ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
27 (2002) 315–325

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

www.elsevier.com/locate/jpba

HPLC analysis of 4-chlorophenyl methyl sulphide and diphenyl sulphide and their corresponding sulphoxides and sulphones in rat liver microsomes

Ivo P. Nnane [a,*], Lyaquatali A. Damani [b]

[a] *Department of Pharmaceutical Sciences, Temple University School of Pharmacy, 3307 North Broad Street, Philadelphia, PA 19140, USA*

[b] *Department of Pharmacy, King's College London, Manresa Road, London SW 3 6LX, UK*

Received 1 March 2001; received in revised form 18 July 2001; accepted 26 July 2001

Abstract

Simple high performance liquid chromatography (HPLC) methods for the analysis of 4-chlorophenyl methyl sulphide (CPMS), diphenyl sulphide (DPS) and their corresponding sulphoxide and sulphone metabolites in rat liver microsomes are described. The assay methods are based on a reversed phase HPLC column (Spherisorb® 5 ODS, 15×0.46 cm) using a mixture of water and tetrahydrofuran (THF) as mobile phase at a flow rate of 0.5 ml/min and ultraviolet detection at 260 nm. The compounds were extracted into diethyl ether (2×5 ml) from rat liver microsomal incubation mixture (2 ml) and the recoveries were more than 80%. The calibration curves for determining the sulphoxide and sulphone of CPMS or DPS were linear ($r \geq 0.995$) in the range of 0–50 μg/ml and the assays were reproducible with low inter- and intra-assay variation of less than 13.5%. The lower limit of quantitation (LOQ) was 0.1 μg/ml for CPMSO and 0.025 μg/ml for $CPMSO_2$, diphenyl sulphoxide (DPSO) and diphenyl sulphone ($DPSO_2$). The HPLC methods were successfully applied to measure enzymically formed CPMSO, $CPMSO_2$, DPSO and $DPSO_2$ in rat liver microsomes and to characterise the Michaelis–Menten kinetics associated with the metabolism of CPMS and DPS and their corresponding sulphoxides. About 20% of the initial CPMS (0.5 mM) concentration in the incubation was converted to the sulphoxide although the sulphone was not detected under these optimum incubation conditions. Similarly, about 15–20% of DPS was converted to the sulphoxide while less than 0.1% of DPS was converted to $DPSO_2$. Eadie–Hofstee plot of CPMS sulphoxidation was biphasic. This suggests that the sulphoxidation of CPMS is a consequence of at least two enzyme systems, one characterized by low affinity and high capacity ($K_m = 0.1$ mM; $V_{max} = 2.1$ nmoles/mg protein/min) and the other by high affinity and low capacity ($K_m = 0.05$ mM; $V_{max} = 1.5$ nmoles/mg protein/min). On the other hand, the Eadie–Hofstee plot of DPS sulphoxidation was monophasic with an apparent V_{max} and K_m of 1.8 nmoles/mg protein/min and 0.036 mM, respectively. © 2002 Published by Elsevier Science B.V.

Keywords: Sulphide; Sulphoxide; Sulphone; HPLC; Metabolism

Abbreviations: CPMS, 4-chlorophenyl methyl sulphide; CPMSO, 4-chlorophenyl methyl sulphone; $CPMSO_2$, 4-chlorophenyl methyl sulphone; $CPPSO_2$, 4-chlorophenyl phenyl sulphone; DPS, diphenyl sulphide; DPSO, diphenyl sulphoxide; $DPSO_2$, diphenyl sulphone; HPLC, high performance liquid chromatography.

* Correspondence author. Tel.: +1-215-707-6917; fax: +1-215-707-3678.

E-mail address: innane@unix.temple.edu (I.P. Nnane).

0731-7085/02/$ - see front matter © 2002 Published by Elsevier Science B.V.
PII: S0731-7085(01)00551-9

1. Introduction

A significant number of sulphur-containing xenobiotics are used as industrial and agricultural chemicals, and medicinal agents [1,2]. Sulphides, sulphoxides and sulphones are some of the more frequently encountered sulphur functionalities in medicinal agents and may determine or influence the biological fate of the parent molecule [3,4]. Sulphides are readily converted to sulphoxides in vitro and in vivo due to the readily accessible lone pair of electrons on the divalent sulphur atom [5]. The psychoactive phenothiazine drugs, for example, are metabolised to their corresponding sulphoxides in various animal species and in man [6]. Sulphoxides, on the other hand, may be oxidised to the corresponding sulphones or reduced to sulphides. The sulphoxide drug, sulphinpyrazone, undergoes both reduction to a sulphide and oxidation to the sulphone after parenteral administration to rats [7].

4-Chlorophenyl methyl sulphide (CPMS) and diphenyl sulphide (DPS) and their corresponding sulphoxide and sulphone derivatives are intermediates in the manufacture of some xenobiotics. These compounds also form sub-structures of more complex xenobiotics. It has been shown, for example, that CPMS is metabolised to $CPMSO_2$ via a transient sulphoxide in lactating cattle and sheep although the enzymology of the biotransformation was not characterized (Fig. 1) [8]. Furthermore, the assay method used in the study did not clearly differentiate between the sulphoxide and sulphone metabolite. Similarly, a previous study had evaluated the reduction of diphenyl sulphoxide (DPSO) to its thioether analogue in vitro using rat and rabbit tissues [9]. However, the validation data for the high performance liquid chromatography (HPLC) analysis of DPS and its corresponding sulphoxide and sulphone was not reported in the paper. Therefore, fully validated HPLC methods for the simultaneous measurements of CPMS or DPS and their corresponding sulphoxides and sulphones were required in order to study the enzymology of the *S*-oxidation of CPMS or DPS and their corresponding sulphoxides.

Two distinct enzyme systems are involved in *S*-oxidation reactions depending on the nucleophilicity of the substrate [1,5]. It has been shown that the flavin-containing monooxygenase (FMO) is responsible for the sulphoxidation of the more nucleophilic aliphatic and alicyclic sulphides to their corresponding sulphoxides while both cytochrome P450 and the FMO are involved in the further oxidation of sulphur atoms that reside within or adjacent to aromatic or heterocyclic ring systems [10,11]. Interestingly, modulation of the liver metabolising enzymes activity using various enzyme inducing agents and inhibitors has profound effects on the levels of the metabolites generated and may provide useful information on the nature of enzymes involved in these biotransformation reactions [12]. Furthermore, the activities of distinct isoforms of cytochrome P450 in mammalian liver are known to be increased following administration of certain foreign chemicals that elicit de novo synthesis of enzyme proteins in animals [13].

CPMS is a simple alkylaryl sulphide of intermediate nucleophilicity. On the other hand, DPS is a

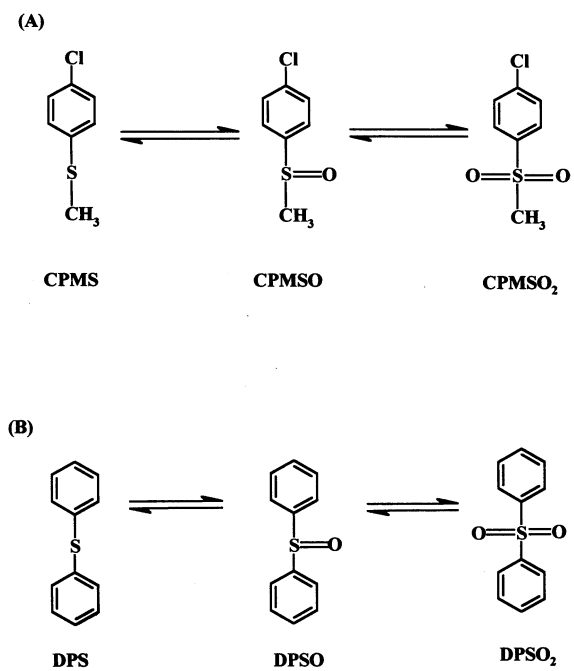

Fig. 1. *S*-Oxidation pathway of CPMS (A) and DPS (B).

diaryl sulphide of reduced nucleophilicity. Preliminary studies in our laboratory suggest that cytochrome P450 and/or the FMO may participate in the metabolic conversion of CPMS or DPS to their corresponding sulphoxides and further to their corresponding sulphones (Fig. 1). Thus, the use of these simple sulphides as model substrates would allow a systematic investigation into the enzymology of the interconversions of the three redox states. Hence, the biotransformation of these sulphides may serve as a useful predictive tool for characterization of the enzymology of more complex sulphur-containing xenobiotics bearing the alkylaryl or diaryl thioether functionality.

This paper describes the development of HPLC assay methods for the simultaneous measurement of the sulphoxide and sulphone metabolites of CPMS or DPS in rat liver microsomal incubations and the application of these methods to study some aspects of the in vitro metabolism of these simple sulphur-containing compounds by rat hepatic microsomes.

2. Experimental

2.1. Chemicals

CPMS (98%), DPS, $CPPSO_2$, phenobarbital, β-naphthoflavone, α-naphthoflavone, orphenadrine, *n*-octylamine, and 3-methylcholanthrene were purchased from Aldrich Chemical Co. (Gillingham, Dorset, UK). Dexamethasone, quinidine and erythromycin were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Clofibrate was a gift from ICI (Macclesfield, Cheshire, UK) and SKF525A was a gift from Smithkline & Beecham (Herts, UK). The corresponding sulphoxide and sulphone of CPMS and DPS were synthesized in our laboratory by methods described previously [14]. The purity of both the sulphoxide and sulphone was more than 98%. Nicotinamide adenine dinucleotide (NADP), glucose-6-phosphate (G6P) and glucose-6-phosphate dehydrogenase (G6PD) were purchased from Boehringer Mannheim (Lewes, Sussex, UK). All other reagents and organic solvents of HPLC or analytical grades were purchased from BDH (Poole, Dorset, UK).

2.2. HPLC apparatus

The isocratic liquid chromatographic system used in this investigation consisted of an LDC/Milton Roy® constametric 3000 solvent delivery system (Riviera Beach, USA) coupled to a Rheodyne injector 7125 (Cotati, CA, USA) and an LDC/Milton Roy® 3000 variable wavelength spectromonitor operated at 260 nm and connected to a C14000 computing integrator (LDC analytical, Stone, UK). Chromatographic separation and quantitation of CPMS, DPS and their corresponding sulphoxide and sulphone was achieved by reversed phase HPLC on a Spherisorb® 5 ODS column (15 × 0.46 cm) obtained from HPLC technology (Macclesfield, Cheshire, UK). The analytical column was protected by a guard column packed with pellicular ODS (Whatman, Maidstone, UK). The mobile phase was composed of a water and tetrahydrofuran (THF) mixture (45:55 v/v) and was pumped at a flow rate of 0.5 ml/min for the study of CPMS biotransformation. The mobile phase composition was changed to THF/water (30:70 v/v) for CPMSO metabolism studies. For DPS metabolism studies, the mobile phase was composed of THF/water (50:50 v/v) and pumped at a flow rate of 0.5 ml/min. The mobile phase composition was changed to THF/water (30:70 v/v) for DPSO metabolism studies.

2.3. Preparation of standards

CPMS and DPS and their corresponding sulphoxide and sulphone metabolites and the internal standards were made up to 1 mg/ml in methanol and stored in the refrigerator (4 °C) until required. From these stock solutions, working solutions of 1, 10 and 100 μg/ml in methanol were prepared for use in the construction of calibration curves.

2.4. Extraction procedure

Screw capped tubes containing the microsomal incubation mixture (2 ml), NaOH (0.1 M, 0.5 ml),

analytes and the internal standard (1 mg/ml, 20 μl) were extracted with diethyl ether (2×5 ml) using a mechanical bench test tube shaker for 10 min and centrifugation at 400 g for 10 min. The organic layers were evaporated to dryness in a water bath at 40 °C. The extracts were reconstituted in a small volume (50 μl) of methanol and an aliquot (10 μl) of the concentrate was injected onto the HPLC column.

2.5. Calibration curves

The calibration curves for quantitation of CPMSO and $CPMSO_2$ were constructed by spiking varying amounts (0–50 μg/ml) into extraction tubes containing boiled microsomes (1 ml), substrate (0.5 mM), cofactors and the internal standard, $CPPSO_2$ or diphenyl sulphone ($DPSO_2$) (20 μl, 1 mg/ml), in a total volume of 2 ml of incubation mixture. $CPPSO_2$ (20 μl, 1 mg/ml) was used as the internal standard for CPMS in vitro metabolism studies, while $DPSO_2$ (20 μl, 1 mg/ml) was used as the internal standard for CPMSO metabolism studies. The mixtures were taken through the extraction procedure; the organic phase was evaporated to dryness and then reconstituted in 50 μl of the mobile phase. An aliquot (10 μl) of the reconstituted sample was injected onto the HPLC column and peak area ratios were plotted against concentration of the analytes.

Similarly, the calibration curves for DPSO and $DPSO_2$ were constructed by spiking varying amounts (0–50 μg/ml) into extraction tubes containing boiled microsomes (1 ml), substrate (0.5 mM), cofactors and the internal standard, $CPPSO_2$ or CPMSO (20 μl, 1 mg/ml), in a total volume of 2 ml incubate. $CPPSO_2$ (20 μl, 1 mg/ml) was used as the internal standard for DPS in vitro metabolism studies while CPMSO was used as the internal standard for DPSO in vitro metabolism studies. The mixtures were taken through the extraction procedure, the organic phase was evaporated to dryness and the reconstituted sample was injected onto the HPLC column and peak area ratios were plotted against concentration of the analytes.

2.6. Assay validation

To determine the precision and accuracy of the assay, known concentrations of CPMSO and $CPMSO_2$ or DPSO and $DPSO_2$ were spiked into simulated inactivated microsomal incubations and taken through the extraction procedure. The study was repeated on five separate occasions and the coefficient of variation (CV), a measure of precision, and the mean percentage difference, MD(%), a measure of accuracy, were calculated.

2.7. Stability of CPMS, DPS and their corresponding sulphoxides and sulphones

To determine the stability of CPMS, DPS and their sulphoxides and sulphones in rat microsomal fractions before extraction, aliquots (100 μg/ml) of the analytes were spiked into extraction tubes containing inactivated microsomal fractions (1 ml, 4 mg/ml of microsomal protein), Tris–KCl buffer (pH 7.4, 0.25 M, 1 ml) and NaOH (0.5 ml, 1 M). The tubes were stored at 4 or -20 °C before analysis. The tubes were analysed at various time intervals (up to 12 weeks) to determine the stability of the analytes. Prior to analysis, the tubes were spiked with the appropriate internal standard and taken through the analytical procedure as outlined above.

2.8. Animal treatment and preparation of hepatic microsomes

Male Wistar rats (250 ± 30 g) were obtained from Bantin and Kinman Limited (Aldbrough, Hull, UK) and maintained in a controlled environment (20 °C, 50% relative humidity and 12 h light/12 h dark cycles) for at least 5 days prior to use. The animals were maintained on standard laboratory diet (RME1) obtained from SDS limited (Witham, Essex, UK) and allowed free access to water. The animals were divided into groups of six rats each and treated with the following compounds once each day for 3 consecutive days; β-naphthoflavone (100 mg/kg), or 3-methylcholanthrene (25 mg/kg) in corn oil, phenobarbital (80 mg/kg) in saline, dexamethasone (100 mg/kg) in distilled water containing 2% tween 80

or clofibrate (200 mg/kg) in saline. The control groups were treated with the corresponding vehicles alone. The animals were starved overnight before tissue preparation. All animals were sacrificed by cervical dislocation and the livers immediately excised into beakers containing ice-cold Tris buffer (0.1 M, pH 7.4) containing KCl (0.1 M) and liver microsomes prepared by a standard ultracentrifugation method as follows. Briefly, homogenates of rat liver were prepared in two volumes of Tris–KCl (0.1 M, pH 7.4) buffer using a Sorvall Omnimix® homogeniser (Dupont Instruments, Herts, UK) and a teflon-glass Potter–Elvehjem® homogeniser (Voss, Essex, UK). The crude homogenate was centrifuged at 10 000 g for 20 min at 4 °C using a Sorvall superspeed RC2-B (Dupont Instruments, Herts, UK) refrigerated centrifuge. The microsomal fraction was separated from the cytosol by centrifugation of the post-mitochondrial supernatant at 100 000 g for 1 h using an MSE AP Pegasus® 65 refrigerated ultracentrifuge. The microsomal pellet was re-suspended in Tris–KCl (0.1 M, pH 7.4) equivalent to 0.5 g wet weight per ml and further centrifuged at 100 000 g for 1 h to obtain the 'washed' microsomal pellet. The 'washed' microsomal pellet was finally suspended in Tris–KCl (0.1 M, pH 7.4), equivalent to 0.5 g wet weight tissue per ml of buffer, and stored at −80 °C prior to use. The microsomal protein content was estimated by the colorimetric method reported by Lowry and colleagues [15]. The difference between the absorbance at 450 and 490 nm of the sodium dithionite (5 mg) reduced microsomal sample and the reduced carbon monoxide complexed sample was measured and used to estimate the microsomal cytochrome P450 content [16].

2.9. Incubation procedures

Incubations were carried out in duplicate with liver microsomes from untreated and pre-treated rats in 25 ml Erlenmeyer flasks at 37 °C using a shaking water bath (Grant Instruments Limited, Cambridge, UK). A typical incubation mixture consisted of a cofactor solution (0.5 ml), microsomal fraction (1 ml, 0–10 mg microsomal protein) and substrate (20 μl) in acetone plus 0.480 μl distilled water, 0–5 mM) in a total volume of 2 ml. The cofactor solution consisted of $NADP^+$ (1 mM), G6P (5 mM), G6PD (1 unit) and magnesium chloride (10 mM, 50% w/w aqueous solution) in sodium phosphate buffer (0.2 M, pH 7.4). Prior to incubation, the cofactors were pre-incubated for 5 min to allow the generation of NADPH, at 37 °C. Metabolism was initiated by the addition of microsomal tissue and terminated by adding NaOH (0.5 ml, 0.1 M) to the incubation mixture. Incubations were also carried out with microsomes from untreated animals in the presence of various metabolic inhibitors and activators using CPMS, CPMSO, DPS or DPSO as substrate, under the optimum incubation conditions. Potential inhibitors or activators were prepared in water with a minimum (20 μl) of acetone and different concentrations were added to the incubation mixtures with appropriate control incubations set up.

2.10. Data analysis

Linear regression analysis on Sigma plot for Windows was used to evaluate calibration curves. The Michaelis–Menten rate equation for a multicomponent enzyme system used to evaluate the enzyme kinetics in this study was as follows:

$$v = \frac{V_{\max(1)}[S]}{K_{m(1)} + [S]} + \frac{V_{\max(2)}[S]}{K_{m(2)} + [S]} + \cdots + \frac{V_{\max(n)}[S]}{K_{m(n)} + [S]}$$

where [S] is substrate concentration, v is the rate of product formation, V_{max} is the maximum rate of product formation and K_m is the Michaelis–Menten constant. The above equation was rearranged according to the Eadie–Hofstee transformation ($v = V_{\max(1)} - K_{m(1)}v/[S] + V_{\max(2)} - K_{m(2)}v/[S] + \ldots + V_{\max(n)} - K_{m(n)}v/[S]$). The method of residuals was used to generate straight lines equations which were used for estimation of V_{max} and K_m. One way analysis of variance (ANOVA) or Students' t-test, as appropriate, on SigmaStats for Windows was used to obtain probability values (P) and compare different treatment groups. A P-value of less than 0.05 was considered statistically significant.

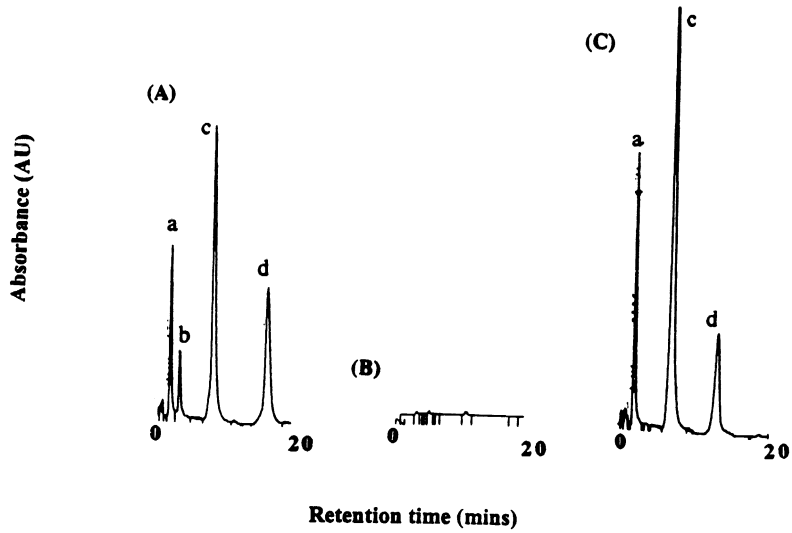

Fig. 2. A typical HPLC chromatogram of authentic compounds (A), an extract of inactivated microsomes (B), and CPMS incubation with rat hepatic microsomes (C). (a) CPMSO, (b) $CPMSO_2$ (c) $CPPSO_2$ (d) CPMS. HPLC column: Spherisorb® 5 ODS (15 × 0.46 cm); Mobile phase: water/THF mixtures (45:55 v/v), flow rate of 0.5 ml/min. The mobile composition was changed to THF/water (30:70 v/v) for CPMSO metabolism studies.

3. Result and discussion

Satisfactory separation of the sulphoxides and sulphones of CPMS and DPS was achieved by using a reversed phase Spherisorb® 5 ODS column (15 × 0.46 cm) and appropriate mixtures of water and THF as mobile phase. Predictably, the more polar sulphoxides and sulphones were eluted first. In contrast, the lipophilic CPMS or DPS was retained longer on the Spherisorb® 5 ODS column. The internal standards, 4-chlorophenyl phenyl sulphone ($CPPSO_2$), $DPSO_2$ or CPMSO were also well resolved from the other peaks of interest. The chromatograms showed sharp and symmetrical peaks for all analytes (Figs. 2 and 3). The recoveries of the sulphoxide and sulphone of CPMS or DPS and the internal standard, $CPPSO_2$, from the microsomal incubations were more than 80% when extracted into diethylether (Table 1). The microsomal extracts were free of endogenous substances that might interfere with the assay (Figs. 2 and 3). Furthermore, CPMSO, DPSO, $CPMSO_2$ and $DPSO_2$ standard solutions were stable for well over 3 months at 4 °C and more than 98% of the original concentration of CPMS and DPS in the standard solutions was accounted for 12 weeks after preparation of the stocks (data not shown). The sulphoxides and sulphones of CPMS and DPS were also stable in microsomal incubations stored at −20 °C for 4 weeks (data not shown). The calibration curves for determining the sulphoxide and sulphone of CPMS or DPS were linear ($r \geq 0.995$) in the range of 0–50 μg/ml and the assay methods were reproducible with low inter- and intra-assay variation of less than 13.5%. The linear regression equations used to assess the formation of CPMSO and $CPMSO_2$ from CPMS (as substrate) in vitro were $y = 0.033x + 0.001$ ($r^2 = 0.999$) and $y = 0.012x + 0.001$ ($r^2 = 0.995$), respectively. The linear regression equation used to assess the formation of $CPMSO_2$ from CPMSO (as substrate) in vitro was $y = 0.0094x + 0.002$ ($r^2 = 0.996$). Similarly, the linear regression equations used to assess the formation of DPSO and $DPSO_2$ from DPS (as substrate) in vitro were $y = 0.020x + 0.016$ ($r^2 = 0.998$) and $y = 0.012x + 0.016$ ($r^2 = 0.999$), respectively. The linear regression equation used to assess the formation of $DPSO_2$ from DPSO (as substrate) in vitro was $y = 0.0086x + 0.013$ ($r^2 = 0.996$). The precision of the assay as indicated by the CV and the accuracy

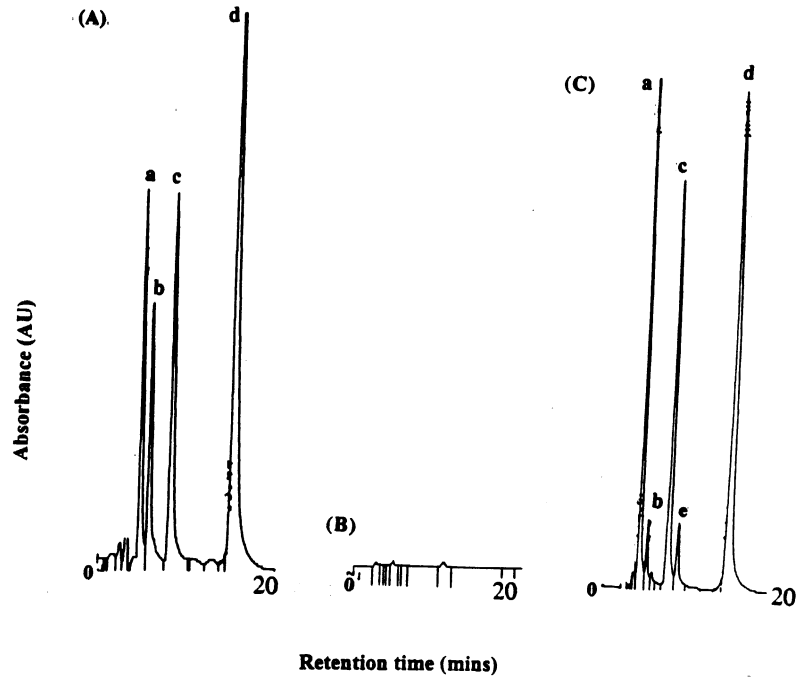

Fig. 3. A typical HPLC chromatogram of authentic compounds (A), an extract of inactivated microsomes (B), and DPS incubation with rat hepatic microsomes (C). (a) DPSO, (b) $DPSO_2$ (c) $CPPSO_2$ (d) DPS (e) hydroxyDPS. HPLC column: Spherisorb® 5 ODS (15×0.46 cm); Mobile phase: water/THF mixtures (50:50 v/v), flow rate of 0.5 ml/min. The mobile composition was changed to THF/water (30:70 v/v) for DPSO metabolism studies.

based on MD(%) were satisfactory since the values of these indicators were not more than 13.5% at the concentrations examined (Table 2). The lower limit of detection (LOD) was 0.1 μg/ml for CPMSO and 0.025 μg/ml for $CPMSO_2$, DPSO and $DPSO_2$. The assay methods were simple, fairly rapid and allowed accurate estimation of enzymically formed sulphoxides and sulphones of CPMS and DPS in vitro.

About 20% of the initial CPMS concentration was converted to the sulphoxide under the optimum incubation conditions used. However, the sulphone of CPMS was not detected under these incubation conditions. Eadie–Hofstee plot of CPMS sulphoxidation was approximately biphasic, an indication of two-enzyme kinetics (data not shown). This suggests that the sulphoxidation of CPMS is a consequence of at least two enzyme systems [17], one characterized by low affinity and high capacity ($K_m = 0.1$ mM; $V_{max} = 2.1$ nmoles/mg protein/min) and the other by high affinity and low capacity ($K_m = 0.05$ mM; $V_{max} = 1.5$ nmoles/mg protein/min). When CPMSO was used as the substrate, about 2% of the initial concentration was converted to the corresponding sulphone. However, the corresponding sulphide, CPMS, was not detected under these incubation conditions. The Eadie–Hofstee plot of the bio-

Table 1
Extraction of CPMSO, $CPMSO_2$, DPS, $DPSO_2$ and CPMSO from rat microsomal fractions

Compound	Recovery (%)	CV (%)
CPMSO	80 ± 3	3.75
$CPMSO_2$	90 ± 5	5.5
$CPPSO_2$	85 ± 2	2.3
DPSO	88 ± 3	3.4
$DPSO_2$	94 ± 5	5.3

Recoveries were performed at a concentration of 20 μg/ml for all analytes. Values are expressed as mean ± SD of three separate determinations.

Table 2
Validation of the HPLC assay methods for the determination of CPMSO, $CPMSO_2$, DPSO and $DPSO_2$ in rat hepatic microsomes

Analyte	Sample concentration (μg/ml)	Within-day analysis			Between-day analysis		
		Measured concentration (μg/ml)	CV (%)	MD (%)	Measured concentration (μg/ml)	CV (%)	MD (%)
CPMSO	5	5.34 ± 0.4	6.5	6.8	5.30 ± 0.2	0.4	5.9
	10	10.7 ± 1.5	7.1	7.0	9.9 ± 0.70	7.4	−0.8
	20	20.4 ± 1.3	6.5	2.10	21.8 ± 0.8	3.8	9.1
	50	50.6 ± 4.6	9.2	0.0	50.0 ± 2.0	4.0	0.0
$CPMSO_2$	10	10.8 ± 0.6	5.5	8.0	10.5 ± 1.4	13.3	5.0
	20	21.0 ± 2.4	11.6	5.0	20.1 ± 0.7	3.6	0.5
	30	30.5 ± 1.72	5.6	1.7	30.2 ± 1.7	5.7	0.7
	40	40.2 ± 0.3	0.6	0.5	40.0 ± 4.2	10.6	0.0
DPSO	5	5.30 ± 0.5	10.0	3.0	5.30 ± 0.2	0.4	5.9
	10	9.7 ± 0.5	5.2	−3.0	9.8 ± 0.8	8.2	−2.0
	20	19.4 ± 1.3	6.7	−3.0	20.8 ± 0.8	3.8	4.0
	40	37.5 ± 3.9	10.4	6.25	40.0 ± 2.0	5.0	0.0
$DPSO_2$	10	10.6 ± 0.7	7.0	6.0	11.0 ± 0.5	4.5	10.0
	20	22.0 ± 2.0	9.1	10.0	20.0 ± 0.6	3.0	0.0
	30	29.5 ± 0.72	2.4	1.7	29.2 ± 1.0	3.4	2.7
	40	40.2 ± 0.3	0.6	0.5	40.0 ± 4.2	10.6	0.0

Values are expressed as mean ± SD of three separate determinations. CV = (S.D./mean) × 100,

$$\mathrm{MD}(\%) = \left(\frac{\text{mean concentration} - \text{spiked concentration}}{\text{spiked concentration}}\right) \times 100.$$

transformation of CPMSO was also biphasic with K_{m1} and V_{max1} of 0.4 mM and 0.3 nmoles/mg protein/min, respectively, and K_{m2} and V_{max2} of 0.08 mM and 0.15 nmoles/mg protein/min, respectively.

The effects of various cytochrome P450 inducers on the microsomal *S*-oxidation of CPMS and CPMSO were evaluated to ascertain the participation of cytochrome P450 in these metabolic reactions. The sulphoxidation of CPMS by rat liver microsomes was increased ($P < 0.01$) by about 2.5-fold after the animals were pretreated with phenobarbital and by 30% ($P < 0.05$) after pretreatment with 3-methylcholanthrene but not with β-naphthoflavone, dexamethasone or clofibric acid (Table 3). The hepatic microsomal cytochrome P450 and protein content increased twofold after pretreatment with phenobarbital. Similarly, microsomes from rats pretreated with phenobarbital produced a significant ($P < 0.01$) increase in the *S*-oxidation of CPMSO by approximately twofold. The other inducers tested had no significant effect on the *S*-oxidation of CPMSO. These results suggest that the sulphoxidation of CPMS to CPMSO and the further oxidation of the sulphoxide to the corresponding sulphone are mediated by a phenobarbital-inducible form of cytochrome P450. The effects of incorporation of various metabolic inhibitors and activators on the in vitro *S*-oxygenation of CPMS and CPMSO are shown in Table 4. In the case of CPMS sulphoxidation, the addition of *n*-octylamine, SKF525A, 1-naphthylthiourea, orphenadrine and quinine in the incubation medium inhibited the enzyme activity in a concentration dependent manner. Maximum inhibitory effects were observed when the inhibitor concentration was 5 μM (Table 4). Addition of *n*-octylamine, 1-naphthylthiourea, orphenadrine, quinine and SKF525A in the incubation media was also effective at inhibiting

the *S*-oxidation of CPMSO. *n*-Octylamine is a potent inhibitor of cytochrome P450 but also an activator of FMO [1]. SKF525A, orphenadrine and quinine are known potent inhibitors of cytochrome P450 while 1-naphthylthiourea is a known inhibitor of FMO [1,18]. Thus, the observed inhibitory actions of these compounds suggest that the sulphoxidation of CPMS and CPMSO is mediated by both cytochrome P450 and FMO. This is in agreement with another literature report where it was shown that the metabolism of a simple alkylaryl sulphides was mediated by both cytochrome P-450 and the FMO [19].

The results obtained with orphenadrine and quinine indicate that the major phenobarbitone-inducible form of cytochrome P450 (CYP2B1) and CYP2D1, respectively, in rat liver, are the predominant contributors to the *S*-oxygenation of CPMS and CPMSO. Orphenadrine has been shown to be a potent inhibitor of CYP2B1, the major phenobarbital-inducible isoform of cytochrome P450 [18]. Quinidine is a documented competitive inhibitor of CYP2D1, also known as debrisoquine 4-hydroxylase [20]. However, further studies with monoclonal antibodies and purified forms of these isozymes are necessary to provide definitive evidence for the role of these cytochrome P-450 isoforms in the *S*-oxygenation of this simple sulphide and sulphoxide. Inclusion of other isozyme-specific inhibitors of cytochrome P450 such as ethanol (CYP2E1), erythromycin (CYP3A1) and α-naphthoflavone (CYP1A1) in the incubation mixtures did not alter the microsomal *S*-oxidation of CPMS or CPMSO to any significant extent (Table 4).

The formation of the sulphoxide and sulphone of DPS was dependent upon the presence of the NADPH regenerating system and the microsomal fraction. About 15–20% of DPS was converted to the sulphoxide while less than 0.1% of DPS was present in the form of $DPSO_2$, under the optimum incubation conditions used. A small fraction of DPS (less than 0.1%) was detected in the form of a hydroxylated derivative of the sulphide (Fig. 3) although the position of hydroxylation on the aromatic ring system of DPS has not been ascertained. Thus, the predominant metabolic pathway of this model substrate was demonstrated to be sulphoxidation with negligible secondary oxidation to the sulphone in vitro. Eadie–Hofstee analysis of DPS sulphoxidation was monophasic, indicating the involvement of a single enzyme in the oxidation reaction. Under the conditions described, the apparent V_{max} and K_m for the sulphoxidation of DPS determined from Eadie–Hofstee plot (data not shown) were 1.8 ± 0.15 nmoles/mg protein/min and 0.036 ± 0.002 mM, respectively. The formation of DPSO by rat liver microsomal fraction appeared to saturate above a substrate concentration of 0.5 mM.

Table 3
The effects of various cytochrome P450 inducers on the *S*-oxidation of CPMS, CPMSO, DPS and DPSO in vitro

Inducers	Activity (nmoles/mg protein/min)				CYP450 (nmoles/mg protein)
	CPMS	CPMSO	DPS	DPSO	
Control	1.8 ± 0.20	0.26 ± 0.03	1.5 ± 0.17	3.01 ± 0.08	0.36 ± 0.02
Phenobarbital	4.5 ± 0.29*	0.54 ± 0.03*	3.4 ± 0.2**	6.03 ± 0.4*	0.73 ± 0.03
β-Naphtholavone	2.0 ± 0.10	0.24 ± 0.02	1.2 ± 0.05*	2.25 ± 0.30	0.45 ± 0.02
3-Methyl-cholanthrene	2.3 ± 0.08	0.27 ± 0.01	1.4 ± 0.08	2.90 ± 0.31	0.51 ± 0.01
Dexamethasone	1.8 ± 0.07	0.25 ± 0.02	1.5 ± 0.11	3.03 ± 0.10	0.65 ± 0.04
Clofibrate	2.0 ± 0.06	0.26 ± 0.01	1.6 ± 0.09	2.98 ± 0.17	0.58 ± 0.02

The incubation conditions were: microsomal protein content = 4 mg/ml; incubation times were: 15 min for CPMS or DPS, 30 min for CPMSO, and 20 min for DPSO metabolism studies. Substrate concentrations were; 0.5 mM for CPMS, 2.5 mM for CPMSO, 0.25 mM for DPS and 1 mM for DPSO metabolism studies.

* $P < 0.05$.

** $P < 0.01$.

Table 4
The effects of various metabolic inhibitors on the *S*-oxidation of CPMS, CPMSO, DPS and DPSO in vitro

Inhibitors	Activity (nmoles/mg protein/min)			
	CPMS	CPMSO	DPS	DPSO
Control	1.80 ± 0.20	0.26 ± 0.03	1.50 ± 0.17	3.01 ± 0.08
n-Octylamine	1.01 ± 0.12**	0.13 ± 0.005**	0.20 ± 0.01**	0.02 ± 0.001**
1-Naphthylthiourea	1.4 ± 0.13*	0.12 ± 0.001**	0.90 ± 0.05**	1.8 ± 0.20*
SKF525A	0.95 ± 0.05**	0.16 ± 0.01*	0.08 ± 0.01**	0.15 ± 0.02**
Orphenadrine	0.98 ± 0.06**	0.17 ± 0.02**	0.30 ± 0.02**	0.15 ± 0.008**
Quinidine	0.70 ± 0.02**	0.13 ± 0.01**	0.02 ± 0.07**	0.03 ± 0.002**
α-Naphthoflavone	1.75 ± 0.04	0.24 ± 0.06	1.40 ± 0.03	2.80 ± 0.20
Erythromycin	1.8 ± 0.20	0.26 ± 0.03	1.50 ± 0.17	3.01 ± 0.08
Ethanol	1.8 ± 0.20	0.26 ± 0.03	1.50 ± 0.17	3.01 ± 0.08

The incubation conditions were: microsomal protein content = 4 mg/ml; incubation times were: 15 min for CPMS or DPS, 30 min for CPMSO, and 20 min for DPSO metabolism studies. Substrate concentrations were: 0.5 mM for CPMS, 2.5 mM for CPMSO, 0.25 mM for DPS and 1 mM for DPSO metabolism studies. Inhibitor concentrations were 5 mM and microsomal cytochrome P450 content was 0.36 ± 0.02 nmoles/mg protein.

* $P < 0.05$.

** $P < 0.01$.

About 10–12% of DPSO was converted to the sulphone. However, the corresponding sulphide, DPS, was not detected under these incubation conditions. Thus, the predominant metabolic pathway of DPSO was demonstrated to be *S*-oxidation to the sulphone with no reduction to the sulphide in vitro. Under the conditions described, the apparent V_{max} and K_m for the s-oxidation of DPSO determined from Eadie–Hofstee plot (data not shown) were 2.9 ± 0.15 nmoles/mg protein/min and 0.041 ± 0.003 mM, respectively.

The effects of various cytochrome P450 inducers on the microsomal *S*-oxidation of DPS and DPSO were evaluated to ascertain the participation of cytochrome P450 in these metabolic reactions. The sulphoxidation of DPS by rat liver microsomes was increased ($P < 0.01$) by about twofold after the animals were pretreated with phenobarbital but was unchanged after pretreatment with 3-methylcholanthrene, dexamethasone or clofibric acid (Table 3). Pre-treatment with β-naphthoflavone caused a small but significant decrease in DPS sulphoxidation. These results suggest that the major inducible form of cytochrome P450 plays a major role in DPS sulphoxidation. Similarly, microsomes from rats pretreated with phenobarbital demonstrated a significant ($P < 0.01$) increase in the *S*-oxidation of DPSO by twofold. The other inducers tested had no significant effect on the *S*-oxidation of DPSO although DPSO *S*-oxidation was slightly lower in microsomes from rats pretreated with β-naphthoflavone. These results suggest that the sulphoxidation of DPS to DPSO and the further oxidation of the sulphoxide to the corresponding sulphone are mediated by a phenobarbital-inducible form of cytochrome P450. In the case of DPS sulphoxidation, the addition of *n*-octylamine, SKF525A, 1-naphthylthiourea, orphenadrine and quinine in the incubation medium inhibited the enzyme activity in a concentration dependent manner with maximum inhibition occurring at 5 mM (Table 4). Addition of *n*-octylamine, SKF525A, 1-naphthylthiourea, orphenadrine and quinine in the incubation media was also effective at inhibiting the *S*-oxidation of DPSO. The results from the present investigation suggest the involvement of cytochrome P450 2B1 (CYP2B1) in the *S*-oxidation of DPS and DPSO. This is in agreement with another literature report where it was shown that the metabolism of a simple diaryl sulphide was mediated by cytochrome P450 monooxygenase [10].

The results obtained with orphenadrine and quinine suggest that the major phenobarbitone-inducible form of cytochrome P450 (CYP2B1) and CYP2D1, respectively, in rat liver, are the pre-

dominant contributors to the *S*-oxygenation of DPS and DPSO. Inclusion of other isozyme-specific inhibitors of cytochrome P450 such as ethanol (CYP2E1), erythromycin (CYP3A1) and α-napththoflavone (CYP1A1) in the incubation mixtures did not alter the microsomal *S*-oxidation of DPS or DPSO (Table 4).

In conclusion, simple HPLC assay methods have been developed for monitoring CPMS, DPS and their corresponding sulphoxides and sulphones in microsomal incubations and used successfully to elucidate the enzymology of CPMS, DPS and their corresponding sulphoxides in vitro. CPMS, DPS and their corresponding sulphoxides may be used as probes for assessing the activity of the microsomal monooxygenases in vivo and in vitro and the information generated with these simple sulphur-containing compounds may be extrapolated to more complex xenobiotics bearing the alkylaryl or diaryl sulphide and sulphoxide functionalities.

Acknowledgements

The work described in this paper has been supported in part by a Cameroon government scholarship awarded to I.P.N. and a Medical Research Council grant awarded to L.A.D.

References

[1] D.M. Ziegler, TIPS 11 (1990) 321.
[2] L.A. Damani (Ed.), Sulphur-Containing Drugs and Related Organic Compounds, vol. 1, part A, Ellis Horwood Ltd., Chichester, 1989.
[3] S.G. Dahl, M. Hyorth, E. Hough, Mol. Pharmacol. 21 (1982) 409.
[4] S.C. Mitchell, Drug Metab. Drug Interact. 6 (1988) 245.
[5] D.M Ziegler, in: W.B. Jacoby (Ed.), Enzymatic Basis of Detoxification, vol. 1, Academic Press, New York, 1980, p. 201.
[6] L.J. Traficante, J. Siekierski, G. Sakalis, S. Gershon, Biochem. Pharmacol. 28 (1979) 621.
[7] A.G. Renwick, S.P. Evans, T.W Sweatman, J. Cumberland, C.F. George, Biochem. Pharmacol. 31 (1982) 2649.
[8] D.D. Oehler, G.W. Ivie, Arch. Environ. Contam. Toxicol. 12 (1983) 227.
[9] S.C. Lee, A.G. Renwick, Biochem. Pharmacol. 49 (11) (1995) 1557.
[10] A.A. Hoodi, L.A. Damani, J. Pharm. Pharmacol. 36 (Suppl.) (1984) 629.
[11] I.P. Nnane, L.A. Damani, Xenobiotica 29 (1999) 1101.
[12] L.A. Damani, I.P. Nnane, Drug Metab. Drug Interact. 13 (1996) 1.
[13] F.J. Gonzalez, Pharmac. Ther. 45 (1990) 1.
[14] M. Madesclaire, Tetrahedron 42 (1986) 5459.
[15] O.H Lowry, N.S. Roseborough, A.L. Farr, R.S. Randall, J. Biol. Chem. 193 (1951) 265.
[16] G.G. Gibson, P. Skett, Introduction to Drug Metabolism, Chapman and Hall, London, 1986.
[17] W. Lenk, Biochem. Pharmacol. 25 (1976) 997.
[18] M. Murray, G.F. Reidy, Pharmacol. Rev. 42 (1990) 85.
[19] D.J. Waxman, D.R. Light, C. Walsh, Biochemistry 21 (1982) 2499.
[20] S. Kobayashi, S. Murray, D. Watson, D. Sesardic, D.S. Davis, A.R. Boobis, Biochem. Pharmacol. 38 (1989) 2795.