Characterisation of theophylline metabolism in human liver microsomes

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1 A radiometric high performance liquid chromatographic method is described for the assay of theophylline metabolism *in vitro* by the microsomal fraction of human liver.

2 Formation of the three metabolites of theophylline (3-methylxanthine, 1-methylxanthine and 1,3-dimethyluric acid) were linear with protein concentrations to 4 mg ml⁻¹ and with incubation times up to 180 min.

3 The coefficients of variation for the formation of 3-methylxanthine, 1-methylxanthine and 1,3-dimethyluric acid were 1.2%, 1% and 1.6%, respectively.

4 Theophylline is metabolised by microsomal enzymes with a requirement for NADPH.

5 The mean ($n = 7$) K_m values for 1-demethylation, 3-demethylation and 8-hydroxylation were 545, 630 and 788 μM , respectively, and the mean V_{max} values were 2.65, 2.84 and 11.23 pmol min⁻¹ mg⁻¹, respectively.

6 There was a high correlation between the K_m and V_{max} values for the two demethylation pathways suggesting that the demethylations are performed by the same enzyme.

7 Overall the *in vitro* studies are consistent with the *in vivo* results which suggest the involvement of two cytochrome P-450 isozymes in the metabolism of theophylline.

Keywords theophylline metabolism cytochrome P-450 *in vitro*

Introduction

The hepatic cytochrome P-450 system catalyses the oxidation of a wide variety of xenobiotics, including human therapeutic agents. The multiplicity of the cytochrome P-450 system has been well established in animals (Guengerich *et al.*, 1982; Aoyama *et al.*, 1982). More recently the isolation and purification of nine isozymes (Wang *et al.*, 1983; Cresteil *et al.*, 1985) of cytochrome P-450 from human liver has provided direct biochemical evidence for the multiplicity of cytochrome P-450 in man.

In 1957 the first comprehensive *in vivo* investigation of the metabolism of the methylxanthines (theophylline, theobromine and caffeine) was

reported by Cornish & Christman (1957). The metabolic pathways for theophylline in man have been confirmed in a number of studies (Grygiel & Birkett, 1981; Grygiel *et al.*, 1984; Ogilvie, 1978). The major route is the 8-hydroxylation to 1,3-dimethyluric acid (DMU) which accounts for 45–55% of the total theophylline clearance. The other metabolic products are 3-methylxanthine (3MX) and 1-methyluric acid (1MU) which represent 13–16% and 20–25% of theophylline clearance, respectively. Subsequently it has been shown that 1MU is formed from 1-methylxanthine (1MX) by a reaction mediated by xanthine oxidase (Birkett *et al.*,

1983). Although the metabolic pathways involved in theophylline metabolism have been identified and quantified, relatively little information exists about the enzymes involved. In 1976 Lohman & Miech reported that theophylline was metabolised to DMU and 1MU by rat liver slices and that this activity was blocked by classical cytochrome P-450 inhibitors and stimulated by pretreatment with P-450 inducing agents. Further, the sub-cellular localisation of this enzymatic activity supported the involvement of the cytochrome P-450 system as theophylline metabolism was located in the microsomal fraction but not in the mitochondrial or cytosolic fractions. No 3MX was formed in the rat liver slice preparation and this is consistent with the hypothesis that unlike man and rabbits, rats cannot demethylate theophylline in the 1 position.

In man, the enzymes involved in theophylline metabolism have been investigated by *in vivo* studies using known inducers and inhibitors of the P-450 system (Grygiel & Birkett, 1981; Grygiel *et al.*, 1984; Robson *et al.*, 1984; Miners *et al.*, 1985; Grygiel *et al.*, 1979). These studies provide inferential evidence that cytochrome P-450 system is the enzyme involved in the metabolism of theophylline in man and are consistent with the more direct evidence in rat liver slices and rat microsomes (Lohman & Miech, 1976). We have postulated on the basis of data from *in vivo* studies (Birkett *et al.*, 1985) that there are at least two isozymes of cytochrome P-450 involved in the metabolism of theophylline, one isozyme predominantly involved in the 1- and 3-demethylations and another isozyme predominantly performing the 8-hydroxylation. Although the involvement of two isozymes of cytochrome P-450 in these pathways can to some extent be differentiated, particularly by inhibitors, their regulation must be closely linked as there is a high degree of correlation between all three pathways in healthy control subjects (Birkett *et al.*, 1985). To gain further information on the metabolism of theophylline in man, an *in vitro* assay has been developed and used to study the kinetics of theophylline metabolism in human liver microsomes.

Methods

Chemicals

All organic solvents and other chemicals were of analytical reagent grade. NADP, glucose-6-phosphate (G-6-P) and glucose-6-phosphate dehydrogenase (G-6-PD) were purchased from Sigma Chemical Co. (St Louis, MO).

8-[¹⁴C]-theophylline with a specific activity of 45.0 mCi mmol⁻¹ was purchased from New England Nuclear. The 8-[¹⁴C]-theophylline was purified by high performance liquid chromatography (h.p.l.c.) by injecting 100 µl aliquots onto a C-18 reversed phase (4 mm × 15 cm) column, with a flow rate of 1.75 ml min⁻¹ of elution solvent (acetonitrile 6%, methanol 6%, 10 mM acetic acid, pH 3.5). Under these conditions the retention time for theophylline was 3.5 min. The eluent under the theophylline peak was collected, pooled and extracted five times with redistilled dichloromethane (volume ratio of 1:10). The organic phase was evaporated to dryness and the purified 8-[¹⁴C]-theophylline reconstituted in 5% ethanol/water to give a final concentration of 0.96 mM. Radiometric purity of the 8-[¹⁴C]-theophylline was greater than 99.5% on subsequent radiometric h.p.l.c. assay.

Human liver samples

Human liver samples were obtained with the consent of relatives from renal transplant donors. The approval of the Flinders Medical Centre Ethical Review Committee was obtained to use such material for drug metabolism studies.

Liver was obtained as soon as practicable (less than 60 min) after circulatory arrest and removal of the kidneys. The liver was cut into small slices (< 1 cm) and immediately frozen in liquid nitrogen and stored at -80° C. Two human liver samples were obtained from the human liver bank of Professor U. Meyer, Basel, Switzerland. Table 1 contains details of the renal donors. All liver donors were negative for hepatitis B and HIV antibodies.

Microsomes were prepared by the differential centrifugation method as previously described by McManus *et al.* (1987) and stored in aliquots at -80° C in 0.1 M phosphate buffer, pH 7.4 containing 20% (v/v) glycerol at a protein concentration of 15–25 mg ml⁻¹. Microsomal protein concentrations were determined by the method of Lowry *et al.* (1951) with bovine serum albumin as a standard. Microsomal cytochrome P-450 concentrations were determined by the method of Omura & Sato (1964).

Theophylline metabolism

A standard 0.5 ml incubation mixture contained microsomal protein 2 mg, phosphate buffer 0.1 M, pH 7.4 and 12.5 nmol 8-[¹⁴C]-labelled theophylline plus unlabelled theophylline to give a final concentration of 25 to 500 µM. All reactions were started by the addition of NADPH-generating system (1 mM NADP, 10 mM G-6-P,

Table 1 Liver donor details

Patient	Sex	Age (years)	Cytochrome P-450 (nmol mg ⁻¹ protein)	Cause of death	Drug history
F5	M	62	0.47	Gunshot	Phenytoin ¹ Dexamethasone ² Non-smoker
F6	M	18	0.34	Motor vehicle accident	Non-smoker
F7	F	44	0.41	Subarachnoid haemorrhage	Dexamethasone ³ Non-smoker
F8	M	24	0.32	Subarachnoid haemorrhage	Smoker
F9	M	18	0.34	Motor vehicle accident	Non-smoker
B5	M	13	0.39	Skiing accident	Non-smoker
B12	*	*	0.35	*	*

* Details not available.

¹ 500 mg day⁻¹ for 9 days.

² 48 mg day⁻¹ for 9 days.

³ 24 mg day⁻¹ for 1 day.

5 mM MgCl₂, 2 iu G-6-P dehydrogenase, phosphate buffer 0.1 M, pH 7.4) and carried out in air at 37°C in a metabolic shaker for 2 h. Generating system was omitted from blanks and replaced by an equal volume of buffer. Reactions were stopped by cooling in an ice bath with the addition of 50 µl 5 M hydrochloric acid. Authentic standards of the theophylline metabolites 3MX, 1MX and DMU were added to visualise absorbance peaks during chromatography. The aqueous reaction mixture was saturated with 400 mg ammonium sulphate and extracted with dichloromethane: isopropanol 80:20 v/v (2 × 10 ml). The pooled extracts were evaporated under nitrogen and the residue dissolved in 0.1 ml mobile phase for chromatographic analysis. Extraction efficiency under these conditions for theophylline and each of its metabolites was greater than 95%.

Chromatography

Theophylline was separated from its metabolites using h.p.l.c. Reconstituted sample (85 µl) was injected onto a reversed-phase C-18 column (4 mm × 15 cm). The initial mobile phase composition was 1% methanol and 1% acetonitrile in 0.01 M acetic acid, pH 3.5. Absorbance was monitored at 280 nm. At 13 min, the mobile phase composition was programmed over 30 s to

7.5% acetonitrile, 7.5% methanol and 85% 0.01 M acetic acid, pH 3.5. With a mobile phase flow rate of 2.0 ml min⁻¹ the retention times of 3MX, 1MX, DMU and theophylline were 6.75, 8, 11.5 and 16.5 min, respectively. Column eluent was collected in aliquots and counted on a liquid scintillation counter (Beckman Model LS 3801). Chromatography was performed using a Beckman 342 gradient liquid chromatograph modular system, consisting of a 420 system controller, two 114 solvent delivery modules, a 340 organiser and a 160 selectable wavelength detector.

Intra-assay coefficients of variation for 3MX, 1MX and DMU formation were 1.2, 1, and 1.6%, (*n* = 6) respectively, and the between day coefficients of variation with the same batch of microsomes were 8, 14 and 8%, respectively (*n* = 10). The limit of sensitivity was defined as less than 500 d min⁻¹ (with background subtracted) in a metabolite peak.

Kinetic analysis

Values for *K_m* and *V_{max}* were calculated by MKMODEL, an extended least squares modelling program (Holford, 1985), using initial parameter estimates obtained from Eadie-Hofstee plots of the data.

Analysis of variance and Spearman's rank correlations were used to analyse the data. *K_m*

values were log transformed prior to analysis to normalise the data.

Results

Microsomes from all human livers metabolised theophylline by 1- and 3-demethylation and 8-hydroxylation to form 3MX, 1MX and DMU, respectively. An unidentified polar compound, representing about 0.1% of the total radioactivity, was present in all microsomal incubations in the second eluent fraction (1.5 to 3.0 min) as shown in Figure 1. There was no activity in the absence of the NADPH generating system.

Linearity of the formation of 3MX, 1MX and DMU by microsomal fractions of human liver

Figure 1 Radiochromatogram of theophylline and its metabolites after extraction from a microsomal incubation mixture. The solid line is the UV absorbance trace showing the elution times for theophylline and metabolites. The percentage of total radioactivity recovered as each metabolite and theophylline is represented by the hatched bars. 1, 3-methylxanthine; 2, 1-methylxanthine; 3, 1,3-dimethyluric acid; 4, theophylline.

with respect to time was investigated at a concentration of 150 μM theophylline concentration. Protein concentration was 4 mg ml^{-1} . The appearances of 3MX, 1MX and DMU were linear to 180 min (Figure 2). The effects of microsomal protein concentration on 3MX, 1MX and DMU formation are shown in Figure 3. The concentration of theophylline was 150 μM and the incubation time was 120 min. Activity for the production of 3MX, 1MX and DMU increased linearly with protein concentration up to 4 mg ml^{-1} , the highest concentration studied. At the lowest substrate concentration used (50 μM), less than 1% of theophylline was converted to products.

Representative Lineweaver-Burk and Eadie-Hofstee plots for one human liver are shown in Figures 4 and 5. The Michaelis-Menten parameters (K_m and V_{max}) for all seven livers are listed in Table 2. The proportions of the V_{max} values for each pathway as a percentage of the total reaction velocity were $16.1\% \pm 5.6$, $16.8\% \pm 4.5$ and $67.2\% \pm 9.7$ for 3-demethylation, 1-demethylation and 8-hydroxylation, respectively. Within an individual, the K_m values for the formation of 3MX and 1MX were similar and tended to differ from the K_m for the formation of DMU.

Statistical analysis using log transformation for analysis of variance showed that there was no significant difference between the K_m values for

Figure 2 Effect of incubation time on the appearance of 3MX (Δ), 1MX ($\bullet$) and DMU ($\blacksquare$). The microsomal protein concentration was 4 mg ml^{-1} . Theophylline concentration was 150 μM .

Figure 3 Effect of microsomal protein concentration on the rate of appearance of 3MX (▲), 1MX (●) and DMU (■). The incubation time was 120 min and the theophylline concentration was 150 μM .

Figure 5 Eadie-Hofstee plot for the rate of appearance of 3MX (▲), 1MX (●) and DMU (■) by microsomes from subject F6.

the two demethylations but there was a difference between the K_m s for the 8-hydroxylation and 1-demethylation ($P < 0.05$) and between 8-hydroxylation and 3-demethylation ($P = 0.06$). Constraining the model to require equal K_m s for the 1- and 3-demethylations, gave fits to the data as good as, or better than, the unconstrained model. Constraining the model to make the K_m

for either demethylation pathway the same as that for 8-hydroxylation resulted in a poorer fit to the data. The Spearman's rank correlation coefficients for the K_m and $V_{\max}$ data are shown in Table 3. There were positive correlations between all K_m values except for 1-demethylation vs 8-hydroxylation which was not statistically significant. There was a high degree of correlation

Figure 4 Lineweaver-Burk plot for the rate of appearance of 3MX (▲), 1MX (●) and DMU (■) by microsomes from subject F6.

Table 2 Michaelis–Menten parameters for *in vitro* theophylline metabolism

Liver	3MX	K_m (μM) 1MX	DMU	V_{max} ($pmol\ min^{-1}\ mg\ protein^{-1}$)		
				3MX	1MX	DMU
F5	484	713	1445	0.87	1.63	18.4
F6	521	566	737	2.2	2.8	8.8
F7	399	464	517	1.8	1.7	7.7
F8	577	645	778	2.3	2.3	9.4
F9	884	1224	1086	5.0	4.9	15.8
B5	112	94	123	0.61	0.53	1.91
B12	839	752	834	5.8	6.0	16.6
Mean	545	636	788	2.65	2.84	11.23
s.d.				1.9	1.9	5.9
95% confidence limits	396–549	443–614	550–762			

Table 3 Correlations between theophylline metabolic pathways

	Spearman rank correlation coefficient	
	K_m	V_{max}
Formation of: 3MX vs 1MX	0.89	0.96
3MX vs DMU	0.64	0.46
1MX vs DMU	0.89	0.42

The critical value of the Spearman rank correlation coefficient for $P < 0.05$ is 0.714.

between the V_{max} values for the two demethylations but neither the 3- nor 1-demethylation was correlated with the 8-hydroxylation.

Discussion

Although the *in vitro* metabolism of theophylline has been demonstrated in rat liver microsomes (Lohman & Miech, 1976) this is the first report of the *in vitro* metabolism of theophylline by human liver microsomes. In the absence of a NADPH generating system no products were formed. This is consistent with the *in vitro* animal data (Lohman & Miech, 1976) and the *in vivo* human data supporting the involvement of the cytochrome P-450 enzyme system in the metabolism of theophylline to 3MX, 1MX and DMU. The metabolic profile *in vitro* is similar to that *in vivo* where 50–60% is metabolised by 8-hydroxylation, 15–20% by 3-demethylation and 20–25% by 1-demethylation. No 1-methyluric acid (1MU) was detected which is consistent with the proposal that, *in vivo*, 1MU is formed from 1MX by xanthine oxidase which is not located in the microsomal fraction used in these *in vitro* studies.

Theophylline is a low intrinsic clearance drug and to demonstrate and characterise the *in vitro* metabolism of theophylline in human liver microsomes a highly sensitive radiometric h.p.l.c. assay was developed. To calculate K_m , the range of substrate concentrations should ideally range from 0.2 to 5 times the estimated K_m value. The range of theophylline concentrations used in the present studies was limited by the specific activity of 8- $[^{14}C]$ -theophylline available but there was an eight-fold difference between the lowest and highest velocities which allowed K_m to be estimated with reasonable precision. There was an 8- to 10-fold interindividual variation in the K_m values. Within an individual, the K_m values for the formation of 3MX and 1MX tended to be similar and to differ from the K_m for the formation of DMU. This is consistent with the *in vivo* inhibition and induction studies (Grygiel & Birkett, 1981; Grygiel *et al.*, 1979, 1984; Robson *et al.*, 1984; Miners *et al.*, 1985) suggesting that the 1- and 3-demethylation are predominantly performed by the same P-450 isozyme and that the 8-hydroxylation is performed by a different P-450 isozyme. The correlations between the kinetic parameters for the pathways are also consistent with the involvement of at least two isozymes of cytochrome P-450 in the metabolism of theophylline. The V_{max} value is directly proportional to the amount of enzyme present under linear reaction conditions which applied in this study. The high correlation between the V_{max} values for 3MX and 1MX production are consistent with these reactions being carried out by the same isozyme. The lack of correlation between V_{max} for DMU formation and those for 1MX and 3MX production strongly suggests that a different isozyme is at least partly responsible for 8-hydroxylation. There were significant correlations between K_m s for 1-demethylation and

3-demethylation and between K_m s for 3-demethylation and 8-hydroxylation. The significance of the K_m correlations is not clear particularly as the K_m s for the three pathways are relatively similar. The interindividual variation in the K_m values is difficult to reconcile with the predominant involvement of one isozyme in each of the pathways. However, similar interindividual variations in K_m values have been reported for other substrates, particularly those exhibiting genetic polymorphism which are presumed to be predominantly metabolised by one P-450 isozyme. A two-fold range has been reported for extensive metabolisers of mephenytoin (Meier *et al.*, 1985), and a large standard error of the mean has been reported for phenacetin K_m (Boobis *et al.*, 1981). A membrane environment varying between individuals may contribute to the range in the K_m values reported in this and other studies.

Subject F5 is unusual in that the 8-hydroxylation activity of liver F5 was selectively increased so that the ratios of V_{max} values for 8-hydroxylation compared to the demethylations were three- to four-fold higher than with the other livers. Liver F5 was obtained from a patient treated with dexamethasone and phenytoin and it is possible that the 8-hydroxylation pathway was selectively induced in this patient. The F7 liver

was obtained from a patient also treated with dexamethasone but for a shorter duration, and did not show the same increase in 8-hydroxylation compared to F5. It is possible that the increased 8-hydroxylation activity in the liver F5 was due to the phenytoin but a contribution from dexamethasone cannot be excluded.

The K_m values for all pathways, except liver B5, are well above the usual therapeutic range which is consistent with the fact that saturation kinetics are infrequent in clinical practice. The *in vivo* K_m values reported by Dahlqvist *et al.* (1984) for six subjects also demonstrated a large interindividual variation. The calculated mean *in vivo* K_m values for all three pathways were lower than the *in vitro* values determined in this study.

The data presented are consistent with the involvement of two isozymes of cytochrome P-450 in the metabolism of theophylline, but definitive evidence awaits the isolation and characterisation of these cytochrome P-450 isozymes.

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