JPP Journal of Pharmacy and Pharmacology

JPP 2005, 57: 641–650

Received September 9, 2004
Accepted February 2, 2005
DOI 10.1211/0022357056046
ISSN 0022-3573

Influence of the *Ginkgo* extract EGb 761 on rat liver cytochrome P450 and steroid metabolism and excretion in rats and man

Shyam S. Chatterjee, Cees J. A. Doelman, Michael Nöldner, Anton Biber and Egon Koch

Abstract

Extracts from leaves of *Ginkgo biloba* L. are among the most used herbal medicinal products worldwide. Based on in-vitro tests and studies in rats, concern has been expressed that intake of *Ginkgo* extracts may affect hepatic metabolism of xenobiotics and cause drug interactions, although no evidence for modulation of cytochrome P450 (CYP450) enzyme activity was obtained in human trials. Because of these contradictory findings, we investigated the effects of the standardised extract EGb 761 on hepatic CYP450 in rats. EGb 761 (100 mg kg^{-1} daily, p.o., for 4 days) strongly increased liver CYP450 content and altered the ex-vivo biotransformation of androstendione, as well as metabolism of endogenous steroids. However, in human subjects no effect on the urinary steroid profile was observed after intake of EGb 761 for 28 days (240 mg daily). These results indicate that the effects of EGb 761 on drug metabolising enzymes are specific for rats and may not be extrapolated to man.

Introduction

Extracts from the leaves of *Ginkgo biloba* L. are among the most used herbal remedies world-wide. They are mainly utilised for the treatment of vascular and cognitive disorders, such as Alzheimer's disease, vascular dementia and peripheral arterial occlusive disease. A standardised product, termed EGb 761, is the pharmacologically, toxicologically and clinically most well studied *Ginkgo* extract. Results of numerous studies in experimental animals, controlled clinical trials and postmarketing surveillance data justify its medicinal use and reveal that it is a well tolerated remedy with an extremely wide safety margin (DeFeudis 1998). However, in recent years concern has been expressed that intake of *Ginkgo* extracts may affect hepatic drug metabolising enzyme systems and interfere with the biotransformation of co-medication. Inhibition of activity of rat and human cytochrome P450 (CYP) isoenzymes by different *Ginkgo* extracts has repeatedly been reported (Budzinski et al 2000; Mohutsky & Elmer 2000; Umegaki et al 2002; Zou et al 2002; Gaudineau et al 2004; von Moltke et al 2004). Besides inhibition of enzyme activity in-vitro, a transient decrease of CYP3A-mediated metabolism of diltiazem has been observed following a single oral or intravenous application of a *Ginkgo* extract in rats (Ohnishi et al 2003). However, after daily treatment of rats for periods between 5 days and 4 weeks the mRNA and protein content, as well as activity of various CYP isoenzymes, in the liver was increased (Shinozuka et al 2002; Umegaki et al 2002; Yang et al 2003). Due to enhanced metabolism, the hypotensive effect of nicardipine and the hypnotic action of phenobarbital were attenuated after co-administration with a *Ginkgo* extract (Shinozuka et al 2002; Kubuto et al 2004). These later observations indicate that during chronic exposure, the expression of CYP enzymes is enhanced in a compensatory manner.

Indeed, observations made during safety and chronic toxicity studies with EGb 761 in the mid 1980s revealed that daily oral application of the extract at a dose of 100 mg kg^{-1} to rats for 3 or 4 days consistently increased antipyrine clearance.

Dr Willmar Schwabe Pharmaceuticals, Willmar-Schwabe-Str. 4, 76227 Karlsruhe, Germany

Shyam S. Chatterjee, Michael Nöldner, Anton Biber, Egon Koch

Streekziekenhuis Koningin Beatrix, Beatrixpark 1, 7100 Winterswijk, Netherlands

Cees J. A. Doelman

Correspondence: E. Koch, Dr Willmar Schwabe GmbH & Co. KG, Department of Pharmacology, Willmar-Schwabe-Strasse 4, 76227 Karlsruhe, Germany. E-mail: egon.koch@schwabe.de

However, a subsequent study in human subjects, conducted to assess the potential therapeutic relevance of these preclinical findings, did not demonstrate any change in the elimination half-life of antipyrine after intake of 400 mg EGb 761 for 13 days (Duche et al 1989). Similarly, oral administration of EGb 761 twice a day at a dose of 120 mg for 14 days had no effect on the metabolism of dextromethorphan, a probe substrate for CYP2D6, when compared with baseline (Markowitz et al 2002). This observation was recently confirmed with respect to CYP2D6, although a slight reduction of the Cmax value for alprazolam, a substrate for CYP3A4, was obtained in this study. However, as the half-life of alprazolam elimination was not significantly different after EGb 761, the authors exclude a hepatic CYP3A4 induction (Markowitz et al 2003). In a further study in man, modulation of CYP enzyme activity was investigated after intake of a *Ginkgo* extract (240 mg daily) for 28 days. Using a probe-drug cocktail for CYP3A4, CYP1A2, CYP2E1 and CYP2D6, no effect of the extract was detected (Gurley et al 2002). Likewise, in a double-blind, crossover study with a group of middle-aged outpatients on stable long-term warfarin treatment, intake of a *Ginkgo* extract (100 mg) for four weeks did not influence blood coagulation parameters (Engelsen et al 2002).

It is now well recognised that several CYP isoenzymes play a pivotal role in the biosynthesis and oxidative metabolism of steroid hormones (Lee et al 2001; Dubey et al 2004), and that inappropriate modulation of their activity can have toxicological consequences (Omiecinski et al 1999). Previously it has been reported that *Ginkgo* extracts can have modulating effects on steroid metabolism (Amri et al 1996). Therefore, the rat experiments described in this communication were conducted to evaluate potential effects of EGb 761 on steroid metabolism and excretion. Results of these experiments reveal that the extract not only induces hepatic CYP content but also alters the ex-vivo biotransformation of androstendione, and the urinary steroid profile of male, as well as female, rats. Consequently, a study in human subjects was initiated to evaluate the potential therapeutic relevance of these findings. To this end, the urinary steroid profiles were analysed before and after 28 days intake of EGb 761 at a daily dose of 240 mg.

Materials and Methods

Ginkgo biloba extract EGb 761

The standardised *Ginkgo biloba* extract EGb 761, which contains 22–27% flavonol glycosides, 5–7% terpene trilactones and < 5 ppm ginkgolic acids, was obtained from the manufacturer (Dr Willmar Schwabe GmbH & Co. KG, Karlsruhe, Germany) and was of the same quality as the extract used in clinical trials and contained in commercialised products (e.g., Tebonin forte). For animal experiments, it was suspended in agar (0.2%) and was administered orally in a volume of 10 mL kg^{-1}. The placebo and EGb 761 tablets used in the study in human subjects were prepared by the Department of Galenical Development of Dr Willmar Schwabe GmbH & Co. KG. All other reagents and chemicals used were purchased from appropriate commercial suppliers and were of highest purity available.

Evaluation of hepatic enzyme induction

Experimental animals

Male and female Sprague-Dawley rats (Janvier Le Genest, St Isle, France) were maintained under standardised environmental conditions (ambient temperature 21 ± 1°C, relative humidity 50–60%, 12-h light–dark cycle) for at least 14 days before starting an experiment. They were housed in macrolon cages and had free access to food (Altromin, Lage, Germany) and tap water. All experiments were conducted in accordance with German animal welfare laws and were approved by a regional ethical committee.

Microsomal enzyme induction

Male rats, 205–215 g, were used in this experiment with 4 rats in each group. The rats in the first group served as controls and were treated orally by gavage with the vehicle (0.2% agar) once daily for three consecutive days. The other three groups were similarly treated with EGb 761 (100 mg kg^{-1} daily), methylcholanthrene (80 mg kg^{-1} daily) or phenobarbital (100 mg kg^{-1} daily). On the fourth day (i.e., 24 h after the last treatment) the rats were sacrificed and microsomes were prepared from the livers according to the method described by Lake (1987) with minor modifications. In short, the livers of the CO_2-anaesthetised rats were perfused in-situ via the portal vein with cold (4°C) saline. The livers were then weighed and homogenised (Potter; teflon pestle; 10 strokes) in cold (4°C) sodium phosphate buffer (20 mM, pH 7.4) containing 1.15% KCl. The homogenates were centrifuged (4°C) at 9000 *g* for 20 min and the resultant supernatant was recentrifuged (4°C, 40 000 *g*, 60 min). Microsomal pellets obtained after the last centrifugation were stored until analysis (suspended in 0.1 M sodium phosphate buffer) at −80°C. All analyses were completed within 8 weeks after preparation. The following parameters were quantified according to published methods: protein (Lowry et al 1951), cytochrome P450 (Schenkman & Jansson 1998), cytochrome b5 (Lake 1987), 7-ethoxycoumarin-*O*-deethylase (ECOD; Waxman & Chang 1998), 7-methoxyresorufin-*O*-demethylase (MROD; Kennedy & Stephanie 1994), 7-ethoxyresorufin-*O*-deethylase (EROD; Kennedy & Stephanie 1994), 7-pentoxyresorufin-*O*-deethylase (PROD; Kennedy & Stephanie 1994) and 7-benzyloxyresorufin-*O*-dealkylase (BROD; Kennedy & Stephanie 1994).

Steroid metabolism in microsomes

Preparation of microsomes

The effects of four daily doses of EGb 761 (100 mg kg^{-1} daily, p.o.) on microsomal androstendione metabolism (ex-vivo) were compared with those of a similar treatment with vehicle only (0.2% agar, 10 mL kg^{-1}). For

these experiments, two groups of 10 male rats, 240–270 g, were used and the microsomes were prepared on day 5 (i.e., 24 h after the last administration) as described above.

Incubation with [4-^{14}C]-4-androsten,3,17-dione
The incubation medium (1.2 mL) contained an NADPH regenerating system in Tris-HCl buffer (50 mM, pH 7.8, 37°C) and androstendione (200 μM spiked with 4-^{14}C-labelled molecule). The NADPH regenerating system consisted of (in mM): NADP-Na 1; glucose-6-phosphate 6.7, glucose-6-phosphate dehydrogenase 1 Unit and $MgSO_4$ 6.7. The reaction was started by adding an appropriate dilution of microsomes (final CYP concentration 0.3 μM). After incubation for 10 min the reaction was stopped by adding 50% trichloroacetic acid and the tubes were centrifuged to obtain a clear supernatant. The supernatant was directly injected for reversed-phase HPLC analysis. The metabolites were separated on a LiChrocart C18; 125 × 4 mm, 5 μm column (Merck, Darmstadt, Germany) using a 20–70% gradient (during 60 min) of acetonitrile containing 85% H_3PO_4 (3 mL L^{-1}) in water acidified with 85% H_3PO_4 (3 mL L^{-1}). For detection and quantification, a radioactivity detector (LP 506C; Berthold, Wildbad, Germany) equipped with a YG cell was used.

Steroid metabolism and excretion experiments

Collection of urine from EGb 761-treated rats
The effect of three daily doses of EGb 761 (100 mg kg^{-1} daily, p.o.) on urinary steroid profiles of male and female rats was compared with that of vehicle (0.2% agar suspension, 10 mL kg^{-1} daily). Each experimental group consisted of 6 rats. Immediately after the treatment on days 1–3, the rats were individually placed in metabolic cages for urine collection. After 8 h of urine collection, the rats were returned to their home cages and supplied with food and water. On the fourth day, the rats were killed and their livers were removed and weighed. All urine samples were kept frozen (−20°C) until analysis.

Collection of urine samples from human subjects
Human urine samples were collected during a randomised double blind, parallel group, placebo-controlled clinical study with EGb 761 (Cieza et al 2003). This study complied with the principles of the Declaration of Helsinki and was approved by an ethical committee. All participants gave their informed consent. Urine samples from 31 placebo- and 34 EGb 761-treated healthy subjects (age 50–65 years) were available for analysis. EGb 761 was administered at a dose of 240 mg daily (in two equally divided daily doses) for 28 days. Urine samples (24 h) were collected twice, the first one the day before the start of treatment (day 0) and the second one on day 28, the ultimate day of the treatment. The urine samples were frozen (−20°C) until steroid profiling was done. Results of this study were not known to the analyst until the steroid profiling data were finally available.

Steroid profile in urine samples
The standard chromatographic technique (Schmidt et al 1985; Weykamp et al 1989) developed in Dr Weykamp's laboratory for steroid profiling in human urine was used for the human study as well as for the rat experiment. The following steroids were quantified in all samples: androsterone (A); etiocholanolone (E); dehydroepiandrosterone (DHEA); 11-keto-androsterone (11-KA); 11-keto-etiocholanolone (11-KE); 11-OH-androsterone (OHA); 11-OH-etiocholanolone (OHE), pregnantriol (P3); 11-deoxy-tetrahydrocortisone (THS), tetrahydrocortisone (THE), tetrahydrocortisol (THF) and allo-tetrahydrocortisol (aTHF). Unknown peaks in chromatograms were characterised by retention time and peak height.

Statistical methods

All results are given as mean ± standard deviation (s.d.). As indicated in the table and figure legends, for statistical analysis of data we used the *t*-test (paired or unpaired) or one-way analysis of variance followed by a Tukey's multiple comparison test as appropriate (GraphPad Prism 3.00; GraphPad Software, San Diego, CA). Generally, a value of $P < 0.05$ was considered as proof of statistical significance.

Results

Microsomal CYP induction

Preliminary dose finding studies indicated that 100 mg kg^{-1} EGb 761 or phenobarbital administered for 3 consecutive days could be high enough doses sufficient for quantifying their CYP-inducing effects in rats, and that for similar purposes 80 mg kg^{-1} methylcholanthrene daily would be appropriate. The minimum CYP-inducing dose of EGb 761 suggested by these efforts was about 50 mg kg^{-1} daily. As 100 mg kg^{-1} of the extract is often used in pharmacological studies and is close to the equivalent human therapeutic dose, this dosage was considered appropriate for this investigation.

It was the aim of this study to characterise the activity profile of CYP enzymes in the hepatic microsomes from EGb 761-treated rats and compare them with those from vehicle-, phenobarbital- or methylcholanthrene-treated rats. The body weights and other parameters quantified in this experiment are summarised in Tables 1 and 2. These results confirm that EGb 761 induces hepatic microsomal CYP and that 100 mg kg^{-1} of the extract administered daily for only 3 days drastically altered the metabolic activity of liver tissue in rats. However, it must be noted that all three treatments induced an individual pattern of changes (Table 2). These observations reveal that EGb 761 is not a methylcholanthrene-like agent and that despite certain commonality with phenobarbital, the hepatic-enzyme-inducing activity profile of the extract is also not identical to that of this widely used anticonvulsant.

Table 1 Body weight, liver weight and protein content in liver homogenates of rats treated for three days with vehicle, EGb 761, methylcholanthrene or phenobarbital

Group	Dose (mg kg^{-1})	Body weight (g)	Liver (g)	Protein (mg (g liver)$^{-1}$)
Control	—	230.5 ± 4.2	10.1 ± 1.2	165.6 ± 11.6
EGb 761	100	231.5 ± 12.8	11.2 ± 0.9	158.8 ± 29.2
Methylcholanthrene	80	217.3 ± 8.4	10.1 ± 1.8	162.1 ± 24.9
Phenobarbital	100	221.3 ± 13.0	13.0 ± 0.8*	156.9 ± 8.8

The values are means ± s.d. of 4 rats used in each group. *$P < 0.05$ compared with the control and methylcholanthrene group (one-way analysis of variance followed by Tukey's multiple comparison test).

Table 2 CYP-related enzyme activity and other parameters quantified in hepatic microsome of rats treated orally for three consecutive days with vehicle (0.2% agar, 10 mL kg^{-1} daily), EGb 761 (100 mg kg^{-1} daily), methylcholanthrene (80 mg kg^{-1} daily) or phenobarbital (100 mg kg^{-1} daily)

Parameter	Control		EGb 761		Methylcholanthrene		Phenobarbital	
	A	B	A	B	A	B	A	B
Protein (μg (g liver)$^{-1}$)	17.8 ± 2.8	—	20.1 ± 2.9	—	19.3 ± 2.4	—	20.0 ± 4.6	—
CYP (nmol (mg protein)$^{-1}$)	698 ± 15	—	965 ± 124	—	913 ± 34	—	1153 ± 362[a]	—
Cytochrome b5	277 ± 17	397 ± 30	301 ± 14	314 ± 27	343 ± 20[a]	376 ± 24	313 ± 42	290 ± 83[a]
ECOD	1142 ± 118	1637 ± 169	2569 ± 314	2667 ± 138	4029 ± 292[a]	4420 ± 398[a,b]	3664 ± 2008[a]	2970 ± 978[a,c]
MROD	45.3 ± 1.7	64.9 ± 1.8	48.1 ± 9.7	49.6 ± 5.1	91.4 ± 2.0[a,b]	100.2 ± 4.6[a,b]	41.9 ± 7.9[c]	41.9 ± 24.8[c]
EROD	40.3 ± 11.8	57.9 ± 17.9	46.6 ± 7.9	48.0 ± 3.0	226.4 ± 1.2[a,b]	248.2 ± 8.4[a,b]	49.3 ± 6.7[c]	45.4 ± 12.0[c]
BROD	12.6 ± 8.3	18.0 ± 11.6	66.0 ± 11.5[a]	68.6 ± 10.6[a]	21.5 ± 2.1	23.5 ± 2.0[b]	68.5 ± 41.1[a,c]	54.1 ± 30.6
PROD	12.3 ± 4.1	17.7 ± 5.9	25.4 ± 1.3[a]	26.7 ± 4.1[a]	20.9 ± 2.4	22.8 ± 1.8	23.7 ± 8.9[a]	20.1 ± 3.1

The values are means ± s.d. of 4 rats used in each group. Enzyme activity given in column A represents pmol (mg protein)$^{-1}$, whereas that in column B is pmol (μmol CYP)$^{-1}$. [a]$P < 0.05$ compared with the control group; [b]$P < 0.05$ compared with the EGb 761 group; [c]$P < 0.05$ compared with the methylcholanthrene group (one-way analysis of variance followed by Tukey's multiple comparison test).

Steroid metabolism in hepatic microsomes

It is well established that regio- and stereospecific hydroxylation of steroidal molecules provides a sensitive indicator for specific CYP isoenzymes (Wood et al 1983; Van der Hoeven 1984; Khatsenko et al 1990; Jansen & Fluiter 1992; Lee et al 2003) and the usefulness of androstendione as a substrate for rat liver microsomal studies has also been reported (Khatsenko et al 1990).

Incubation of microsomes from control rats with androstendione for 10 min always gave rise to 6 major metabolites and the same was also the case with microsomes from rats treated with EGb 761. Although the mean amount of androstendione metabolised in the EGb 761-treated group was somewhat lower (45.0%; range 24.3–69.4%) than that in the control group (50.5%; range 29.8–78.9%) this difference was not statistically significant. The pattern of metabolites observed with the microsomes of the two groups of rats were, however, not identical (Table 3). Quantitatively, the sequence of metabolites observed in EGb 761-treated rats was A > B > F > others > C = D = E, whereas for the control group this sequence was F > B > others > A > C > D = E. In addition, the mean value for metabolite A in the EGb 761 group was significantly higher and those for C, D, E and F in this group were significantly lower than the corresponding values in the control group. Since the concentration of CYP was the same in all incubations, these results indicate that EGb 761 treatment activates or induces some androstendione metabolising enzymes in the liver and at the same time inhibits or reduces the activity of others.

Urinary excretion experiments

Although we have not yet been successful in identifying the microsomal steroid metabolising enzymes potentially modulated by EGb 761, results of ongoing studies strongly suggested that its modulating effects on steroid metabolism in male and female rats are not identical. Consequently, in the following experiments the effects of the extract were studied in male as well as female animals.

Urinary steroid profile in rats

Since the chromatographic system used to quantify urinary steroid profiles in rats was not specifically developed

Table 3 Effect of EGb 761 treatment on liver microsomal ^{14}C-androstendione metabolism in rats

Metabolites	Control		EGb 761	
	Relative area (%)	% of all metabolites	Relative area (%)	% of all metabolites
A	5.3 ± 1.6	11.0 ± 2.8	15.0 ± 5.7$^{\#\#\#}$	33.1 ± 7.0$^{\#\#\#}$
B	10.8 ± 4.1	23.3 ± 11.1	14.3 ± 6.4	31.2 ± 5.2
C	4.9 ± 3.1	8.9 ± 3.1	2.3 ± 1.4$^{\#}$	4.8 ± 1.4$^{\#\#}$
D	3.4 ± 2.2	6.3 ± 2.4	2.0 ± 1.2	4.1 ± 1.2*
E	2.9 ± 0.6	6.5 ± 3.1	1.7 ± 0.4$^{\#\#\#}$	4.0 ± 1.5$^{\#}$
F	15.4 ± 7.2	30.4 ± 10.5	5.4 ± 2.0$^{\#\#\#}$	14.2 ± 8.5$^{\#\#}$
Others	7.7 ± 5.3	13.6 ± 7.5	4.3 ± 3.2	8.6 ± 4.5
Androstendione metabolised (%)	50.5 ± 15.0		45.0 ± 15.2	

See text for details. The metabolites A, B, C, D, E and F are defined by their retention times in HPLC corresponding to 15.4, 17.1, 21.4, 22.2, 28.4 and 36.4 min, respectively. Others represent the sum of all other metabolites in the chromatogram. Given are the means ± s.d. for 10 rats per group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, compared with the corresponding control value (unpaired *t*-test).

for studies in this species, only some major steroidal metabolites could be reliably quantified in this experiment. As can be seen from the data summarised in Table 4A, the major quantifiable steroid excreted in male control rats was 11-KA. The level of this steroid in urine samples from EGb 761-treated male rats was below the detection level on all three days. Contrarily, the urinary level of DHEA was below the detection level for control male rats on all three days, but they were significantly elevated after EGb 761 treatment, as was the case for the, as yet undefined, steroidal molecule with an RRT of 2.23 min. Since this steroidal metabolite has not been detected in any, later analysed, male or female human urinary samples, it seems to be a steroidal metabolic product normally excreted by female rats only. As is apparent from Table 4B, excretion of this as yet undefined molecule increases drastically in female rats after EGb 761 treatment, and is one of the quantitatively major steroidal metabolites in female rats. All other steroidal molecules quantified in female rat urine were reduced after EGb 761 treatment. Here, again, all observed effects of the extract treatment were apparent already after the first application. Therefore, it appears that not all effects of the extract on rat steroid metabolism are necessarily due to its microsomal drug-metabolising enzyme-inducing activity alone, and that the reported inhibitory action of EGb 761 on some of these enzymes could also be involved in its observed effects. In any case, these observations clearly suggest that the urinary steroid profile could be a convenient index for judging potential modulating effects of EGb 761 on steroid hormone metabolism or on their circulating levels.

Urinary steroid profile in human subjects

Mean age, body weight, 24-h urinary volume and creatinine excretion rates in samples collected from the subjects before and after treatment with EGb 761 or placebo are shown in Table 5. It should be noted that values from only 22 males and 33 females are included. Although a total of 65 subjects took part in this study, urine samples from 5 of

Table 4A Effect of EGb 761 on urinary steroid excretion in male rats

Day	Group	Body weight (g)	Liver weight (g)	Urine volume (mL)	DHEA	11-KA	Unknown (RRT 2.23 min)
1	Control	237.8 ± 4.4	—	5.7 ± 0.8	0	0.07 ± 0.05	0
	EGb 761	236.3 ± 5.1		6.0 ± 1.3	0.10 ± 0.00$^{\#\#\#}$	0$^{\#\#\#}$	3.48 ± 1.14$^{\#\#\#}$
2	Control	246.5 ± 5.2	—	6.0 ± 1.8	0	0.10 ± 0.00	0
	EGb 761	242.8 ± 3.1		6.3 ± 0.8	0.07 ± 0.05$^{\#}$	0$^{\#\#\#}$	3.98 ± 3.26$^{\#}$
3	Control	256.0 ± 1.3	—	7.2 ± 2.4	0	0.05 ± 0.05	0
	EGb 761	253.8 ± 4.9		5.7 ± 0.5	0.08 ± 0.04$^{\#\#\#}$	0$^{\#}$	4.68 ± 3.12$^{\#\#}$
4	Control	263.7 ± 4.1	12.1 ± 0.7	—	—	—	—
	EGb 761	263.5 ± 5.5	12.7 ± 0.7				

DHEA, dehydroepiandrosterone; 11-KA, 11-keto-androsterone. All values are means ± s.d. (6 rats per group) and are expressed as μmol (mmol creatinine)$^{-1}$. Values of 0 indicate that most values in these groups were below the detection level. The levels of all other steroid molecules quantifiable by the applied method were below the detection levels. In addition, the body and liver weights of the rats and the urine volumes collected on days 1, 2 and 3 during 8 h after treatment are given. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, compared with the corresponding value of the control group (unpaired *t*-test).

Table 4B Effect of EGb 761 on urinary steroid excretion in female rats

Day	Group	Body weight (g)	Liver weight (g)	Urine volume (mL)	11-KA	11-KE	11-OHA	Unknown (RRT 2.23 min)
1	Control	210.7 ± 8.7	—	6.8 ± 1.8	0.15 ± 0.08	0.08 ± 0.05	0.15 ± 0.05	0.13 ± 0.22
	EGb 761	208.8 ± 5.6		$4.3 \pm 1.4^{\#}$	$0.10 \pm 0.00^{\#\#\#}$	$0^{\#\#\#}$	$0.07 \pm 0.05^{\#}$	$6.98 \pm 4.26^{\#\#}$
2	Control	212.5 ± 10.7	—	6.7 ± 1.5	0.17 ± 0.05	0.08 ± 0.04	0.15 ± 0.05	0.18 ± 0.22
	EGb 761	208.7 ± 5.4		5.2 ± 1.3	$0.08 \pm 0.04^{\#}$	$0^{\#\#\#}$	$0.05 \pm 0.05^{\#}$	$5.57 \pm 1.59^{\#\#\#}$
3	Control	215.7 ± 9.0	—	6.5 ± 1.4	0.12 ± 0.08	0.07 ± 0.05	0.12 ± 0.04	0.18 ± 0.13
	EGb 761	214.0 ± 6.6		5.0 ± 1.1	0.05 ± 0.05	$0^{\#}$	$0.05 \pm 0.05^{\#}$	$6.42 \pm 5.10^{\#}$
4	Control	218.2 ± 10.3	9.8 ± 1.0	—	—	—	—	
	EGb 761	217.8 ± 7.0	9.5 ± 0.8					

11-KA, 11-keto-androsterone; 11-KE, 11-keto-etiocholanolone; 11-OHA, 11-OH-androsterone. All values are means ± s.d. (6 rats per group) and are expressed as μmol (mmol creatinine)$^{-1}$. Values of 0 indicate that most values in these groups were below the detection level. The levels of all other steroid molecules quantifiable by the applied method were below the detection levels. In addition, the body and liver weights of the rats and the urine volumes collected on days 1, 2 and 3 during 8 h after treatment are given. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ compared with the corresponding value of the control group (unpaired *t*-test).

Table 5 Age, body weight, 24-h urinary volume and creatinine excretion in human subjects treated with placebo or EGb 761 for 28 days at a dose of 240 mg daily

Parameter	Placebo				EGb 761			
	Female (n = 14)		Male (n = 12)		Female (n = 19)		Male (n = 10)	
	Day 0	Day 28	Day 0	Day 28	Day 0	Day 28	Day 0	Day 28
Age (years)	56.3 ± 3.5		57.6 ± 3.1		56.7 ± 3.7		55.3 ± 4.0	
Body weight (kg)	59.3 ± 5.7		80.3 ± 5.7^{b}		65.9 ± 10.6		78.2 ± 9.5^{b}	
Urine volume (mL)	2138.9 ± 709.1	1815.0 ± 741.0	2166.7 ± 930.4	2067.5 ± 1009.1	2088.4 ± 717.9	1890.0 ± 570.9	2093.0 ± 702.0	1844.0 ± 842.8^{a}
Creatinine (mmol/24 h)	8.5 ± 2.0	6.9 ± 2.0^{a}	12.7 ± 3.2^{b}	11.7 ± 2.1^{b}	7.9 ± 2.3	7.8 ± 1.9	13.8 ± 4.1^{b}	11.6 ± 3.0^{b}

Values are means ± s.d. $^{a}P < 0.05$ compared with the corresponding value on day 0 (paired *t*-test); $^{b}P < 0.05$ compared with the corresponding value in female subjects (unpaired *t*-test).

them were not properly collected, stored or transported. Creatinine excretion values, calculated by the Cockorft–Gault equation (Pugia et al 1998), for 5 other subjects were not within ±70% of the expected values for healthy subjects. These subjects with abnormal creatinine clearance were evenly distributed in the four groups completing the study. Thus, abnormal creatinine clearance was detected in 1 male and 2 females in the placebo group and 1 male and female each in the EGb 761 group. As it was our aim to evaluate potential effects of EGb 761 on urinary steroid excretion pattern only, data generated for these few subjects were not used for final statistical analysis.

Values summarised in Figure 1A and 1B clearly demonstrate differences in the urinary excretion pattern of males and females. Not only creatinine excretion rates were lower in females, but also those of A, P3, aTHF and OHE were significantly different between males and females. The last named value was higher for females whereas the others were higher in males. These observations demonstrate the validity of the method used for our purposes, and reconfirm the well-known differences in urinary steroid excretion profiles of male and female healthy persons (Shamim et al 2000).

Similar gender-dependent differences in urinary excretion patterns persisted on day 28 in both placebo- and EGb 761-treated groups, but statistically significant effects of the treatment on excretion rates of any quantified steroidal molecule were not detected, although on day 0 for A and THE a significant difference between placebo and EGb 761 male subjects was observed.

Discussion

Long-term clinical use and extensive pre-clinical studies indicate that the standardised *Ginkgo* extract EGb 761 is an extremely well tolerated herbal remedy (DeFeudis 1998). However, due to a PAF-antagonistic effect of *Ginkgo* extracts or their observed hepatic CYP function modulating effects in rats, concern has been expressed that

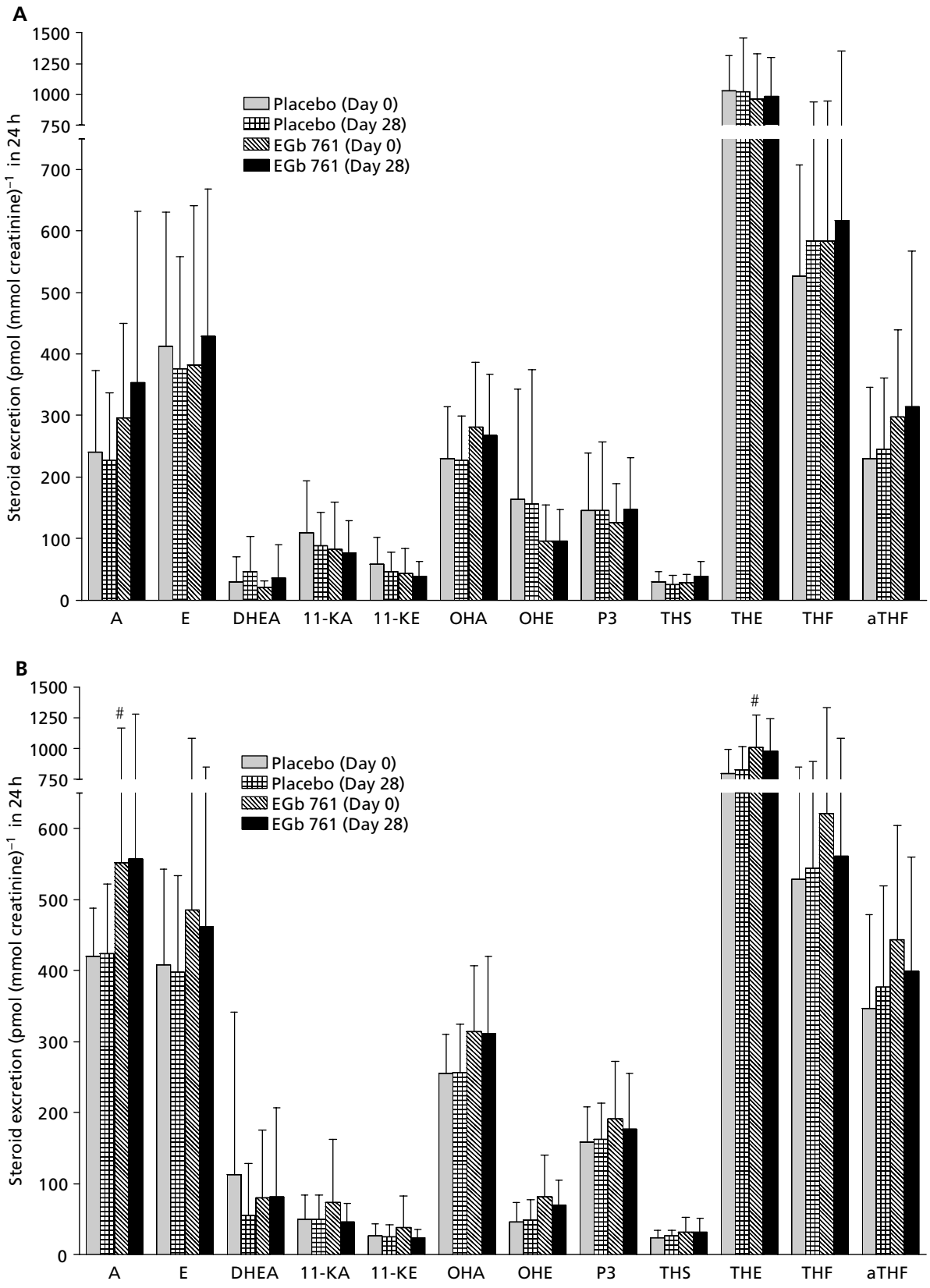

Figure 1 Urinary steroid excretion pattern (mean ± s.d.) in female (A) and male (B) subjects treated with placebo or EGb 761 for 28 days at a dose of 240 mg daily for 28 days. All steroid metabolite values are given as pmol (mmol creatinine)$^{-1}$ excreted in 24 h. Further details on the subjects are given in Table 5. $^{\#}P < 0.05$ compared with the corresponding value in placebo-treated subjects (unpaired *t*-test).

their concomitant use may alter bioavailability of other co-administered drugs. Although during recent years several cases of haemorrhage have been reported to occur in coincidence with the use of *Ginkgo* extract-based products, a clear causality between extract intake and drug–drug interactions could not be established (Juretzek et al 2002). Likewise, a number of human studies found no evidence for interference of orally administered *Ginkgo* extracts with the activity of hepatic drug metabolising enzymes. Thus, after intake of 400 mg EGb 761 for 13 days no change in the elimination half-life of antipyrine, which involves several CYP isoenzymes, was observed in human subjects (Duche et al 1989). In another human study, no modulation of CYP3A4, CYP1A2, CYP2E1 or CYP2D6 enzyme activity was detected after intake of a *Ginkgo* extract (240 mg daily) for 28 days (Gurley et al 2002). Similarly, in a double-blind, crossover study with a group of middle-aged patients on stable long-term warfarin treatment, intake of a *Ginkgo* extract (100 mg) for four weeks did not influence INR values (Engelsen et al 2002). Finally, using dextromethorphan and alprazolam as probes for CYP2D6 and CYP34A, respectively, no evidence for alteration in the metabolism of co-administered medications primarily depending on these two pathways for elimination was obtained in a study with 12 healthy subjects after intake of EGb 761 at a dose of 120 mg twice daily for 14 days (Markowitz et al 2003). Taken together, these observations strongly suggest that even higher therapeutic doses of EGb 761 do not alter hepatic CYP-dependent drug metabolising enzymes in man. Our finding that the extract does not alter urinary steroid profile in healthy subjects adds further circumstantial evidence in support of this suggestion.

In striking contrast to the above-mentioned human clinical studies, in-vitro inhibition of rat and human CYP isoenzymes has been reported in several recent publications (Budzinski et al 2000; Mohutsky & Elmer 2000; Umegaki et al 2002; Zou et al 2002; Gaudineau et al 2004; von Moltke et al 2004). However, it should be mentioned that the phytochemical composition of many of the *Ginkgo* extracts used for these studies is not known or has not been reported. In addition, investigations in-vitro have many limitations as extract constituents introduced into the system may not be bioavailable or may not circulate as free parent compound after resorption. Furthermore, plant extracts frequently contain compounds, such as tannins, which unspecifically interact with proteins.

In several rat studies, an increased hepatic content of mRNA and protein, as well as activity of various CYP isoenzymes, has been observed (Shinozuka et al 2002; Umegaki et al 2002; Yang et al 2003; Kubuto et al 2004). Similarly, our studies reveal that daily administration of EGb 761 at a dose of $100\,mg\,kg^{-1}$ for only 3 days is sufficient to drastically alter the metabolic activity of liver tissue of treated rats. The hepatic-enzyme-inducing activity profile of the extract was found to be similar, but not identical, to that of the anticonvulsant phenobarbital, which primarily induces CYP2B. These observations are in agreement with findings of other groups (Umegaki et al 2002; Kubuto et al 2004). In addition, and unlike in human subjects, the urinary steroid profile was drastically altered in rats after EGb 761 treatment (Table 4A, B). At present, definitive interpretation of this effect of the extract in terms of its modulating potential on drug-metabolising enzymes is difficult. However, it can not be ignored that its inducing effect on rat hepatic microsomal CYP has consistently been observed, and that steroid metabolism is regulated by several CYP isoenzymes. This study demonstrates, indeed, that the extract causes drastic changes in the microsomal metabolic profile of a steroidal molecule, androstenedione, too (see Table 3). Therefore, it seems reasonable to assume that modulating effects of EGb 761 on rat microsomal enzymes are involved in its observed effects on urinary steroid profile. It must also be noted that EGb 761 not only induced certain, as yet undefined, CYP isoenzymes, but also decreased the formation of some androstendione metabolites in the ex-vivo experiment. In addition, a reduced level of certain excreted steroidal molecules in the urine of EGb 761-treated rats was observed. Thus, it seems certain that after repeated daily doses, the activity of certain steroid-metabolising enzymes is reduced and that of some others is simultaneously induced. Reported inhibitory effects of *Ginkgo* extracts on several CYP isoenzymes, and our observations that the urinary steroid profile is altered even after a single dose of EGb 761, are in agreement with this conclusion.

The experiments described in this communication constitute part of an ongoing project, the ultimate goal of which is to test the therapeutic relevance of pharmacological and toxicological observations made with EGb 761 in rats as experimental animals. The medicinal uses of this extract became known to the western world only as a consequence of therapeutic observations made initially during the 1970s (DeFeudis 2003). Since then, the results of many controlled clinical trials and post-marketing surveillance data have not only reconfirmed the initial therapeutic observations, but also have indicated diverse other therapeutic possibilities offered by this standardised extract. However, many current suggestions on its mode(s) of action are often based on observations made in rat experiments conducted after higher oral doses of the extract only. Taken together, the results of the experiments described in this communication strongly suggest that extrapolation of pre-clinical pharmacological, as well as toxicological, observations made with the extract in rats must be made with caution.

Conclusion

These findings not only indicate that the extrapolation of pharmacological data generated with *Ginkgo* extracts in rats to the therapeutic situation in patients is limited but also question the validity of toxicological data generated in this species for evaluation of its human toxicological potential. In view of the fact that after $100\,mg\,kg^{-1}$ EGb 761 daily, at least two parameters of toxicological relevance (i.e. hepatic enzyme induction

and metabolism of steroids) are reproducibly altered in rats, we recommend that this species should not be used for evaluating drug interactions and health hazards of *Ginkgo* extracts.

References

Amri, H., Ogwuegbu, S. O., Boujrad, N., Drieu, K., Papadopoulos, V. (1996) In vivo regulation of peripheral-type benzodiazepine receptor and glucocorticoid synthesis by Ginkgo biloba extract EGb 761 and isolated ginkgolides. *Endocrinology* **137**: 5707–5718

Budzinski, J. W., Foster, B. C., Vandenhoek, S., Arnason, J. T. (2000) An in vitro evaluation of human cytochrome P450 3A4 inhibition by selected commercial herbal extracts and tinctures. *Phytomedicine* **7**: 273–282

Cieza, A., Maier, P., Pöppel, E. (2003) Effects of Ginkgo biloba on mental functioning in healthy volunteers. *Arch. Med. Res.* **34**: 373–381

DeFeudis, F. V. (1998) *Ginkgo biloba extract (EGb 761). From chemistry to the clinic*. Ullstein Medical, Wiesbaden

De Feudis, F. V. (2003) A brief history of EGb 761 and its therapeutic uses. *Pharmacopsychiatry* **36** (Suppl. 1): S2–S7

Dubey, R. K., Tofovic, S. P., Jackson, E. K. (2004) Cardiovascular pharmacology of estradiol metabolites. *J. Pharmacol. Exp. Ther.* **308**: 403–409

Duche, J. C., Barre, J., Guinot, P., Duchier, J., Cournot, A., Tillement, J. P. (1989) Effect of Ginkgo biloba extract on microsomal enzyme induction. *Int. J. Clin. Pharm. Res.* **9**: 165–168

Engelsen, J., Nielsen, J. D., Winther, K. (2002) Effect of coenzyme Q10 and Ginkgo biloba on warfarin dosage in stable, long-term warfarin treated outpatients. A randomised, double blind, placebo-crossover trial. *Thromb. Haemost.* **87**: 1075–1076

Gaudineau, C., Beckerman, R., Welbourn, S., Auclair, K. (2004) Inhibition of human P450 enzymes by multiple constituents of the Ginkgo biloba extract. *Biochem. Biophys. Res. Commun.* **318**: 1072–1078

Gurley, B. J., Gardner, S. F., Hubbard, M. A., Williams, D. K., Gentry, W. B., Cui, Y., Ang, C. Y. (2002) Cytochrome P-450 phenotypic ratios for predicting herb-drug interactions in humans. *Clin. Pharmacol. Ther.* **72**: 276–287

Jansen, E., Fluiter, P. (1992) Detection of the enzymatic activity of cytochrome P-450 enzymes by high-performance liquid chromatography. *J. Chromatogr.* **580**: 325–346

Juretzek, W., Kaddour, H. H., Habs, M. (2002) Blutungsgefahr unter Ginkgo? *Der Hausarzt* **19**: 1–6

Kennedy, S. W., Stephanie, P. (1994) Simultaneous measurement of cytochrome P4501A catalytic activity and total protein concentration with a fluorescence plate reader. *Anal. Biochem.* **222**: 217–223

Khatsenko, O. G., Gulyaeva, F., Mishin, V. M., Lyakhovich, V. V. (1990) Estimation of the catalytic activity of multiple forms of cytochrome P-450 in liver microsomes of Wistar rats according to the androstendione metabolism. *Biochemistry-USSR* **55**: 228–232

Kubuto, Y., Kobayashi, K., Tanaka, N., Nakamura, K., Kunitomo, M., Umegaki, K., Shinozuka, K. (2004) Pretreatment with Ginkgo biloba extract weakens the hypnosis action of phenobarbital and its plasma concentration in rats. *J. Pharm. Pharmacol.* **56**: 401–405

Lake, B. G. (1987) Preparation and characterization of microsomal fractions for studies on xenobiotic metabolism. In: Snell, K., Mullock, B. (eds) *Biochemical toxicology*. IRL Press, Oxford, pp 183–215

Lee, A. J., Kosh, J. W., Conney, A. H., Zhu, B. T. (2001) Characterization of the NADPH-dependent metabolism of 17ß-estradiol to multiple metabolites by human liver microsomes and selectively expressed human cytochrome P450 3A4 and 3A5. *J. Pharmacol. Exp. Ther.* **298**: 420–432

Lee, A. J., Cai, M. X., Thomas, P. E., Conney, A. H., Zhu, B. T. (2003) Characterization of the oxidative metabolites of 17beta-estradiol and estrone formed by 15 selectively expressed cytochrome P450 isoforms. *Endocrinology* **144**: 3382–3398

Lowry, O. H., Rosebrough, N. S., Farr, A. L., Randall, R. J. (1951) Protein measurement with folin phenol reagent. *J. Biol. Chem.* **193**: 265–275

Markowitz, J. S., DeVane, C. L., Donovan, J. L., Risch, S. C. (2002) An assessment of CYP2D6 inhibitory effects of Ginkgo biloba in normal volunteers [Abstr. P.4.E.057]. *Int. J. Neuropsychopharmacol.* **5** (Suppl. 1): S192

Markowitz, J. S., Donovan, J. L., DeVane, C. L., Sipkes, L., Chavin, K. D. (2003) Multiple-dose administration of Ginkgo biloba did not affect cytochrome P-450 2D6 or 3A4 activity in normal volunteers. *J. Clin. Psychopharmacol.* **23**: 576–581

Mohutsky, M. A., Elmer, G. (2000) Inhibition of cytochrome P-450 in vitro by the herbal product Ginkgo biloba. *41st Annual Meeting of the American Society of Pharmacognosy* Abstr. P-144

Ohnishi, N., Kusuhara, M., Yoshioka, M., Kuroda, K., Soga, A., Nishikawa, F., Koishi, T., Nakagawa, M., Hori, S., Matsumoto, T., Yamashita, M., Ohta, S., Takara, K., Yokoyama, T. (2003) Studies on interactions between functional foods or dietary supplements and medicines. I. Effects of Ginkgo biloba leaf extract on the pharmacokinetics of diltiazem in rats. *Biol. Pharm. Bull.* **26**: 1315–1320

Omiecinski, C. J., Remmel, R. P., Hosagrahara, V. P. (1999) Concise review of the cytochrome P450s and their role in toxicology. *Toxicol. Sci.* **48**: 151–156

Pugia, M. J., Lott, J. A., Luke, K. E., Shihabi, Z. K., Wians, J. F. H., Philips, L. (1998) Comparison of instrument-read dipsticks for albumin and creatinine in urine with visual results and quantitative methods. *J. Clin. Lab. Anal.* **12**: 280–284

Schenkman, J. B., Jansson, I. (1998) Spectral analysis of Cytochromes P450. In: Phillips, I. R., Shephard, E. A. (eds) *Methods in molecular biology, vol. 107, cytochrome P450 protocols*. Humana Press, Totowa, pp 25–33

Schmidt, N. A., Borburgh, H. J., Penders, T. J., Weykamp, C. W. (1985) Steroid profiling – an update. *Clin. Chem.* **31**: 637–639

Shamim, W., Yousufuddin, M., Bakhai, A., Coats, A. J. S., Honour, J. W. (2000) Gender differences in the urinary excretion rates of cortisol and androgen metabolites. *Ann. Clin. Biochem.* **37**: 770–774

Shinozuka, K., Umegaki, K., Kubota, Y., Tanaka, N., Mizuno, H., Yamauchi, J., Nakamura, K., Kunitomo, M. (2002) Feeding of Ginkgo biloba extract (GBE) enhances gene expression of hepatic cytochrome P-450 and attenuates the hypotensive effect of nicardipine in rats. *Life Sci.* **70**: 2783–2792

Umegaki, K., Saito, K., Kubota, Y., Sanada, H., Yamada, K., Shinozuka, K. (2002) Ginkgo biloba extract markedly induces pentoxyresorufin O-dealkylase activity in rats. *Jpn. J. Pharmacol.* **90**: 345–351

Van der Hoeven, T. (1984) Assay of hepatic microsomal testosterone hydroxylases by HPLC. *Anal. Biochem.* **138**: 57–65

von Moltke, L. L., Weemhoff, J. L., Bedir, E., Khan, I. A., Harmatz, J. S., Goldmann, P., Greenblatt, D. J. (2004)

Inhibition of human cytochromes P450 by components of Ginkgo biloba. *J. Pharm. Pharmacol.* **56**: 1039–1044

Waxman, D. J., Chang, T. K. H. (1998) Use of 7-ethoxy coumarin to monitor multiple enzymes in the human CYP1, CYP2 and CYP3 families. In: Phillips, I. R., Shephard, E. A. (eds) *Methods in molecular biology, vol. 107: cytochrome P450 protocols*. Humana Press, Totowa, pp 175–179

Weykamp, C. W., Penders, T. J., Schmidt, N. A., Borburgh, A. J., van de Calseyde, J. F., Walthers, B. J. (1989) Steroid profiling of urine: reference values. *Clin. Chem.* **35**: 2281–2284

Wood, A., Ryan, D., Thomas, P., Levin, W. (1983) Regio- and stereoselective metabolism of two C19 steroids by five highly purified and reconstituted rat hepatic cytochrome P-450 isozymes. *J. Biol. Chem.* **258**: 8839–8847

Yang, X. F., Wang, N. P., Lu, W. H., Zeng, F. D. (2003) Effects of Ginkgo biloba extract and tanshinone on cytochrome P-450 isozymes and glutathione transferase in rats. *Acta Pharmacol. Sin.* **24**: 1033–1038

Zou, L., Harkey, M. R., Henderson, G. L. (2002) Effects of herbal components on cDNA-expressed cytochrome P-450 enzyme catalytic activity. *Life Sci.* **71**: 1579–1589