marked effect. In contrast, 3TFMeOBFC metabolism was markedly induced by BNF treatment, with much lower levels of induction being produced by NaPB, PCN and DEX. At all three substrate concentrations studied 3TFMeOBFC was found to be a substrate for cDNA-expressed CYP1A1, whereas only low rates of metabolism were observed with the CYP1A2, CYP2B1, CYP3A1 and CYP3A2 preparations. Overall, 3TFMeOBFC appears to be a useful probe substrate for CYP1A1 in rat liver.

The metabolism of 7BQ to 7HQ was also studied. Previous work has demonstrated that 7BQ is metabolized to 7HQ by rat liver microsomes and that enzyme activity is inducible by NaPB, but not by the CYP1A inducer 20-methylcholanthrene (Mayer *et al.* 1990). In the present investigations, 7BQ metabolism was markedly induced by PCN and DEX. Treatment with NaPB, BNF, MCP and ISN also produced small increases in 7BQ metabolism at substrate concentrations of 2.5 and 25 μM . 7BQ was a substrate for cDNA-expressed CYP3A2 and to a lesser extent for CYP3A1 and the other cDNA-expressed CYP forms studied. 7BQ may be a useful probe for CYP3A form induction in rat liver microsomes.

Of the probe, substrates examined in this study only the metabolism of BFC and 7BQ have been previously studied in rat liver microsomes (Mayer *et al.* 1990, Price *et al.* 2000). However, some data is available on the metabolism of BFC, BFBFC and 7BQ by human liver microsomes (Renwick *et al.* 2000, 2001). In the present study, BFBFC and 7BQ appeared to be relatively specific substrates for CYP3A forms, whereas BFC was a probe substrate for CYP1A and CYP2B forms. The CYP specificity of BFBFC and 7BQ in rat liver is identical to that of human liver, with both probes being considered to be relatively specific substrates for CYP3A4 (Renwick *et al.* 2001). That BFC exhibits some specificity for CYP1A forms in rat liver also correlates with a recent study in human liver microsomes. In this latter investigation BFC was found to be preferentially metabolized by CYP1A2 at low substrate concentrations, but is also a substrate for CYP3A4 at higher substrate concentrations (Renwick *et al.* 2000). These results suggest that good probes for rat CYP3A forms may also have a similar specificity for human hepatic CYP3A forms.

Apart from BFC, the other six HFC derivatives studied consisted of three pairs of isomers (figure 1). For BFBFC and BTBFC the 2,5-substitution appears to confer CYP3A specificity whereas a 3,5-substitution abolishes metabolism by rat liver microsomes. While the CYP specificity of 2TFMeOBFC was not established in the present work, it is clear that 3-substitution confers CYP1A1 specificity. For 2TFBFC and 3TFBFC the 3-substitution appears to confer CYP2B specificity, whereas 2-substitution results in a mixture of CYP2B and CYP3A specificity. While these studies have focussed on the metabolism of the HFC derivatives and 7BQ by CYP1A, CYP2B and CYP3A forms, these compounds may also be substrates for other CYP forms in rat liver. For example, BFC is also metabolized by cDNA-expressed CYP2C6 and CYP2C13, but not by CYP2C12 (Price *et al.*

2000). However, the HFC derivatives and 7BQ would be unlikely to be substrates for CYP4A forms as they appear only to be involved in the metabolism of endogenous substrates (Lake and Lewis 1996). Indeed, none of the compounds studied appeared to be substrates for either CYP2E or CYP4A forms as their metabolism was not markedly stimulated by treatment with either ISN or MCP, respectively.

One specific aim of the present investigations was to identify a suitable fluorescent probe substrate for rat hepatic CYP3A forms. In this context, both BFBFC and 7BQ appear to be relatively specific for CYP3A forms in rat liver. Of these two compounds, BFBFC may be preferable as it is extensively metabolized by both CYP3A1 and CYP3A2, whereas 7BQ is more extensively metabolized by CYP3A2 than by CYP3A1. Certainly, the use of either BFBFC or 7BQ as fluorescent probes for CYP3A forms in a 96-well plate format is much more rapid than determining testosterone 6 β -hydroxylase activity. This latter procedure involves separation of 6 β -hydroxytestosterone from testosterone and other testosterone metabolites with quantification by either a UV or a radio-metric detector. Recently, Chauret *et al.* (1999) reported that the compound 3-(3,4-difluorobenzyloxy)-5,5-dimethyl-4-(4-methylsulfonylphenyl)-(5*H*)-furan-2-one was a specific substrate for CYP3A4/5 in human liver microsomes. One advantage of both BFBFC and 7BQ over this substrate is that the presence of NADPH does not interfere with the formation of either the HFC or the 7HQ product.

With respect to 3TFMeOBFC, this probe appears to be relatively specific for CYP1A1, with only very low rates of metabolism being detectable with the cDNA-expressed CYP1A2 preparation. As such this probe may be more specific for CYP1A1 than 7ER. While 7ER and 7-methoxyresorufin may be considered as probe substrates for CYP1A1 and CYP1A2, respectively (Nerurkar *et al.* 1993), studies with purified rat CYP1A1 and CYP1A2 have demonstrated that both substrates are metabolized to some extent by both CYP1A forms (Burke *et al.* 1994).

Apart from being useful fluorescent probe substrates for rat liver microsomes, the HFC derivatives studied and 7BQ may also be useful substrates for *in vitro* rat CYP induction studies with cultured hepatocytes. For example, BFC has been recently reported to be a good fluorescent probe for monitoring CYP1A1 and CYP2B induction in rat hepatocytes cultured in a 96-well plate format (Price *et al.* 2000). In addition, preliminary experiments suggest that 3TFMeOBFC and BFBFC may be useful probes for CYP1A and CYP3A induction in cultured rat hepatocytes (Price, Renwick and Lake, unpublished observations). Additional studies are required to confirm and extend these findings and also to assess the usefulness of 2TFBFC, 3TFBFC and 7BQ as fluorescent probe substrates for induction studies in cultured rat hepatocytes.

Acknowledgements

The authors are grateful to MSD Research Laboratories (Harlow, UK) for the provision of a research grant.

References

- BURKE, M. D., THOMPSON, S., ELCOMBE, C. R., HALPERT, J., HAAPARANTA, T. and MAYER R. T., 1985, Ethoxy-, pentoxy- and benzyloxyphenoxazones and homologues: a series of substrates to distinguish between different induced cytochromes P-450. *Biochemical Pharmacology*, **34**, 3337–3345.
- BURKE, M. D., THOMPSON, S., WEAVER, R. J., WOLF, C. R. and MAYER, R. T., 1994, Cytochrome P450 specificities of alkoxyresorufin O-dealkylation in human liver microsomes. *Biochemical Pharmacology*, **48**, 923–936.
- BUTERS, J. T. M., SCHILLER, C. D. and CHOU, R. C., 1993, A highly sensitive tool for the assay of cytochrome P450 enzyme activity in rat, dog and man. Direct fluorescence monitoring of the deethylation of 7-ethoxy-4-trifluoromethylcoumarin. *Biochemical Pharmacology*, **46**, 1577–1584.
- CHAURET, N., TREMBLAY, N., LACKMAN, R. L., GAUTHIER, J.-Y., SILVA, J. M., MAROIS, J., YERGEY, J. A. and NICOLL-GRIFFITH, D. A., 1999, Description of a 96-well plate assay to measure cytochrome P4503A inhibition in human liver microsomes using a selective fluorescent probe. *Analytical Biochemistry*, **276**, 215–226.
- CLARKE, S. E., 1998, *In vitro* assessment of human cytochrome P450. *Xenobiotica*, **28**, 1167–1202.
- CONNEY, A. J., 1986, Induction of microsomal cytochrome P-450 enzymes: the first Bernard B Brodie lecture at Pennsylvania State University. *Life Sciences*, **39**, 2493–2518.
- CRESPI, C. L., MILLER, V. P. and PENMAN, B. W., 1997, Microtiter plate assays for inhibition of human, drug-metabolizing cytochromes P450. *Analytical Biochemistry*, **248**, 188–190.
- CRESPI, C. L., MILLER, V. P. and PENMAN, B. W., 1998, High throughput screening for inhibition of cytochrome P450 metabolism. *Medical Chemistry Research*, **8**, 457–471.
- DELUCA, J. G., DYSART, G. R., RASNICK, D. and BRADLEY, M. O., 1988, A direct, highly sensitive assay for cytochrome P-450 catalyzed O-deethylation using a novel coumarin analog. *Biochemical Pharmacology*, **37**, 1731–1739.
- DONATO, M. T., GÓMEZ-LECHÓN, M. J. and CASTELL, J. V., 1993, A microassay for measuring cytochrome P450IA1 and P450IIB1 activities in intact human and rat hepatocytes cultured on 96-well plates. *Analytical Biochemistry*, **213**, 29–33.
- GÓMEZ-LECHÓN, M. J., DONATO, M. T., PONSODA, X., FABRA, R., TRULLENQUE, R. and CASTELL, J. V., 1997, Isolation, culture and use of human hepatocytes in drug research. In J. V. Castell and M. J. Gómez-Lechón (eds), *In Vitro Methods in Pharmaceutical Research* (London: Academic Press), pp. 129–153.
- GRASSO, P. and HINTON, R. H., 1991, Evidence for and possible mechanisms of non-genotoxic carcinogenesis in rodent liver. *Mutation Research*, **248**, 271–290.
- GRAY, T. J. B., LAKE, B. G., BEAMAND, J. A., FOSTER, J. R. and GANGOLLI, S. D., 1983, Peroxisome proliferation in primary cultures of rat hepatocytes. *Toxicology and Pharmacology*, **67**, 15–25.
- JAPENGA, A. C., DAVIES, S., PRICE, R. J. and LAKE, B. G., 1993, Effect of treatment with pyrazine and some derivatives on cytochrome P450 and some enzyme activities in rat liver. *Xenobiotica*, **23**, 169–179.
- LAKE, B. G., 1987, Preparation and characterisation of microsomal fractions for studies of xenobiotic metabolism. In K. Snell and B. Mullock (eds), *Biochemical Toxicology: A Practical Approach* (Oxford: IRL), pp. 183–215.
- LAKE, B. G., 1995, Mechanisms of hepatocarcinogenicity of peroxisome-proliferating drugs and chemicals. *Annual Review of Pharmacology and Toxicology*, **35**, 483–507.
- LAKE, B. G., 1997, *In vitro* methods. In R. S. McCuskey and D. L. Earnest (eds), *Comprehensive Toxicology*, vol. 9: *Hepatic and Gastrointestinal Toxicology* (Oxford: Pergamon), pp. 233–249.
- LAKE, B. G. and LEWIS, D. F. V., 1996, The CYP4 family. In C. Ioannides (ed.), *Cytochromes P450: Metabolic and Toxicological Aspects* (Boca Raton: CRC Press), pp. 271–297.
- LAKE, B. G., RENWICK, A. B., CUNNINGHAME, M. E., PRICE, R. J., SURRY, D. and EVANS, D. C., 1998, Comparison of the effects of some CYP3A and other enzyme inducers on replicative DNA synthesis and cytochrome P450 isoforms in rat liver. *Toxicology*, **131**, 9–20.
- LIN, J. H. and LU, A. Y. H., 1998, Inhibition and induction of cytochrome P450 and the clinical implications. *Clinical Pharmacokinetics*, **35**, 361–390.
- LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. and RANDALL, R. J. 1951, Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry*, **193**, 265–275.
- LUBET, R. A., SYI, J.-L., NELSON, J. O. and NIMS, R. W., 1990, Induction of hepatic cytochrome P-450 mediated alkoxyresorufin O-dealkylase activities in different species by prototype P-450 inducers. *Chemico-Biological Interactions*, **75**, 325–399.
- MAUREL, P., 1996, The CYP3 family. In C. Ioannides (ed.), *Cytochromes P450: Metabolic and Toxicological Aspects* (Boca Raton: CRC Press), pp. 241–270.
- MAYER, R. T., NETTER, K. J., HEUBEL, F., HAHNEMANN, B., BUCHHEISTER, A., MAYER, G. K. and BURKE, M. D., 1990, 7-Alkoxyquinolines: new fluorescent substrates for cytochrome P450 monooxygenases. *Biochemical Pharmacology*, **40**, 1645–1655.

- NELSON, D. R., KOYMANS, L., KAMATAKI, T., STEGEMAN, J. J., FEYEREISEN, R., WAXMAN, D. J., WATERMAN, M. R., GOTOH, O., COON, M. J., ESTABROOK, R. W., GUNSALUS, I. C. and NEBERT, D. W., 1996, P450 superfamily: update on new sequences, gene mapping, accession numbers and nomenclature. *Pharmacogenetics*, **6**, 1-42.
- NERURKAR, P. V., PARK, S. S., THOMAS, P. E., NIMS, R. W. and LUBET, R. A., 1993, Methoxyresorufin and benzyloxyresorufin: substrates preferentially metabolised by cytochromes P4501A2 and 2B, respectively, in the rat and mouse. *Biochemical Pharmacology*, **46**, 933-943.
- NIMS, R. W. and LUBET, R. A., 1996, The CYP2B subfamily. In C. Ioannides (ed.), *Cytochromes P450: Metabolic and Toxicological Aspects* (Boca Raton: CRC press), pp. 135-160.
- NIMS, R. W., DEVOR, D. E., HENNEMAN, J. R. and LUBET, R. A., 1987, Induction of alkoxyresorufin O-dealkylases, epoxide hydrolase, and liver weight gain: correlation with tumor-promoting potential in a series of barbiturates. *Carcinogenesis*, **8**, 67-71.
- OKEY, A. B., 1990, Enzyme induction in the cytochrome P-450 system. *Pharmacology and Therapeutics*, **45**, 241-298.
- PARKINSON, A., 1996, Biotransformation of xenobiotics. In C. D. Klaassen (ed.), *Casarett and Doull's Toxicology: The Basic Science of Poisons*, 5th edn (New York: McGraw-Hill) pp. 113-186.
- PELKONEN, O., MÄENPÄÄ, J., TAAVITSAINEN, P., RAUTIO, A. and RAUNIO, H., 1998, Inhibition and induction of human cytochrome P450 (CYP) enzymes. *Xenobiotica*, **28**, 1203-1253.
- PRICE, R. J., SURRY, D., RENWICK, A. B., MENESES-LORENTE, G., LAKE, B. G. and EVANS, D. C., 2000, CYP isoform induction screening in 96-well plates: use of 7-benzyloxy-4-trifluoromethylcoumarin as a substrate for studies with rat hepatocytes. *Xenobiotica*, **30**, 781-795.
- REINKE, L. A., SENTER, S. H. and RIKANS, L. E., 1985, Comparison of ethanol and imidazole pretreatments on hepatic monooxygenase activities in the rat. *Research Communications in Chemistry, Pathology and Pharmacology*, **47**, 97-106.
- RENWICK, A. B., LEWIS, D. F. V., FULFORD, S., SURRY, D., WILLIAMS, B., WORBOYS, P. D., CAI, X., WANG, R. W., PRICE, R. J., LAKE, B. G. and EVANS, D. C., 2001, Metabolism of 2,5-bis(trifluoromethyl)-7-benzyloxy-4-trifluoromethylcoumarin by human hepatic CYP isoforms: evidence for selectivity towards CYP3A4. *Xenobiotica*, **31**, 187-204.
- RENWICK, A. B., SURRY, D., PRICE, R. J., LAKE, B. G. and EVANS, D. C., 2000, Metabolism of 7-benzyloxy-4-trifluoromethylcoumarin by human hepatic cytochrome P450 isoforms. *Xenobiotica*, **30**, 955-969.
- RODRIGUES, A. D., 1999, Integrated cytochrome P450 reaction phenotyping. Attempting to bridge the gap between cDNA-expressed cytochromes P450 and native human liver microsomes. *Biochemical Pharmacology*, **57**, 465-480.
- SONDERFAN, A. J., ARLOTTO, M. P., DUTTON, M. R., McMILLEN, S. K. and PARKINSON, A., 1987, Regulation of testosterone hydroxylation by rat liver microsomal cytochrome P450. *Archives of Biochemistry and Biophysics*, **255**, 27-41.
- TANAKA, E., 1998, Clinically important drug-drug interactions: role of cytochrome P450 enzymes. *Journal of Clinical Pharmacology and Therapeutics*, **23**, 403-416.
- WHITE, I. N. H., 1988, A continuous fluorimetric assay for P-450-dependent mixed function oxidases using 3-cyano-7-ethoxycoumarin. *Analytical Biochemistry*, **172**, 304-310.
- WHYSNER, J., ROSS, P. M. and WILLIAMS, G. M., 1996, Phenobarbital mechanistic data and risk assessment: enzyme induction, enhanced cell proliferation, and tumor promotion. *Pharmacology and Therapeutics*, **71**, 153-191.
- WRIGHTON, S. A., VANDENBRANDEN, M., STEVENS, J. C., SHIPLEY, L. A., RING, B. J., RETTIE, A. E. and CASHMAN, J. R., 1993, *In vitro* methods for assessing human hepatic drug metabolism: their use in drug development. *Drug Metabolism Reviews*, **25**, 453-484.

Rapid conversion of tea catechins to monomethylated products by rat liver cytosolic catechol-*O*-methyltransferase

BAO TING ZHU†*, USHMA K. PATEL‡, MAY XIAOXIN CAI‡, ANTHONY J. LEE† and ALLAN H. CONNEY‡

† Department of Basic Pharmaceutical Sciences, College of Pharmacy, University of South Carolina, Columbia, SC 29208, USA

‡ Laboratory for Cancer Research, Department of Chemical Biology, College of Pharmacy, Rutgers University, Piscataway, NJ 08854, USA

Received 28 February 2001

1. The metabolic *O*-methylation of several catechol-containing tea polyphenols by rat liver cytosolic catechol-*O*-methyltransferase (COMT) has been studied.

2. When (–)-epicatechin was used as substrate, its *O*-methylation showed dependence on incubation time, cytosolic protein concentration, incubation pH and concentration of *S*-adenosyl-*L*-methionine. The *O*-methylation of increasing concentrations of (–)-epicatechin followed typical Michaelis–Menten kinetics, and the apparent K_m and V_{max} were 51 μM and 2882 pmol mg protein^{–1} min^{–1}, respectively, at pH 7.4, and were 17 μM and 2093 pmol mg protein^{–1} min^{–1}, respectively, at pH 10.0.

3. Under optimized conditions for *in vitro* *O*-methylation, (–)-epicatechin, (+)-epicatechin and (–)-epigallocatechin were rapidly *O*-methylated by rat liver cytosol. In comparison, (–)-epicatechin gallate and (–)-epigallocatechin gallate were *O*-methylated at significantly lower rates under the same reaction conditions.

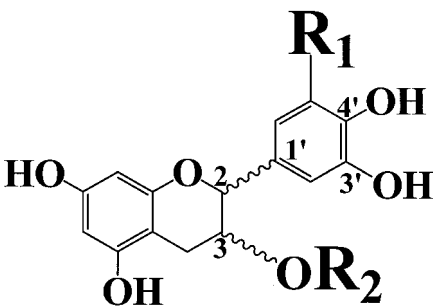
4. COMT-catalysed *O*-methylation of (–)-epicatechin and (–)-epigallocatechin was inhibited in a concentration-dependent manner by *S*-adenosyl-*L*-homocysteine, a demethylated product of *S*-adenosyl-*L*-methionine. The IC₅₀ was ~10 μM .

5. In summary, the results showed that several catechol-containing tea polyphenols were rapidly *O*-methylated by rat liver cytosolic COMT. These observations raise the possibility that some of the biological effects of tea polyphenols may be exerted by their *O*-methylated products or may result from their potential inhibition of the COMT-catalysed *O*-methylation of endogenous catecholamines and catechol oestrogens.

Introduction

Catechol-*O*-methyltransferase (COMT), an enzyme ubiquitously present in high activity in rodents and humans, catalyses the metabolic *O*-methylation of endogenous catecholamines and catechol oestrogens (Axelrod and Tomchick 1958, Axelrod 1966, Guldborg and Marsden 1975, Thakker and Creveling 1990, Zhu and Conney 1998). In addition to the endogenous catechol substrates, COMT also catalyses metabolic *O*-methylation of many catechol-containing xenobiotics (Axelrod and Tomchick 1958, Axelrod 1966, Guldborg and Marsden 1975, Thakker and Creveling 1990, Zhu *et al.* 1994). Zhu *et al.* (1994) reported that quercetin and fisetin, two catechol-containing dietary flavonoids, are rapidly *O*-methylated by cytosolic COMT, with rates two to three orders of magnitude higher than for endogenous catecholamines.

* Author for correspondence; e-mail: BTZhu@cop.sc.edu



Tea polyphenols	Stereochemistry	R ₁	R ₂
(-)-Epicatechin	2 <i>R</i> , 3 <i>R</i>	—H	—H
(+)-Epicatechin	2 <i>S</i> , 3 <i>S</i>	—H	—H
(-)-Epigallocatechin	2 <i>R</i> , 3 <i>R</i>	—OH	—H
(-)-Epicatechin gallate	2 <i>R</i> , 3 <i>R</i>	—H	
(-)-Epigallocatechin gallate	2 <i>R</i> , 3 <i>R</i>	—OH	

Figure 1. Chemical structures of several catechol-containing dietary tea polyphenols used.

Like dietary quercetin and fisetin, several of the common tea polyphenols, such as (–)-epicatechin, (+)-epicatechin, (–)-epigallocatechin, (–)-epicatechin gallate and (–)-epigallocatechin gallate (figure 1), also contain a catechol structure and share certain degrees of structural similarity to them. These catechol-containing tea components constitute a major fraction of the water-extractable polyphenols contained in the tea leaves (Balentine 1992). Recent studies showed that several of these tea polyphenols (e.g. (–)-epigallocatechin and epicatechins) can be readily absorbed in human subjects after drinking tea, and some of them can reach submicromolar peak concentrations (ranging from 0.1 to 0.4 μM) in the plasma (Lee *et al.* 1995, Unno *et al.* 1996, Yang *et al.* 1998, van het Hof *et al.* 1998, Li *et al.* 2000). Another earlier study using ³H-labelled (–)-epigallocatechin gallate showed that this tea catechin is widely distributed in mouse tissues (Suganuma *et al.* 1998).

We recently reported that the catechol-containing tea polyphenols are rapidly metabolized to monomethyl ethers by the cytosolic COMT present in human term placenta (Zhu *et al.* 2000). We describe here our additional results on the rapid *O*-methylation of tea catechins by rat liver cytosol. These observations raise the possibility that some of the biological effects of tea polyphenols may be caused by their methylated products or may result from their potential inhibition of COMT-catalysed *O*-methylation of endogenous catecholamines and catechol oestrogens.

Materials and methods

Chemicals

(–)-Epicatechin, (+)-epicatechin, (–)-epicatechin gallate, (–)-epigallocatechin, (–)-epigallocatechin gallate, *S*-adenosyl-L-methionine, dithiothreitol and Tris-HCl were purchased from the Sigma Chemical Co. (St Louis, MO, USA). The purities for the tea polyphenols used were 95–98% as determined by

HPLC analysis. [Methyl-³H]S-adenosyl-L-methionine (specific activity 11.2–13.5 Ci mmol⁻¹) was obtained from Perkin Elmer Life Science Products (Boston, MA, USA). All organic solvents used in this study were of HPLC grade and obtained from the Fisher Scientific Co. (Springfield, NJ, USA).

Preparation of rat liver cytosol

Six-week-old female Sprague–Dawley rats (body weight 125–150 g) were purchased from Harlan Sprague–Dawley Laboratory (Houston, TX, USA). After arrival, they were allowed to acclimatize for 1 week before use. The animals were euthanized under CO₂ inhalation, and the livers from five female rats were collected and pooled. Rat livers were immediately minced in 3 vols ice-cold 0.1 M Tris-HCl/KCl solution (pH 7.4) and homogenized with a Polytron homogenizer for 2–3 min. Tissue homogenates were centrifuged at 9000g for 15 min, and the supernatants were pooled and filtered through two layers of cheesecloth to remove lipid clots. The filtrates were recentrifuged at 105 000g (4°C) for 90 min. The supernatants (cytosolic preparations) were stored in 1-ml aliquots at -80°C. The protein concentration was determined by using the BioRad protein assay kit with bovine serum albumin (BSA) as a standard.

Rat liver COMT-catalysed O-methylation of catechol-containing tea polyphenols

The reaction mixture was prepared at ~4°C and consisted of the desired concentration of a tea polyphenol substrate, 0.5 mg rat liver cytosolic protein ml⁻¹, 50 µM S-adenosyl-L-methionine (containing 0.25 µCi [methyl-³H]S-adenosyl-L-methionine), 1.2 mM magnesium chloride, and 1.0 mM dithiothreitol in a final volume of 1 ml Tris-HCl buffer (10 mM, pH 7.4). The reaction was initiated by addition of rat liver cytosolic protein and was carried out at 37°C for 20 min under constant mild shaking. The reaction was arrested by placing the tubes on ice followed immediately by addition of 0.5 ml ice-cold water and extraction of the ³H-containing methylated catechin metabolites with 5 ml ethyl acetate. After centrifugation for 20 min at ~3000g, portions of the upper ethyl acetate phase (2 ml) were removed for measurement of radioactivity in a scintillation counter (Beckman Instruments, model LS 5000TD). Blank values obtained from incubations without rat liver cytosolic protein were determined in each individual assay and subtracted. The blank radioactivity counts were usually <20% of the values obtained from incubations in the presence of rat liver cytosolic protein. By using the ³H-labelled monomethylated products of (-)-epicatechin and (-)-epigallocatechin (isolated by HPLC), the extraction efficiency for the O-methylated (-)-epicatechin and (-)-epigallocatechin was >94%. The rate (velocity) for the O-methylation of various tea polyphenol substrates by rat liver cytosolic COMT was expressed as pmol methylated tea polyphenol formed per mg protein per min⁻¹.

Liquid chromatography-mass spectrometry analysis of methylated tea polyphenols

To determine the structures of the methylated tea polyphenols, 50 µM (-)-epicatechin or (-)-epigallocatechin was first incubated *in vitro* for 30 min with 1.0 mg rat liver cytosolic protein ml⁻¹, 100 µM S-adenosyl-L-methionine, 1.2 mM magnesium chloride, and 1.0 mM dithiothreitol in a final volume of 1 ml Tris-HCl buffer (10 mM, pH 7.4). The reaction mixture was extracted twice with 10 ml ethyl acetate. The organic solvent extracts were combined and dried under a stream of nitrogen, and the residues were redissolved in 100 µl 20% acetonitrile in water by vortex mixing. The mixture was then centrifuged at ~15 000g for 10 min, and a 50-µl aliquot of the supernatant was injected into the HPLC for analysis of methylated tea polyphenol metabolites according to the method for tea catechins described by Lee *et al.* (1995). The incubations described above were first done with tea catechins and [methyl-³H]-S-adenosyl-L-methionine to determine the retention times of the radioactive metabolites. Parallel incubations of the catechins with only non-radioactive S-adenosyl-L-methionine were performed for collection of metabolites for mass spectrometric analysis.

The peaks corresponding to methylated metabolites of (-)-epicatechin or (-)-epigallocatechin were collected from the HPLC column and were dried under a stream of nitrogen. They were then analysed by liquid chromatography-mass spectrometry (Finnegan LCQ; Thermoquest Corp., San Jose, CA, USA) using electrospray-mass spectrometry with an ion trap operated in the negative-ion mode.

Results

Confirmation by mass spectrometry of metabolic formation of monomethylated tea catechins

Incubation of (-)-epigallocatechin with rat liver cytosol in the presence of [methyl-³H]S-adenosyl-L-methionine (0.25 µCi) resulted in formation of a less polar radioactive metabolite peak with a retention time of 22.0 min (figure 2A, B). Similarly, incubation of (-)-epicatechin with rat liver cytosol in the presence of

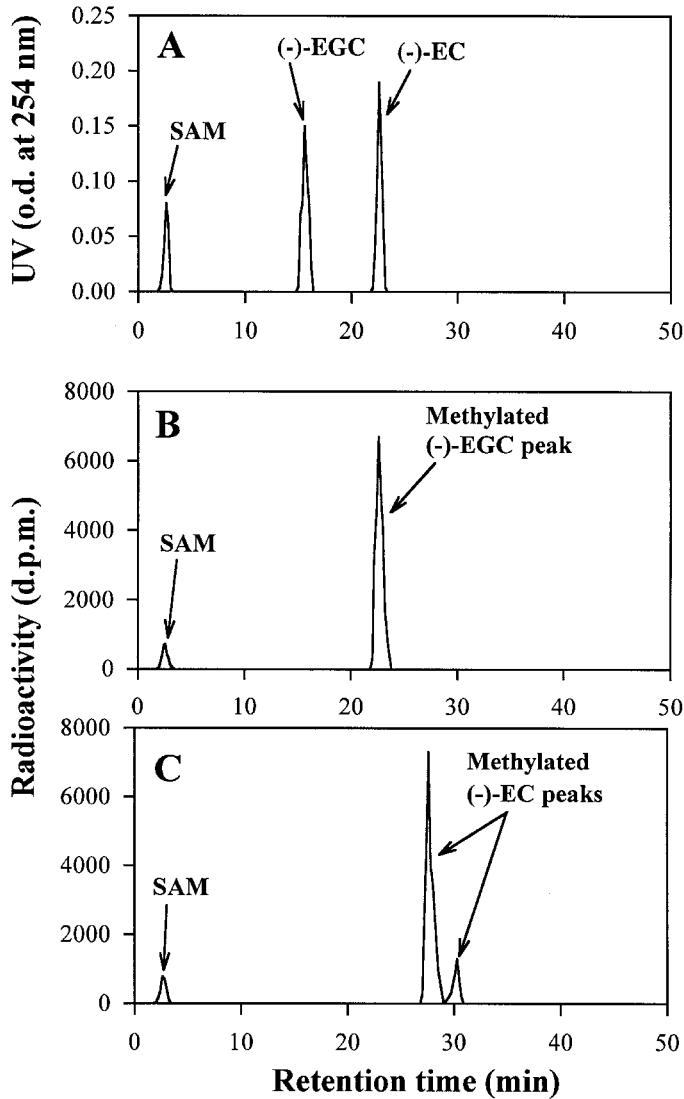


Figure 2. HPLC separation of the methylated metabolites of (–)-epicatechin and (–)-epigallocatechin formed by rat liver cytosol. (A) Representative UV trace for authentic *S*-adenosyl-*L*-methionine (SAM), (–)-epigallocatechin (EGC) and (–)-epicatechin (EC). (B, C) Representative radioactive traces for the methylated metabolites of (–)-epigallocatechin and (–)-epicatechin, respectively. The enzymatic formation and analysis of methylated tea catechins were as described in the Materials and methods.

[methyl-³H]*S*-adenosyl-*L*-methionine (0.25 μCi) resulted in formation of two less polar radioactive metabolite peaks, with retention times of 27.2 and 30.0 min, respectively (figure 2A, C). The ratio of the two radioactive peaks was ~5:1.

For mass spectrometric analysis of the methylated metabolites of tea catechins, we first used the HPLC to isolate sufficient amount of the 22.0-min metabolite peak formed from (–)-epigallocatechin and the 27.2-min metabolite peak formed from (–)-epicatechin. Analysis by liquid chromatography-mass spectrometry (LC/MS) of the metabolite derived from (–)-epigallocatechin (MW = 306) revealed a

base peak (deprotonated ion) with a mass of 319, consistent with the identity of this metabolite as a monomethylated metabolite of (–)-epigallocatechin. Similarly, analysis of the major metabolite derived from (–)-epicatechin (MW = 290) revealed a base peak (deprotonated ion) with a mass of 303, consistent with the identity of this major metabolite as a monomethylated metabolite of (–)-epicatechin.

Kinetic studies on the O-methylation of catechol-containing tea polyphenols by rat liver cytosol

When (–)-epicatechin (30 μM) was used as substrate for metabolic O-methylation by rat liver cytosol, formation of methylated products was dependent on incubation time (figure 3, upper) and cytosolic protein concentration (figure 3, lower). The dependence of O-methylation of a tea catechin on the concentrations

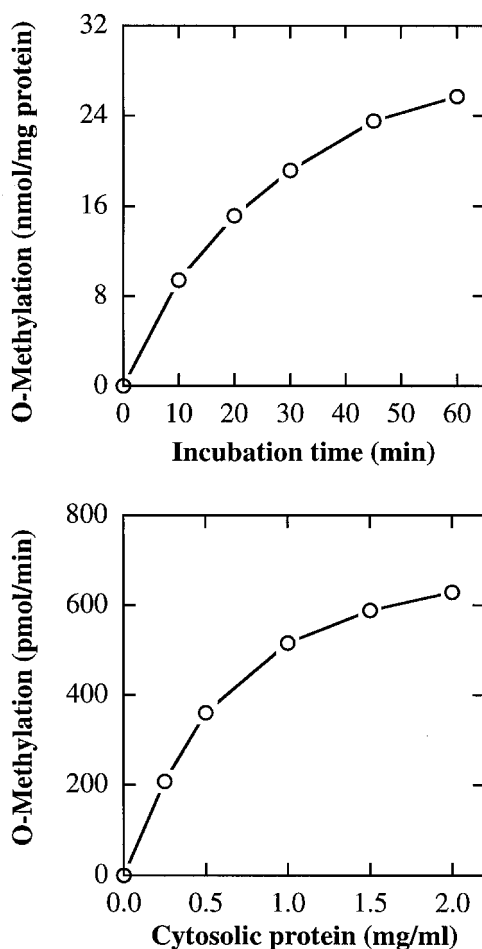


Figure 3. Dependence of hepatic O-methylation of (–)-epicatechin on incubation time (upper) and cytosolic protein concentration (lower). The incubation conditions and HPLC analyses were as described in the Materials and methods. Data are the mean of triplicate determinations.

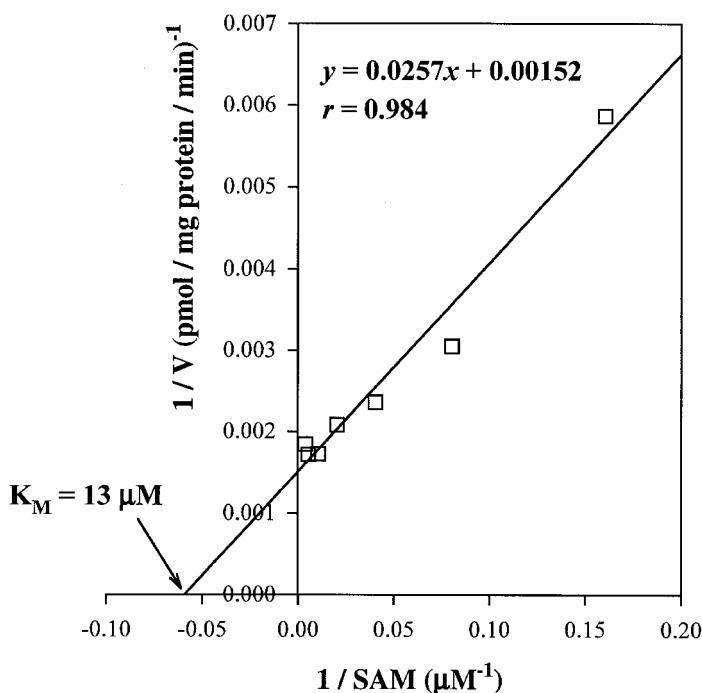


Figure 4. Double-reciprocal plot of increasing concentrations of *S*-adenosyl-L-methionine (SAM) versus the rate of *O*-methylation of (–)-epicatechin. The incubation conditions and HPLC analyses were as described in the Materials and methods. Each point is the mean of triplicate determinations.

of *S*-adenosyl-L-methionine follows typical Michaelis–Menten kinetics, with a $K_m \approx 13 \mu\text{M}$ as calculated from a double-reciprocal plot (figure 4).

We also determined the pH dependence (from 4.5 to 13) for metabolic *O*-methylation of (–)-epicatechin by rat liver cytosol (figure 5). Rates of (–)-epicatechin *O*-methylation by rat liver cytosol were highest at pH > 8.0. This pH dependence pattern is very different from that when human placental cytosol was used as the enzyme source, which showed a characteristic narrow pH optimum at ~7.4 (data not shown).

Under optimized conditions for *in vitro* *O*-methylation, (–)-epicatechin, (+)-epicatechin and (–)-epigallocatechin at a 10 or 100 μM concentration were all rapidly *O*-methylated by rat liver cytosol (table 1). Note that similar rates observed for the enzymatic *O*-methylation of (–)-epicatechin and (+)-epicatechin suggest that different stereochemistry (2*R*,3*R* versus 2*S*,3*S*; figure 1) between the two isomers does not significantly affect the rate of their enzymatic *O*-methylation *in vitro*. In comparison, (–)-epicatechin gallate and (–)-epigallocatechin gallate were *O*-methylated at markedly lower rates under the same reaction conditions (table 1).

We also determined the kinetic parameters (apparent K_m and V_{max}) for the *O*-methylation of (–)-epicatechin by rat liver cytosolic COMT. The rate of metabolic *O*-methylation of increasing concentrations of (–)-epicatechin at pH 7.4 or 10.0 showed typical Michaelis–Menten curve patterns (figure 6). Double-reciprocal plotting of this data showed the apparent K_m and V_{max} were 51 μM

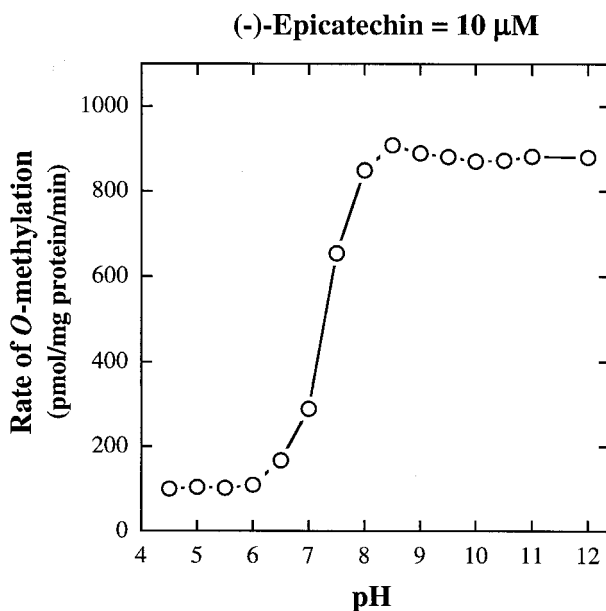


Figure 5. pH dependence of the *O*-methylation of (-)-epicatechin by rat liver cytosol. The indicated pH was the original pH of the 20 mM Tris-HCl stock buffer solution. The incubation conditions and HPLC analyses were as described in the Materials and methods. Each point is the mean of duplicate determinations.

Table 1. Rates of *O*-methylation of several catechol-containing tea polyphenols by rat liver cytosolic COMT at pH 7.4 and 10.0.

Tea polyphenol substrate	Concentration (μ M)	Rate of <i>O</i> -methylation (pmol mg protein ⁻¹ min ⁻¹)	
		pH 7.4	pH 10.0
(-)-Epicatechin	10	713 $\pm$ 19	870 $\pm$ 14
	100	1530 $\pm$ 29	1230 $\pm$ 6
(+) -Epicatechin	10	401 $\pm$ 13	777 $\pm$ 8
	100	1261 $\pm$ 3	1226 $\pm$ 10
(-)-Epigallocatechin	10	793 $\pm$ 18	580 $\pm$ 9
	100	1296 $\pm$ 5	927 $\pm$ 7
(-)-Epicatechin gallate	10	246 $\pm$ 9	145 $\pm$ 1
	100	243 $\pm$ 11	170 $\pm$ 6
(-)-Epigallocatechin gallate	10	262 $\pm$ 3	160 $\pm$ 2
	100	285 $\pm$ 8	160 $\pm$ 3

Incubation mixture consisted of 10 or 100 μ M of a tea polyphenol substrate, 50 μ M [³H-methyl]*S*-adenosyl-L-methionine (containing 0.2 μ Ci), 0.5 mg ml⁻¹ rat liver cytosolic protein, 1 mM dithiothreitol and 1.2 mM MgCl₂ in a final volume of 1.0 ml Tris-HCl buffer (10 mM, pH 7.4 or 10.0). Incubations were carried out at 37°C for 20 min. Each point is the mean $\pm$ SD of triplicate determinations. Measurements for most of the tea polyphenols substrates were repeated three times and similar results were observed.

and 2882 pmol mg protein⁻¹ min⁻¹, respectively, at pH 7.4, and 17 μ M and 2093 pmol mg protein⁻¹ min⁻¹, respectively, at pH 10.0. Similarly, the rate of metabolic *O*-methylation of (-)-epigallocatechin by rat liver cytosol also followed typical Michaelis-Menten kinetics (data not shown). The apparent *K_m* for the

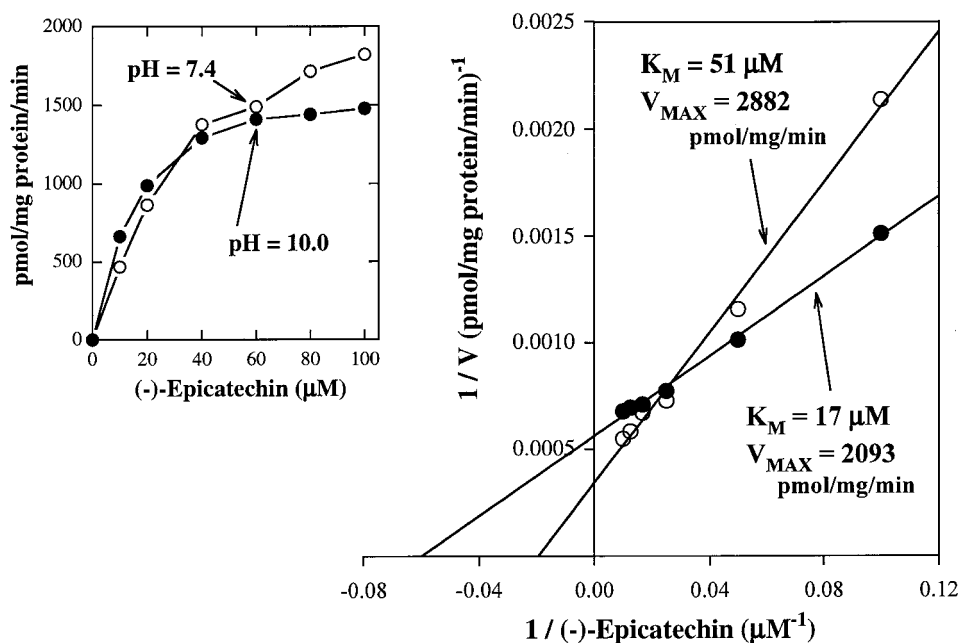


Figure 6. Double-reciprocal plots of the *O*-methylation of (–)-epicatechin by rat liver cytosol. The left inset depicts the relationship between the increasing concentrations of (–)-epicatechin and the rate of its enzymatic *O*-methylation *in vitro*. Open circles represent the data obtained at pH 7.4; solid circles at pH 10.0. The incubation conditions and HPLC analyses were as described in the Materials and methods. Each point is the mean of duplicate determinations.

O-methylation of (–)-epigallocatechin at pH 7.4 was $64 \mu\text{M}$, and the V_{max} was $1832 \text{ pmol mg protein}^{-1} \text{ min}^{-1}$.

Inhibition of rat liver O-methylation of tea polyphenols by S-adenosyl-L-homocysteine

When $5 \mu\text{M}$ (–)-epicatechin or (–)-epigallocatechin was used as substrate, their *O*-methylation by rat liver cytosol was inhibited by *S*-adenosyl-L-homocysteine (the demethylated product of *S*-adenosyl-L-methionine) in a concentration-dependent manner, with an $\text{IC}_{50} \sim 10 \mu\text{M}$. Kinetic analysis showed that *S*-adenosyl-L-homocysteine inhibited the COMT-catalysed *O*-methylation of (–)-epicatechin with a mixed (competitive plus non-competitive) mechanism (data not shown).

Discussion

It is known that polyphenolic dietary compounds (such as tea catechins and bioflavonoids) undergo extensive metabolism in the body to form more water-soluble conjugates, largely glucuronides and sulphates. Recently, we reported that the catechol-containing tea polyphenols could also be rapidly metabolized to monomethyl ethers by the cytosolic COMT present in human term placenta (Zhu *et al.* 2000). In the present study, it was found that several tea polyphenols

were rapidly O-methylated by rat liver cytosolic COMT. The results showed that (–)-epicatechin, (+)-epicatechin, (–)-epigallocatechin, (–)-epicatechin gallate and (–)-epigallocatechin gallate are all substrates for COMT-mediated O-methylation. Under the same conditions for *in vitro* O-methylation, (–)-epicatechin, (+)-epicatechin and (–)-epigallocatechin were rapidly O-methylated by rat liver cytosol, whereas (–)-epicatechin gallate and (–)-epigallocatechin gallate were O-methylated at significantly lower rates.

When (–)-epicatechin was used as a representative substrate, its O-methylation by rat liver cytosolic COMT showed characteristic dependence on the incubation pH, with maximal velocity observed at pH 8–12 (figure 5). Notably, when the same incubation conditions and the same substrate were used, human placental cytosolic COMT showed a narrow pH optimum at ~7.4 (Zhu *et al.* 2000), which is very different from the pH dependence observed in this study with rat liver cytosolic COMT. The reasons for the observed differences in pH dependence are not clear at present.

The results showed that the apparent K_m of *S*-adenosyl-L-methionine (the methyl donor) for the *in vitro* O-methylation of (–)-epicatechin is ~13 μM (figure 2). Since this *S*-adenosyl-L-methionine concentration is within the range of its tissue levels as previously reported for animals (Ueland 1982, Wagner *et al.* 1984, Zhu and Liehr 1996), it suggests that the COMT-catalysed rapid O-methylation of tea catechins is a biochemically feasible reaction *in vivo*.

During the metabolic O-methylation of catechol substrates (such as tea catechins), *S*-adenosyl-L-methionine is converted to *S*-adenosyl-L-homocysteine in equimolar amounts with the monomethyl products formed. It is known that *S*-adenosyl-L-homocysteine is an important feedback inhibitor for COMT-catalysed O-methylation of a variety of catechol substrates (Ueland 1982). The present results also showed that the rapid metabolic O-methylation of tea polyphenols is subject to the inhibitory regulation by *S*-adenosyl-L-homocysteine. This potent inhibition of the O-methylation of tea catechols by *S*-adenosyl-L-homocysteine may effectively prevent the endogenous *S*-adenosyl-L-methionine pool from being readily depleted, which would be important for maintaining the homeostasis of the universal methyl donor *S*-adenosyl-L-methionine during metabolic O-methylation of excess amounts of endogenous and/or exogenous catechols.

Regarding the possible structures of the methylated tea polyphenols, earlier studies with norepinephrine and quercetin (a catechol-containing flavonoid) showed that COMT at pH 7.4 predominantly methylates the *meta*-hydroxyl group of these substrates (Creveling 1970, Creveling *et al.* 1972, Zhu *et al.* 1994). Our HPLC analysis of the methylated products of (–)-epicatechin showed two less polar peaks with ~5:1 ratio (figure 2). Further LC/MS analysis confirmed that the quantitatively major metabolite of (–)-epicatechin was a monomethylated derivative. It is thus suspected that the major monomethylated metabolite of (–)-epicatechin likely is the 3'-O-methyl isomer, with the 4'-O-methyl isomer a minor metabolite (see figure 1 for the 3' and 4' positions). Similarly, it is suspected that the major methylated metabolite of (–)-epigallocatechin would be the 3'-O-methyl isomer. This suggestion is consistent with our chromatographic observation showing that one major monomethylated metabolite (likely the 3'-O-methyl metabolite) was detected on HPLC (figure 2). The largely undetectable formation of 4'-O-methyl metabolite from (–)-epigallocatechin as substrate might be, in part, due to the following two reasons: (1) (–)-epigallocatechin has two identical

meta-hydroxyl groups (i.e. two 3'-hydroxyl groups) that could be *O*-methylated at pH 7.4, and this would quantitatively favour the formation of 3'-*O*-methyl product relative to the 4'-*O*-methyl product when compared to (–)-epicatechin as substrate; and (2) the 4'-hydroxyl group of (–)-epigallocatechin might have lower accessibility by the enzyme than that of (–)-epicatechin.

Several recent studies showed that many common tea polyphenols such as (–)-epigallocatechin and (–)-epicatechin could be readily absorbed in human subjects after drinking tea, and some of them can reach submicromolar concentrations (0.1–0.4 μM) in the plasma (Lee *et al.* 1995, Unno *et al.* 1996, Yang *et al.* 1998, van het Hof *et al.* 1998, Li *et al.* 2000). A recent study using ^3H -labelled (–)-epigallocatechin gallate also showed that this tea catechin is widely distributed in the mouse tissues (Suganuma *et al.* 1998). It is thus expected that a significant fraction of the ingested catechol-containing tea polyphenols would be *O*-methylated in the body (Hackett and Griffiths 1981, 1982, Kuhnle *et al.* 2000). It will be of considerable interest to determine whether there are any important biological actions that are associated with methylated tea polyphenols. Noteworthy, Sano *et al.* (1999) reported that two quantitatively minor methylated catechins isolated from oolong tea showed a stronger anti-allergic activity than an unmethylated catechin. Likewise, it will also be of interest to determine whether tea polyphenols can effectively inhibit the metabolic *O*-methylation of endogenous catecholamines and catechol oestrogens, and to determine whether some of tea's effects are related to inhibition of the methylation metabolism of these endogenous catechols. Effective inhibition of the *O*-methylation of endogenous catecholamines by tea components would be expected to alter their physiological functions as neurotransmitters/neurohormones, and inhibition of COMT-catalysed methylation metabolism of levodopa (the most effective agent for treatment of Parkinson's disease) would markedly increase its bioavailability in the central nervous system and reduce its peripheral side-effects. In the case of catechol oestrogens, inhibition of COMT-catalysed *O*-methylation of endogenous 2- and 4-hydroxylated oestrogens by tea polyphenols may lead to elevated tissue levels of the procarcinogenic 4-hydroxyoestradiol (Liehr 2000) plus decreased tissue levels of the anticarcinogenic 2-methoxyoestradiol (Zhu and Conney 1998). Although our earlier studies showed that chronic administration of dietary quercetin (a strong inhibitor of catechol oestrogen *O*-methylation *in vivo*) enhanced oestradiol-induced kidney tumour formation in male Syrian hamsters (Zhu *et al.* 1994, Zhu and Liehr 1994), the effects of tea polyphenols on hormonal carcinogenesis remain to be determined.

In summary, several catechol-containing tea polyphenols are very rapidly *O*-methylated by rat liver cytosolic COMT. This metabolic *O*-methylation of tea polyphenols is subject to inhibition by *S*-adenosyl-L-homocysteine. The results of the present study call for further research to determine whether some of the important biological actions of tea polyphenols may be caused by their methylated products. Studies are also needed to determine the potential inhibitory effects of tea polyphenols on the COMT-catalysed *O*-methylation of endogenous catecholamines and catechol oestrogens.

Acknowledgements

The authors thank Mao-Jung Lee and Chung S. Yang for advice on the HPLC separation of tea catechins, and Chuan Li, Bozena Winnik and Brian Buckley for

help with the liquid chromatography-mass spectrometry analysis of the O-methylated catechins.

References

- AXELROD, J., 1966, Methylation reactions in the formation and metabolism of catecholamines and other biogenic amines. *Pharmacological Reviews*, **18**, 95–113.
- AXELROD, J. and TOMCHICK, R., 1958, Enzymatic O-methylation of epinephrine and other catechols. *Journal of Biological Chemistry*, **233**, 702–705.
- BALENTINE, D. A., 1992, Manufacturing and chemistry of tea. In C. T. Ho, M. T. Huang and C. Y. Lee (eds), *Phenolic Compounds in Food and their Effects on Health I* (Washington, DC: American Chemical Society Press), pp. 102–117.
- CREVELING, C. R., DELGARD, N., SCHIMIZU, H. and DALY, J. W., 1970, Catechol O-methyltransferase. 3. *m*- and *p*-O-methylation of catecholamines and their metabolites. *Molecular Pharmacology*, **6**, 691–696.
- CREVELING, C. R., MORRIS, N., ONG, H. H. and DALY, J. W., 1972, Catechol O-methyltransferase. IV. Factors affecting *m*- and *p*-methylation of substituted catechols. *Molecular Pharmacology*, **8**, 398–409.
- GULDBERG, H. C. and MARSDEN, C. A., 1975, Catechol-O-methyltransferase: Pharmacological aspects and physiological role. *Pharmacological Reviews*, **27**, 135–206.
- HACKETT, A. M. and GRIFFITHS, L. A., 1981, The metabolism and excretion of 3-O-methyl-(+)-catechin in the rat, mouse, and marmoset. *Drug Metabolism and Disposition*, **9**, 54–59.
- HACKETT, A. M., SHAW, I. C. and GRITHS, L. A., 1982, 3'-O-methyl-(+)-catechin glucuronide and 3'-O-methyl-(+)-catechin sulphate: new urinary metabolites of (+)-catechin in the rat and the marmoset. *Experientia*, **38**, 538–540.
- KUHNLE, G., SPENCER, J. P. E., SCHROETER, H., SHENOY, B., DEBNAM, E. S., SRAI, S. K. S., RICE-EVANS, C. and HAHN, U., 2000, Epicatechin and catechin are O-methylated and glucuronidated in the small intestine. *Biochemical and Biophysical Research Communications*, **277**, 507–512.
- LEE, M.-J., WANG, Z.-Y., LI, H., CHEN, L., SUN, Y., GOBBO, S., BALENTINE, D. A. and YANG, C. S., 1995, Analysis of plasma and urinary tea polyphenols in human subjects. *Cancer Epidemiology, Biomarker, and Prevention*, **4**, 393–399.
- LI, C., LEE, M. J., SHENG, S., MENG, X., PRABHU, S., WINNIK, B., HUANG, B., CHUNG, J. Y., YAN, S., HO, C. T. and YANG, C. S., 2000, Structural identification of two metabolites of catechins and their kinetics in human urine and blood after tea ingestion. *Chemical Research in Toxicology*, **13**, 177–184.
- LIEHR, J. G., 2000, Is estradiol a genotoxic mutagenic carcinogen? *Endocrine Reviews*, **21**, 40–54.
- PISKULA, M. K. and TERAQ, J., 1998, Accumulation of (–)-epicatechin metabolites in rat plasma after oral administration and distribution of conjugation enzymes in rat tissues. *Journal of Nutrition*, **128**, 1172–1178.
- SANO, M., SUZUKI, M., MIYASE, T., YOSHINO, K. and MAEDA-YAMAMOTO, M., 1999, Novel antiallergic catechin derivatives isolated from oolong tea. *Journal of Agricultural and Food Chemistry*, **47**, 1906–1910.
- SUGANUMA, M., OKABE, S., ONIYAMA, M., TADA, Y., ITO, H. and FUJIKI, H., 1998, Wide distribution of [³H](–)-epigallocatechin gallate, a cancer preventive tea polyphenol, in mouse tissue. *Carcinogenesis*, **19**, 1771–1776.
- THAKKER, D. R. and CREVELING, C. R., 1990, O-methylation. In G. J. Mulder (ed.), *Conjugation Reactions in Drug Metabolism* (London: Taylor & Francis), pp. 193–232.
- UELAND, P. M., 1982, Pharmacological and biochemical aspects of S-adenosylhomocysteine and S-adenosylhomocysteine hydrolase. *Pharmacological Reviews*, **34**, 223–253.
- UNNO, T., KONDO, K., ITAKURA, H. and TAKEO, T., 1996, Analysis of (–)-epigallocatechin gallate in human serum obtained after ingesting green tea. *Bioscience, Biotechnology and Biochemistry*, **60**, 2066–2068.
- VAN HET HOF, K. H., KIVITS, G. A. A., WESTSTRATE, J. A. and TIJBURG, L. B. M., 1998, Bioavailability of catechins from tea: the effect of milk. *European Journal of Clinical Nutrition*, **52**, 356–359.
- WAGNER, J., CLAVERIE, N. and DANZIN, C., 1984, A rapid high-performance liquid chromatographic procedure for the simultaneous determination of methionine, ethionine, S-adenosylmethionine, S-adenosylethionine, and the natural polyamines in rat tissues. *Analytical Biochemistry*, **140**, 108–116.
- YANG, C. S., CHEN, L., LEE, M.-J., BALENTINE, D., KUO, M. C. and SCHANTZ, S. P., 1998, Blood and urine levels of tea catechins after ingestion of different amounts of green tea by human volunteers. *Cancer Epidemiology, Biomarkers and Prevention*, **7**, 351–354.
- ZHU, B. T. and CONNEY, A. H., 1998, Is 2-methoxyestradiol an endogenous estrogen metabolite that inhibits mammary carcinogenesis? *Cancer Research*, **58**, 2269–2277.

- ZHU, B. T., EZELL, E. L. and LIEHR, J. G., 1994, Catechol-*O*-methyltransferase-catalyzed *O*-methylation of mutagenic flavonoids: metabolic inactivation as possible reason for their lack of carcinogenicity *in vivo*. *Journal of Biological Chemistry*, **269**, 292–299.
- ZHU, B. T. and LIEHR, J. G., 1994, Quercetin increases the severity of estradiol-induced tumorigenesis in hamster kidney. *Toxicology and Applied Pharmacology*, **125**, 149–158.
- ZHU, B. T. and LIEHR, J. G., 1996, Inhibition of catechol-*O*-methyltransferase-catalyzed *O*-methylation of 2- and 4-hydroxyestradiol by quercetin. *Journal of Biological Chemistry*, **271**, 1367–1363.
- ZHU, B. T., PATEL, U. K., CAI, M. X. and CONNEY, A. H., 2000, *O*-Methylation of tea polyphenols catalysed by human placental cytosolic catechol-*O*-methyltransferase. *Drug Metabolism and Disposition*, **28**, 1024–1030.
- ZHU, B. T., TANEJA, N., LODER, D. P., BALENTINE, D. A. and CONNEY, A. H., 1998, Effects of tea polyphenols and flavonoids on liver microsomal glucuronidation of estradiol and estrone. *Journal of Steroid Biochemistry and Molecular Biology*, **64**, 207–215.

Plasma concentrations of individual tea catechins after a single oral dose in humans

J. M. M. VAN AMELSVOORT*, K. H. VAN HET HOF, J. N. J. J. MATHOT, T. P. J. MULDER, A. WIERSMA and L. B. M. TIJBURG

Unilever Health Institute, Unilever Research, PO Box 114, 3130 AC Vlaardingen, The Netherlands

Received 2 April 2001

1. Ten healthy volunteers ingested 1.5 mmole epicatechin gallate (ECg), epigallocatechin (EGC) or epigallocatechin gallate (EGCg) in a randomized crossover design. After deconjugation, catechins in plasma and 24-h urine samples were determined by high-performance liquid chromatography (HPLC). Antioxidant activity was measured in plasma by determining ferric reducing activity (FRAP).

2. The catechin levels in plasma after ingestion were significantly different: EGC rose quickly with a short elimination half-life ($t_{1/2 \text{ elim}} = 1.7 \text{ h}$), ECg was intermediate in rise but slowest in decline ($t_{1/2 \text{ elim}} = 6.9 \text{ h}$), EGCg was slowest in rise but intermediate in decline ($t_{1/2 \text{ elim}} = 3.9 \text{ h}$). At 24 h, EGC and EGCg had returned to base levels, but ECg was still elevated. Peak maximum varied between 1.3 (EGCg) and $5.0 \mu\text{mol l}^{-1} \text{ (EGC)}$.

3. Very limited interconversion ($\text{ECg} \rightarrow \text{epicatechin}$, $\text{EGCg} \rightarrow \text{EGC}$) occurred indicating that degallation is not required for uptake.

4. Up to 13.6% of the ingested EGC (partly methylated) was excreted in the urine, but ECg or EGCg were not detected.

5. EGC and ECg produced an increase in antioxidant activity in plasma, but with EGCg, no statistically significant effect was found.

6. The pattern of uric acid in plasma showed a clear resemblance with that of FRAP and linear regression analysis indicated a very significant relationship ($R^2 = 0.88$, $p < 0.0001$).

7. It is concluded that tea catechins differ significantly in their pharmacokinetic behaviour.

Introduction

Epidemiological studies indicate that tea consumption may reduce the risk of cardiovascular disease (Hertog *et al.* 1993, Keli *et al.* 1996, Tijburg *et al.* 1997). In addition, there is increasing evidence that green or black tea consumption inhibits various types of cancer in experimental animals (Yang and Wang 1993). These effects may be due to the antioxidant properties of tea flavonoids (Wiseman *et al.* 1997, Leenen *et al.* 2000). Green and black tea originate from the same plant, *Camellia sinensis*, and drying the freshly picked leaves produces green tea. It contains mainly catechins (figure 1), which are flavanol monomers and strong antioxidants *in vitro* (Salah *et al.* 1995). Quantitatively, the most important catechins in green tea are (in descending order): epigallocatechin gallate (EGCg), epigallocatechin (EGC), epicatechin gallate (ECg) and epicatechin

* Author for correspondence; e-mail: Hans-van.Amelsvoort@unilever.com

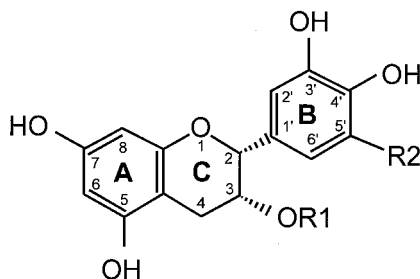


Figure 1. Structures of (–)epicatechins. Epicatechin, R1=H, R2=H; epigallocatechin, R1=H, R2=OH; epicatechin gallate, R1=gallate, R2=H; epigallocatechin gallate, R1=gallate, R2=OH. Gallate = 3,4,5-trihydroxybenzoic acid.

(EC), although ECg is the dominant catechin in some tea cultivars (Shao *et al.* 1995). The analysis of catechins in biological fluids is technically difficult, but in the past few years significant progress has been made (Dalluge and Nelson 2000, Lee *et al.* 2000).

Although recently studies on the plasma concentrations of individual catechins from either green tea (Yang *et al.* 1998, Hof *et al.* 1998, 1999, Chow *et al.* 2001) or black tea (Warden *et al.* 1999) have appeared, there are currently no data from well-designed studies using equimolar doses of single pure catechins in humans (Hollman *et al.* 1997). This information is important because Chen *et al.* (1997) reported that the plasma concentration and elimination of EGCg in rat are affected very significantly by other components in decaffeinated green tea. In contrast, however, Chow *et al.* (2001) reported that very similar plasma EGCg levels were detected when pure EGCg or a decaffeinated green tea preparation containing mainly EGCg were ingested by healthy volunteers. To avoid interference by other compounds present in tea extracts, we now report the post-prandial response of the individual tea catechins (EGCg, EGC, ECg) dosed in equimolar amounts in a crossover design in healthy volunteers.

Materials and methods

The protocol of the study and information brochure for the healthy volunteers were approved by the Medical Ethical Committee of the 'Nederlandse Unilever Bedrijven BV'. Volunteers were recruited by advertisements in the local laboratory newspaper. Inclusion criteria were: age between 18 and 70 years, body mass index between 19 and 32 kg m⁻², blood haemoglobin >7.5 (women) or 8.0 (men) mmol l⁻¹, not on a specific diet, alcohol consumption <21 (women) or 28 (men) units week⁻¹. Those taking vitamin supplements or medicines and smokers, as well as pregnant or lactating women, were excluded from the study. A group of three female and seven male volunteers participated in the study. Their body characteristics were (averages): age 48 years (SD = 13.1), height 1.78 m (0.09), body weight 82 kg (13.6), body mass index 25.8 kg m⁻² (3.05). The tea catechins EGCg, EGC and ECg were kindly supplied by Dr J. R. Lewis of Unilever Research Laboratory Colworth (Sharnbrook, Bedford, UK). When analysed by HPLC using detection at 280 nm, all catechins were >99% pure. The catechins had been isolated from green tea by methanol, ethyl acetate and chloroform solvent extraction, and subsequently purified by column chromatography on Sephadex LH-20 with ethanol elution and double recrystallization from water and final freeze drying and stored at –20°C. The final preparations contained between 5 and 10% (w/w) water.

On the test days, 1.5 mmole of either EGCg, EGC or ECg was dissolved in 150 ml hot water. Antioxidant-free syrup mixture (Euroshopper Burg All In, Ter Aar, The Netherlands) was added to improve palatability. The control treatment consisted of hot water and syrup. The beverage was consumed in <15 min in a fasting state in combination with a standard breakfast. A restricted range of cold foods and hot meals, low in flavonoids and anti-oxidant vitamins, was provided during the

experimental day. In addition, volunteers had been asked not to consume flavonoid-rich foods during the preceding day.

The experimental tea components were consumed in a randomized crossover design with 9-day intervals. Blood samples were taken before and at various times after consumption of the tea compounds until 24 h after consumption. Plasma was prepared by centrifugation at 1500g. Urine was collected and stored at 4°C in containers containing freshly dissolved ascorbic acid and Na₂EDTA as preservatives (final concentrations 0.4% w/v and 0.002% w/v, respectively; Lee *et al.* 1995), and sampled after completion of the 24 h post-prandial period. All samples were stored at -70°C until analysis.

Catechins in plasma and urine samples were deconjugated (removal of any sulphate and/or glucuronic acid groups) by mixing 0.5 ml plasma with 0.1 ml of a solution containing 20% (w/v) ascorbic acid, 0.1% (w/v) Na₂EDTA, 10 mmol l⁻¹ propylgallate (to suppress esterase activity), NaH₂PO₄/NaOH (pH 3.6) and 25 µl of a β-glucuronidase/sulphatase enzyme preparation from *Helix pomatia*, (Sigma Chemical Co., St Louis, MO, USA). After incubation for 40 min at 37°C under an argon atmosphere, the reaction was stopped by addition of 0.5 ml of a 1:1 (v/v) mixture of a 5% (w/v) NaCl solution and methanol. After centrifugation for 5 min at 2500g, the water layer was removed and the pellet re-extracted with 0.5 ml 5% (w/v) NaCl. Next, the combined water layers were extracted with 2 ml ethyl acetate. After re-extraction of the water layer, the combined ethyl acetate layers were stored at -20°C (24 h maximum) until analysis.

Before analysis, the organic solvent was evaporated under a stream of nitrogen gas. The residue was completely dissolved in 0.1 ml methanol/10 mmol l⁻¹ H₃PO₄ solution (1:1 v/v), and a 20-µl sample was injected on an Inertsil 5 ODS-2 column (Chrompack, Middelburg, The Netherlands) operated at 40°C with water/methanol/acetic acid (800/200/5 v/v/v) as mobile phase at a flow rate of 0.5 ml min⁻¹. Post-column detection was performed by mixing the eluent with 1 ml min⁻¹ of a solution containing 56 mmol l⁻¹ 4-dimethyl amino cinnamaldehyde in methanol/perchloric acid (9:1 v/v) (Kivits *et al.* 1997). After development of the colour in a reaction coil (PTFE, 15 m, 0.023-inch internal diameter) the absorbance was read at 635 nm. This colour reaction is specific for flavonoids containing meta-oriented hydroxyl groups in the A-ring and the absence of the carbonyl at C4 (Treuter 1989).

The pure catechins were used as a standard and were analysed without prior deconjugation. Methylated EGC was quantified assuming an extinction coefficient similar to that of free EGC (Da Silva *et al.* 1998, Manach *et al.* 1999). To determine the retention time of the methylated EGC, it was prepared from EGC and *S*-adenosyl-L-methionine (5 mmol l⁻¹) by the action of catechol-*O*-methyltransferase (250 kU l⁻¹, EC 2.1.1.6 from porcine liver *ex* Sigma) in the presence of 1 mmol l⁻¹ glutathione, 25 mmol l⁻¹ MgCl₂, 0.1 mol l⁻¹ Tris-HCl, pH 7.8, at 37°C essentially as described by Zhu *et al.* (1994).

Antioxidant activity was measured by means of ferric reducing activity of plasma (FRAP) (Benzie and Strain 1996), and uric acid was determined according to Town *et al.* (1985).

Pharmacokinetic parameters were calculated using compartment model-independent analysis. Elimination half-lives ($t_{1/2 \text{ elim}}$) were calculated over the range 4–8 h and were based on linear regression analysis. The area under the curve (AUC) was calculated from the actual data points (and not from any fit over 24 h) using the linear trapezoidal rule. The same approach was used for the calculation of the time to reach maximal plasma concentration (T_{max}). Statistical analysis was performed by analysis of variance with treatment as factor and subject as block; differences between treatments were established using Tukey's test. SAS package release 6.12 was used.

Results

The post-prandial plasma curves of the enzymatically deconjugated catechins epigallocatechin (EGC), epigallocatechin gallate (EGCg) and epicatechin gallate (ECg), each given at a dose of 1.5 mmole, are presented in figure 2. The values found with the control drink without catechins did not differ significantly from the starting value and are not shown. There is a clear difference in the kinetics of appearance of the three catechins in the blood (table 1) with EGC showing a rapid rise and decline ($t_{1/2 \text{ elim}} = 1.7 \text{ h}$), ECg being intermediate in rise but slowest in decline ($t_{1/2 \text{ elim}} = 6.9 \text{ h}$), and EGCg rising slowest but with an intermediate rate of elimination ($t_{1/2 \text{ elim}} = 3.9 \text{ h}$). Interestingly, at 24 h, EGC and EGCg had returned to their starting levels, whereas a significant amount of ECg was still present in the blood. The maximum plasma concentration that was reached varied from 1.3 µmol l⁻¹ for EGCg to 3.1 µmol l⁻¹ for ECg, and 5.0 µmol l⁻¹ for EGC (table 1). AUC_{0-24h} followed a different order with EGCg being the smallest

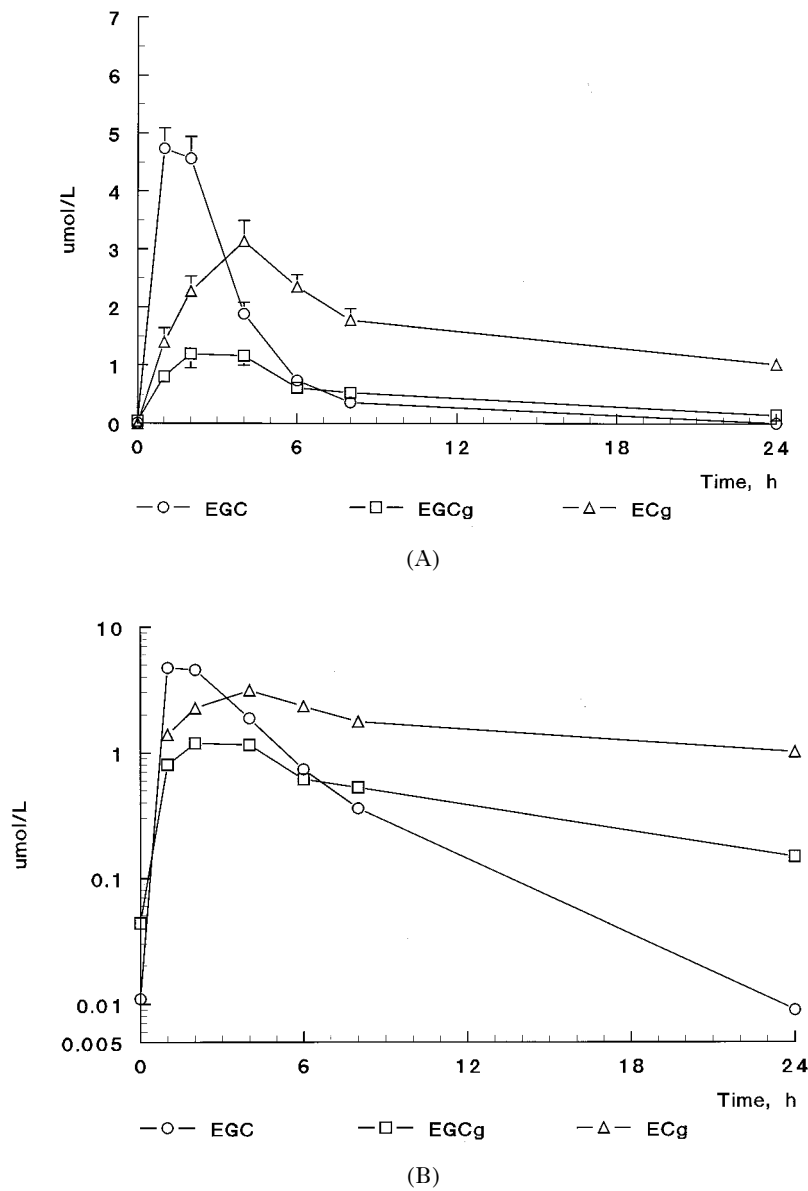


Figure 2. Post-prandial levels of enzymatically deconjugated catechins in blood plasma of volunteers given 1.5 mmol of either epigallocatechin (EGC, open circles), epigallocatechin gallate (EGCg, open squares) or epicatechin gallate (ECg, open triangles) on separate days. (A) Means with SEM on a linear scale, (B) means on a semilogarithmic scale ($n = 9$); EGCg ($n = 8$). The values for the control did not differ significantly from the starting value ($<0.02 \mu\text{mol l}^{-1}$) and hence are not shown.

($12.1 \text{ h} \mu\text{mol l}^{-1}$), EGC intermediate ($20.1 \text{ h} \mu\text{mol l}^{-1}$) and ECg largest ($39.9 \text{ h} \mu\text{mol l}^{-1}$). The observed difference in $\text{AUC}_{0-24\text{h}}$ between ECg and EGCg and their similar elimination rates indicate a much higher bioavailability of ECg.

With all three catechins, the molecular species consumed was also the major constituent found in the plasma. Limited, but clearly detectable, degallation

Table 1. Pharmacokinetic data of the post-prandial curves of catechin concentrations in plasma (means with SEM, $n = 9$; see figures 1 and 2)^a.

Treatment	$C_{\max}$ ($\mu\text{mol l}^{-1}$)	$T_{\max}$ ^b (h)	Area under curve 0–24 h ($\text{h } \mu\text{mol l}^{-1}$)	$t_{1/2}$ of elimination (h)
EGC	5.0 ± 0.32	1.4	20.1 ± 1.55	1.7 ± 0.05
MeEGC	1.9 ± 0.14	2	12.6 ± 0.98	2.5 ± 0.07
MeEGC + EGC	6.7 ± 0.43	1.7	32.8 ± 2.47	2.0 ± 0.05
EGCg ^c	1.3 ± 0.23	2.9	12.1 ± 1.47	3.9 ± 0.71
ECg	3.1 ± 0.35	4	39.9 ± 3.81	6.9 ± 1.95

^a Samples were treated with glucuronidase and sulphatase before analysis.^b Estimated mean, $T_{\max}$, was calculated from the actual data points and not from any curve fit.^c $n = 8$.Table 2. Amounts of epigallocatechin (EGC) and methylated epigallocatechin (Me-EGC) excreted in the urine in 24 h after consumption of 1.5 mmol EGC^a (means with SEM, $n = 9$)^b.

Parameter	$\mu\text{mole } 24 \text{ h}^{-1}$	Percentage of dose ingested
EGC	147 ± 12	9.8 ± 0.8
Me-EGC	57 ± 4	3.8 ± 0.3
EGC + MeEGC	204 ± 16	13.6 ± 1.0

^a With EGCg or ECg, <0.02% of the amount consumed was found in 24 h.^b Samples were treated with glucuronidase and sulphatase before analysis.

occurred after ingestion of EGCg and ECg. After consumption of EGCg, almost all of the catechin was recovered from plasma as EGCg, but 5.1% of the $\text{AUC}_{0-24\text{h}}$ occurred as EGC. After ECg consumption $\sim 3.3\%$ of the catechin detected in plasma was EC and not ECg. With EGC, a separate peak was found in the HPLC trace of the plasma. This peak co-eluted with *O*-methylated EGC (Me-EGC), and hence was assumed identical to that. No additional peaks were seen in the chromatogram after consumption of EGCg or ECg. Since the putative conversion of EGC to Me-EGC leads to an underestimation of the actual amount of EGC absorbed, we also calculated the sum of EGC and MeEGC (figure 3 and table 1). The peak maximum and $\text{AUC}_{0-24\text{h}}$ for the EGC + MeEGC thus increased to 6.7 and $32.8 \text{ h } \mu\text{mol l}^{-1}$, respectively.

With EGCg or ECg, <0.02% of the amount consumed was excreted in urine in 24 h. The amount of EGC excreted in the urine in 24 h is given in table 2. Again part of the EGC occurred in the methylated form. Hence, 13.6% of the ingested dose of EGC was excreted in 24 h, of which 3.8% was methylated and 9.8% was in the non-methylated form.

The changes in FRAP (a measure of the antioxidant activity) in the blood of the volunteers during the full 24 h after ingestion of the catechins or the control drink is presented in figure 4A. EGC induced a significant increase in FRAP levels when compared with control at one ($39.0 \pm 5.2 \mu\text{mol l}^{-1}$ increase relative to starting value versus $11.7 \pm 8.1 \mu\text{mol l}^{-1}$ increase relative to starting value, respectively; $p < 0.01$) and at 2 h (10.6 ± 4.3 increase relative to starting value versus $19.7 \pm 4.0 \mu\text{mol l}^{-1}$ decrease relative to starting value, respectively; $p < 0.01$) after consumption. ECg also induced an increase in FRAP level when compared with control at 2 h after consumption (1.8 ± 7.8 increase relative to starting value versus $19.7 \pm 4.0 \mu\text{mol l}^{-1}$ decrease relative to starting value, respectively; $p < 0.05$). For EGCg, the change

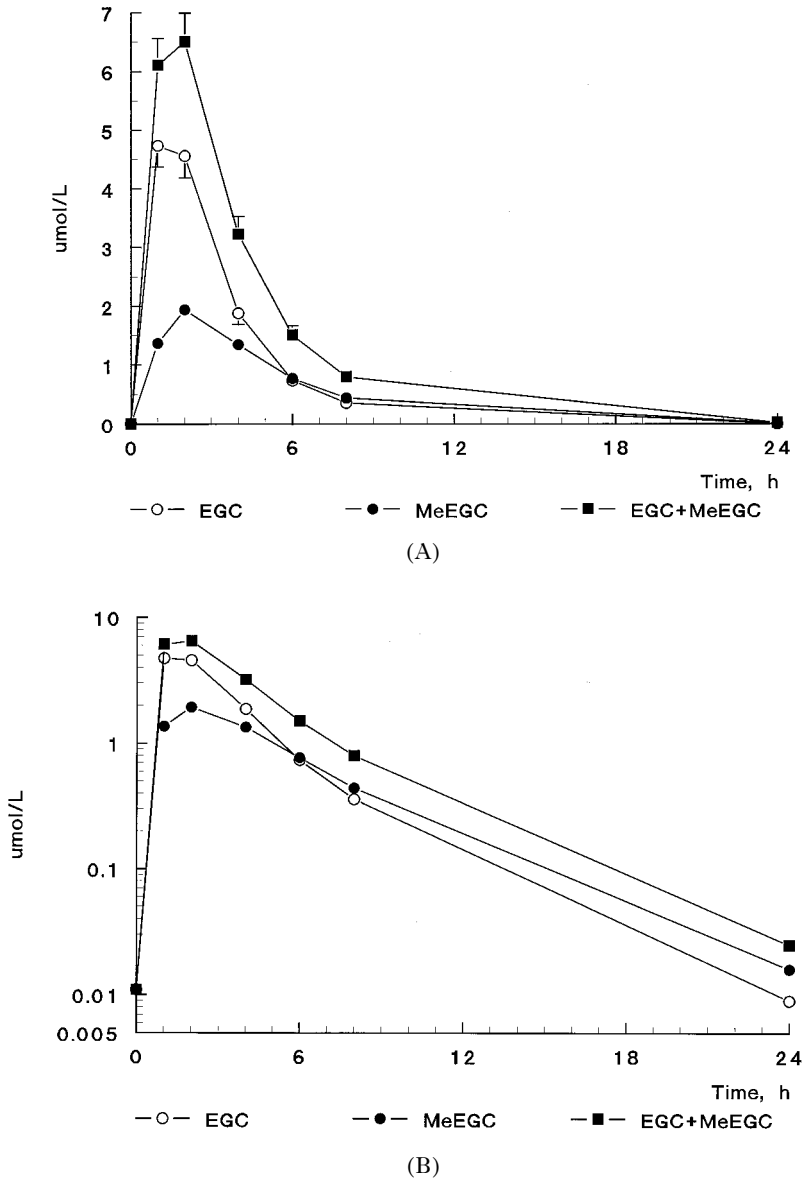
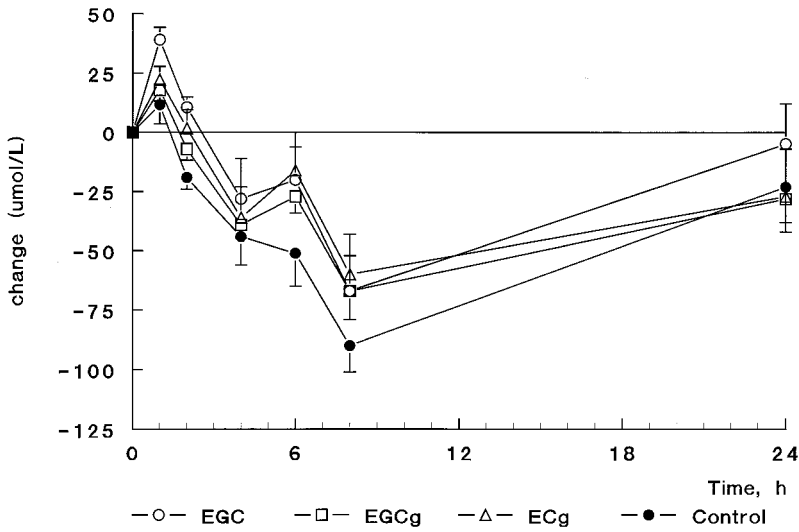
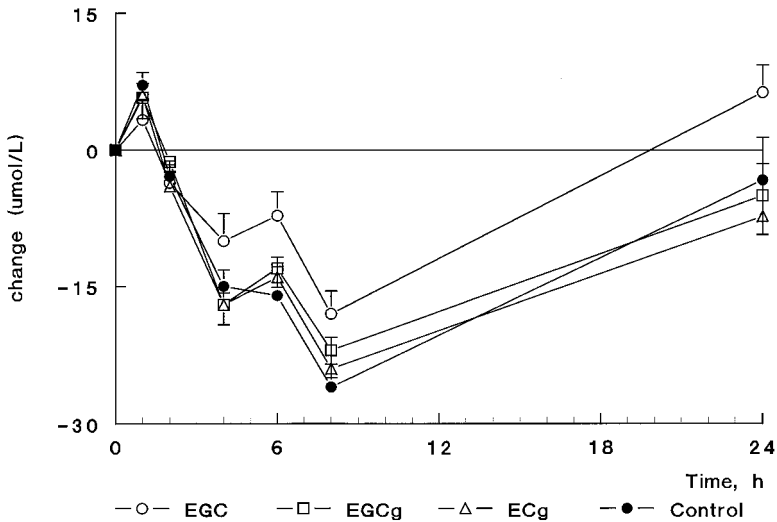


Figure 3. Post-prandial levels of enzymatically deconjugated epigallocatechin (EGC, open circles) and methylated epigallocatechin (Me-EGC, closed circles) and the combination of EGC and Me-EGC (closed squares) in blood plasma of volunteers given 1.5 mmol epigallocatechin. (A) Means with SEM on a linear scale, (B) means on a semilogarithmic scale ($n = 9$).

in FRAP relative to the starting value was never significantly different from control. There were no statistically significant differences in FRAP of the points after 2 h (figure 4A), neither in the $\text{AUC}_{0-24\text{h}}$ (table 3). Since the FRAP data at 6 and 8 h suggested a higher level for the catechins than control (figure 4A), it was calculated the AUC for FRAP and uric acid for 2–8, 4–8 and 6–8 h (table 3; data for 4–8 h not shown). In this case, the FRAP $\text{AUC}_{2-8\text{h}}$ for EGC and FRAP $\text{AUC}_{6-8\text{h}}$ for EGC tended to be higher than control (-152 versus $-298\text{ h}\mu\text{mole l}^{-1}$,



(A)



(B)

Figure 4. (A) Change in ferric reducing activity of plasma (FRAP) ($\mu\text{mol l}^{-1}$) in blood plasma of the volunteers during the 24 h after consumption of the tea catechins (means with SEM, $n = 9$; epigallocatechin gallate, $n = 8$). At 1 h, the value obtained after epigallocatechin was significantly elevated ($p < 0.01$) when compared with the control. At 2 h, both epigallocatechin ($p < 0.01$) and epicatechin gallate ($p < 0.05$) gave significantly higher FRAP levels when compared with the corresponding control. (B) Change in uric acid ($\mu\text{mol l}^{-1}$) in blood plasma of the volunteers after consumption of the tea catechins (means with SEM, $n = 9$; epigallocatechin gallate, $n = 8$). Epigallocatechin, open circles; epigallocatechin gallate, open squares; epicatechin gallate, open triangles; control, closed circles.

and -75.6 versus -140 $\mu\text{mol l}^{-1}$, respectively) but the difference did not reach statistical significance ($0.05 < p < 0.10$).

The changes in uric acid in the blood plasma of the volunteers are presented in figure 4B. At none of the time points was a significant difference detected between

Table 3. Areas under the post-prandial curves (hµmole l⁻¹) of FRAP and uric acid in plasma for the first 2 h, between 2 and 8 h and 6 and 8 h, and the full 24 h (least squares means^a).

Treatment	FRAP				Uric acid			
	0–2 h	2–8 h	6–8 h	0–24 h	0–2 h	2–8 h	6–8 h	0–24 h
EGC	44.3 ^b	–152 ^d	–86.8	–684	1.6	–56.3 ^d	–24.9 ^c	–145
EGCg	15.4	–201	–90.6	–900	5.3	–79.5	–32.5	–274
ECg	23.2 ^c	–161	–75.6 ^d	–833	4.0	–90.8 ^e	–38.3	–338
Control	2.1	–298	–140	–1201	5.7	–92.2	–42.8	–325
Standard Error ^a	5.8	41.7	17.0	190	2.3	9.5	4.3	53.6

^a Least squares means with ‘pooled’ standard error are given.
^b Different from control ($p < 0.001$).
^c Different from control ($p < 0.05$).
^d Different from control ($p < 0.1$).
^e Different from EGC ($p < 0.1$).

the control samples and samples obtained after catechin consumption (figure 4B and table 3).

The pattern of uric acid in plasma showed a clear resemblance with that of FRAP and linear regression analysis indicated a very significant relationship ($\text{FRAP} = 316 + 2.86 \times \text{uric acid}$, $R^2 = 0.88$, $p < 0.001$). Analysis of the changes relative to the starting value of uric acid and FRAP also indicated a very significant relationship ($\text{change in FRAP} = -0.63 + 2.68 \times \text{change in uric acid}$, $R^2 = 0.54$, $p < 0.001$). Statistical analysis of FRAP using uric acid as a co-variable, however, did not result in major changes in the conclusions obtained.

Discussion

This is the first post-prandial study in which individual catechin preparations have been used instead of green tea catechin mixtures. Therefore, the pharmacokinetics of a single catechin in isolation, without interference by other catechins or green tea components, could be studied.

With all three catechins, the molecular species consumed appeared to be the major constituent found in the plasma after enzymatic deconjugation. Following consumption of EGCg, ~5% occurred as EGC in the plasma at peak maximum, and with ECg ~3% occurred as EC. These results indicate that a small, but significant, amount of the gallated catechins were degallated before or after absorption into the body. A total of 13.6% of the ingested dose of EGC was excreted in 24 h, but with EGCg or ECg <0.02% was found in the urine. This suggests that these gallated catechins are not excreted in urine but transported to the liver for excretion into bile. Both bile-excreted and non-absorbed catechins may be metabolized by the bacterial flora present in the large bowel (Das 1971, Griffiths and Barrow 1972, Gott and Griffiths 1987, Bravo *et al.* 1994, Okushio *et al.* 1999) and converted into metabolites that are not detected by the method used (Hollman *et al.* 1997). The same might be true for a significant proportion of the EGC (~86.4%) that could not be recovered from the urine.

After absorption, methylation was found to occur only with EGC, but not with the gallated EGCg or ECg. EC and EGC have been reported to be good substrates

for *O*-methylation by human placental cytosolic catechol-*O*-methyltransferase (Zhu *et al.* 2000). In contrast, ECg and EGCg were methylated at much lower rates by this enzyme (Zhu *et al.* 2000). Probably, the methylation of EGC occurs by catechol-*O*-methyltransferase in the liver or the kidney (Zhu *et al.* 1994). This assumption is supported by the fact that there was ~ 0.5 h delay in reaching the peak maximum of Me-EGC as compared with that of the native substance (figure 3). After absorption, EGC is partly *O*-methylated and excreted to a significant extent (13.6%) in the urine. Free or conjugated ECg and EGCg are not found in the urine and are probably excreted into bile. These findings are in agreement with those of Yang *et al.* (1998) who did find excretion of EGC and EC but not EGCg in the urine of volunteers who ingested different amounts of green tea. In comparison with our data, the latter authors reported slower rates of elimination from the blood (EGC, $t_{1/2 \text{ elim}} = 2.5\text{--}3.4$ versus 1.7 h, and EGCg, $t_{1/2 \text{ elim}} = 5.0\text{--}5.5$ versus 3.9 h). This might be due either to interference between the various catechins in the green tea extract used by Yang *et al.* (1998) during absorption or excretion, or to the relatively large doses of single catechins that we used. Assuming 500 mg tea solids per cup of tea, and about one-third of these representing flavonoids (Balentine *et al.* 1997), it appears that ~ 150 mg flavonoids are consumed per cup, or a total of ~ 600 mg flavonoids per four cups of tea drunk per day. This is close to the amount administered and indicates that the dose of catechin used in the study is not excessively high.

The three catechins studied, EGC, EGCg and ECg, showed a large difference in their post-prandial levels and in the FRAP level they induced in the blood. During the initial hours after ingestion, the order in which they increased FRAP corresponded to the catechin concentration reached. At an equimolar dose of 1.5 mmole, highest post-prandial levels of the catechins were induced by (in descending order): EGC > ECg > EGCg. This order is similar to that observed by Yang *et al.* (1998) for green tea at non-equimolar doses. The catechins EGC and ECg induced significant increases in FRAP, but the influence of EGCg on FRAP was without a statistically significant effect. It is likely that this is due to the low post-prandial level of EGCg that is attained.

It should be noted that a significant proportion of the antioxidant activity in plasma is due to the presence of uric acid (Benzie and Strain 1996). Because the plasma level of uric acid is subject to a circadian rhythm, the net influence of catechins on the plasma FRAP value is obscured in figure 4. However, both ECG and ECg had a significant effect on the 2-h FRAP when uric acid was included as a co-variable in the analysis of variance.

Acknowledgements

The authors thank the volunteers for participating in the study, Dr J. R. Lewis, Unilever Research Laboratory Colworth (Sharnbrook, Bedford, UK) for the supply of isolated tea fractions, and Dr G. S. M. J. E. Duchateau for critically reading the manuscript. The assistance of C. G. Blonk, G. P. Dubbelman, Ms W. M. C. Dubelaar, Ms S. Y. Gielen, G. A. A. Kivits, W. G. L. van Nielen, Ms E. F. Schra, Ms M. Slotboom and J. E. C. van Wijk is gratefully acknowledged.

References

- BALENTINE, D. A., WISEMAN, S. A. and BOUWENS, L. C. M., 1997, The chemistry of tea flavonoids. *Critical Reviews in Food Science and Nutrition*, **37**, 693–704.
- BENZIE, I. F. F. and STRAIN, J. J., 1996, The ferric reducing ability of plasma (FRAP) as a measure of 'antioxidant power': the FRAP assay. *Analytical Biochemistry*, **239**, 70–76.
- BRAVO, L., ABIA, R., EASTWOOD, M. A. and SAURA-CALIXTO, F., 1994, Degradation of polyphenols (catechin and tannic acid) in the rat intestinal tract. Effect of colonic fermentation and faecal output. *British Journal of Nutrition*, **71**, 933–946.
- CHEN, L., LEE, M. J. and YANG, C. S., 1997, Absorption, distribution, and elimination of tea polyphenols in rats. *Drug Metabolism and Disposition*, **25**, 1045–1050.
- CHOW, H. H. S., CAI, Y., ALBERTS, D. S., HAKIM, I., DORR, R., SHAHI, F., CROWELL, J. A., YANG, C. S. and HARA, Y., 2001, Phase I pharmacokinetic study of polyphenols following single-dose administration of epigallocatechin gallate and polyphenon E. *Cancer Epidemiology Biomarkers and Prevention*, **10**, 53–58.
- DA SILVA, E. L., PISKULA, M. and TERAQ, J., 1998, Enhancement of antioxidative ability of rat plasma by oral administration of (–)-epicatechin. *Free Radical Biology and Medicine*, **24**, 1209–1216.
- DALLUGE, J. J. and NELSON, B. C., 2000, Determination of tea catechins. *Journal of Chromatography A*, **881**, 411–424.
- DAS, N. P., 1971, Studies on flavonoid metabolism. Absorption and metabolism of (+)-catechin in man. *Biochemical Pharmacology*, **20**, 3435–3445.
- GOTT, D. M. and GRIFFITHS, L. A., 1987, Effects of antibiotic treatments on the metabolism and excretion of [^{14}C](+)-catechin ([^{14}C](+)-cyanidanol-3) and its metabolite, 3'-O-methyl-(+)-catechin. *Xenobiotica*, **17**, 423–434.
- GRIFFITHS, L. A. and BARROW, A., 1972, The fate of orally and parenterally administered flavonoids in the mammal. *Angiologica*, **9**, 162–174.
- HERTOG, M. G. L., FESKENS, E. J. M., HOLLMAN, P. C. H., KATAN, M. B. and KROMHOUT, D., 1993, Dietary antioxidant flavonoids and risk of coronary heart disease: the Zutphen elderly study. *Lancet*, **342**, 1007–1011.
- HOF, VAN HET, K. H., KIVITS, G. A. A., WESTSTRATE, J. A. and TIJBURG, L. B. M., 1998, Bioavailability of catechins from tea: the effect of milk. *European Journal of Clinical Nutrition*, **52**, 356–359.
- HOF, VAN HET, K. H., WISEMAN, S. A., YANG, C. S. and TIJBURG, L. B. M., 1999, Plasma and lipoprotein levels of tea catechins following repeated tea consumption. *Proceedings of the Society for Experimental Biology and Medicine*, **220**, 203–209.
- HOLLMAN, P. C. H., TIJBURG, L. B. M. and YANG, C. S., 1997, Bioavailability of flavonoids from tea. *Critical Reviews in Food Science and Nutrition*, **37**, 719–738.
- KELI, S. O., HERTO, M. G. L., FESKENS, E. J. M. and KROMHOUT, D., 1996, Dietary flavonoids, antioxidant vitamins and incidence of stroke. The Zutphen study. *Archives of Internal Medicine*, **154**, 637–642.
- KIVITS, G. A. A., VAN DER SMAN, F. J. P. and TIJBURG, L. B. M., 1997, Analysis of catechins from green and black tea in humans: a specific and sensitive assay of total catechins in biological fluids. *International Journal of Food Sciences and Nutrition*, **48**, 387–392.
- LEE, M. J., PRABHU, S., MENG, X. F., LI, C. and YANG, C. C., 2000, An improved method for the determination of green and black tea polyphenols in biomatrices by high-performance liquid chromatography with coulometric array detection. *Analytical Biochemistry*, **279**, 164–169.
- LEE, M. J., WANG, Z. Y., LI, H., CHEN, L., SUN, Y., GOBBO, S., BALENTINE, D. A. and YANG, C. S., 1995, Analysis of plasma and urinary polyphenols in human subjects. *Cancer Epidemiology/Biomarkers and Prevention*, **4**, 393–399.
- LEENEN, R., ROODENBURG, A. J. C., TIJBURG, L. B. M. and WISEMAN, S. A., 2000, A single dose of tea with or without milk increases plasma antioxidant activity in humans. *European Journal of Clinical Nutrition*, **54**, 87–92.
- MANACH, C., TEXIER, O., MORAND, C., CRESPIY, V., RÉGÉRAT, F., DEMIGNÉ, C. and RÉMÉSY, C., 1999, Comparison of the bioavailability of quercetin and catechin in rats. *Free Radical Biology and Medicine*, **27**, 1259–1266.
- OKUSHIO, K., SUZUKI, M., MATSUMOTO, N., NANJO, F. and HARA, Y., 1999, Identification of (–)-epicatechin metabolites and their metabolic fate in the rat. *Drug Metabolism and Disposition*, **27**, 309–316.
- SALAH, N., MILLER, N. J., PAGANGA, G., TIJBURG, L., BOLWELL, G. P. and RICE-EVANS, C., 1995, Polyphenolic flavonoids as scavengers of aqueous phase radicals and as chain-breaking antioxidants. *Archives of Biochemistry and Biophysics*, **322**, 339–346.
- SHAO, W., POWELL, C. and CLIFFORD, M. N., 1995, The analysis by HPLC of green, black and Pu'er teas produced in Yunnan. *Journal of the Science of Food and Agriculture*, **69**, 535–540.
- TIJBURG, L. B. M., MATTERN, T., FOLTS, J. D., WEISGERBER, U. M. and KATAN, M. B., 1997, Tea flavonoids and cardiovascular diseases: a review. *Critical Reviews in Food Science and Nutrition*, **37**, 771–785.

- TOWN, M. H., GEHM, S., HAMMER, B. and ZIEGENHORN, J., 1985, A sensitive colorimetric method for the enzymatic determination of uric acid. *Journal of Clinical Chemistry and Clinical Biochemistry*, **23**, 591.
- TREUTER, D., 1989, Chemical reaction detection of catechins and proanthocyanidins with 4-dimethylaminocinnamaldehyde. *Journal of Chromatography*, **467**, 185–193.
- WARDEN, B. A., BEECHER, G. R., CLEVIDENCE, B. A. and BALENTINE, D. A., 1999, Studies of the bioavailability of the prominent catechins in black tea. *Clinical Chemistry*, **46**(suppl.), A168.
- WISEMAN, S. A., BALENTINE, D. A. and FREI, B., 1997, Antioxidants in tea. *Critical Reviews in Food Science and Nutrition*, **37**, 705–718.
- YANG, C. S. and WANG, Z., 1993, Tea and cancer. *Journal of the National Cancer Institute*, **85**, 1038–1049.
- YANG, C. S., CHEN, L., LEE, M. J., BALENTINE, D., KUO, M. C. and SCHANTZ, S. P., 1998, Blood and urine levels of tea catechins after ingestion of different amounts of green tea by human volunteers. *Cancer Epidemiology Biomarkers and Prevention*, **7**, 351–354.
- ZHU, B. T., EZELL, E. L., LIEHR, J. G., 1994, Catechol-*O*-methyltransferase-catalyzed rapid *O*-methylation of mutagenic flavonoids. Metabolic inactivation as a possible reason for their lack of carcinogenicity *in vivo*. *Journal of Biological Chemistry*, **269**, 292–299.
- ZHU, B. T., PATEL, U. K., CAI, M. X. and CONNEY, A. H., 2000, *O*-Methylation of tea polyphenols catalyzed by human placental cytosolic catechol-*O*-methyltransferase. *Drug Metabolism and Disposition*, **28**, 1024–1030.