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TOLBUTAMIDE HYDROXYLATION BY HUMAN LIVER MICROSOMES

KINETIC CHARACTERISATION AND RELATIONSHIP TO OTHER CYTOCHROME P-450 DEPENDENT XENOBIOTIC OXIDATIONS

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Abstract—Tolbutamide hydroxylation has been investigated in human liver microsomes. Anti-human liver NADPH-cytochrome P-450 reductase IgG inhibited hydroxytolbutamide formation and this metabolite was not formed when NADPH-generating system was omitted from microsomal incubations. Tolbutamide hydroxylation followed Michaelis–Menten kinetics, consistent with the involvement of a single form of cytochrome P-450 in this reaction. Mean apparent K_m and V_{max} values for hydroxytolbutamide formation were 120 ± 41 μM and 0.273 ± 0.066 nmol min^{-1} mg^{-1}, respectively.

A range of clinically used drugs and xenobiotics used as probes for cytochrome P-450 activity in laboratory animals was screened for inhibitory effects on hydroxytolbutamide formation. Caffeine, paraxanthine, theophylline, theobromine, debrisoquine, erythromycin, phenacetin, propranolol, aminopyrine, benzo(*a*)pyrene and 7-ethoxycoumarin were all found not to inhibit tolbutamide hydroxylation. In contrast, sulphaphenazole, phenylbutazone, nifedipine, verapamil, cimetidine, aniline, dextropropoxyphene and mephenytoin were competitive inhibitors of tolbutamide hydroxylation. The respective apparent K_i values for these compounds were 0.12 μM, 11 μM, 15 μM, 118 μM, 140 μM, 182 μM, 225 μM and 375 μM. Sulphinpyrazone inhibited tolbutamide hydroxylation with atypical kinetics. The *in vitro* data is in good agreement with *in vivo* drug interactions with tolbutamide. The data also confirm that tolbutamide hydroxylation is not associated with the cytochromes P-450 responsible for methylxanthine metabolism or with the form responsible for the polymorphic oxidation of debrisoquine.

Tolbutamide (TB; 1-butyl-3-*p*-tolylsulphonylurea) is widely used as an oral hypoglycaemic agent. Oxidation of the tolyl methyl group to form hydroxy-TB is the rate-limiting step in TB elimination in humans and animals [1, 2]. Hydroxy-TB is further metabolised to carboxy-TB by alcohol dehydrogenase and aldehyde dehydrogenase [3]. It appears that a single form of cytochrome P-450 may mediate the formation of hydroxy-TB since there is some evidence [4, 5] for monogenic control of TB metabolism in man. *In vivo* human volunteer studies [5, 6] indicate that the putative cytochrome P-450 involved in TB metabolism is not co-regulated with the form of cytochrome P-450 responsible for the debrisoquine 4-hydroxylation polymorphism. In addition, it has been demonstrated that TB and theophylline metabolism in human volunteers are differentially affected by cigarette smoking [4, 7] and by pretreatment with propranolol [8, 9], sulphaphenazole [10, 11] or sulphinpyrazone [12, 13], suggesting that the form of cytochrome P-450 involved in TB metabolism is distinct to the forms responsible for theophylline metabolism.

In the present study we have determined the effect of an antibody to human liver NADPH-cytochrome P-450 reductase on hydroxy-TB formation in human liver microsomes. Moreover, we have investigated TB kinetics in human liver microsomes and determined the extent of inhibition of *in vitro* hydroxy-TB formation by a range of chemicals known to be substrates or inhibitors of cytochrome P-450 to characterise further the relationship between tolbutamide hydroxylation and other cytochrome P-450 dependent reactions in man.

MATERIALS AND METHODS

Chemicals and reagents. Aminopyrine, aniline HCl, benzo(*a*)pyrene, 7-ethoxycoumarin, paraxanthine and phenacetin were purchased from the Sigma Chemical Co. (St Louis, MO). Other drugs and drug metabolites were obtained from the following sources: tolbutamide and hydroxytolbutamide from Hoechst Aust. (Melbourne, Australia); caffeine, theobromine and theophylline from Hamilton Laboratories (Adelaide, Australia); phenylbutazone, sulphaphenazole and sulphinpyrazone from Ciba-Geigy Aust. (Sydney, Australia); propranolol HCl from I.C.I. Aust. (Melbourne, Australia); chlorpropamide from Pfizer Ltd. (Sydney, Australia); debrisoquine sulphate from Roche Products Ltd. (Sydney, Australia); erythromicin from F. H. Faulding & Co. (Adelaide, Australia); cimetidine from Smith, Kline and French Laboratories (Sydney, Australia); dextropropoxyphene from Protea Pharmaceuticals (Sydney, Australia); nifedipine from

Bayer Pharmaceutical (Sydney, Australia); mephenytoin from Sandoz Ltd. (Basle, Switzerland); and verapamil from Knoll AG (Ludwigshafen, F.R.G.). All other reagents and solvents were of analytical grade. The polyclonal antibody to human liver NADPH-cytochrome P-450 reductase was prepared as previously described [14].

Liver samples. Human liver samples were obtained from renal transplant donors; relevant details of the donors of livers used in the present study (F5–F9) have been published elsewhere [15]. The donor of liver F5 received phenytoin and dexamethasone while the donor of liver F7 received dexamethasone alone before removal of the kidneys. With the exception of the donor of liver F8, all donors were non-smokers. Liver samples were stored at −80° until used. Microsomes were prepared by differential centrifugation as previously described [15]. The approval of the Flinders Medical Centre Ethical Review Committee was obtained to use renal transplant donor liver for drug metabolism studies.

Measurement of hydroxy-TB formation in human liver microsomes. A standard 1.0 ml incubation contained microsomal protein (0.3 mg) in phosphate buffer (0.1 M, pH 7.4), NADPH-generating system (1 mM NADP, 10 mM glucose-6-phosphate, 2 IU glucose-6-phosphate dehydrogenase and 5 mM $MgCl_2$ in phosphate buffer, 0.1 M, pH 7.4) and TB (25–2000 μM). All reactions were started by the addition of NADPH-generating system and were carried out in air in 37° in a metabolic shaker for 1.5 hr. Generating system was omitted from the blanks and replaced by an equal volume of buffer. Reaction rate was shown to be linear with time to 3 hr and for microsomal protein concentrations to at least 1.6 mg.ml^{-1} (range studied 0.05–1.6 mg.ml^{-1}). The reaction was stopped by the addition of 1.0 ml of 0.15 M phosphoric acid and cooling on ice. Excess TB was removed from an aliquot (1.8 ml) of the incubation mixture by extraction with 8.0 ml of hexane–chloroform–isoamylalcohol (1000:250:5). The organic phase was discarded and, after addition of "internal" standard (0.1 ml of chlorpropamide, 10 mg.l^{-1}), the aqueous phase was re-extracted with diethyl ether (8.0 ml). The aqueous phase was then frozen in an acetone–dry ice bath and the organic phase transferred to a clean conical tube and evaporated to dryness. The residue was reconstituted in 0.12 ml of mobile phase and a 0.05 ml aliquot injected onto the high performance liquid chromatograph.

The chromatograph used was fitted with a Nova-Pak C18 4 micron reversed-phase column (15 cm × 3.9 mm, i.d.; Waters-Millipore) and operated at ambient temperature. Hydroxy-TB and the internal standard, chlorpropamide, were monitored by UV absorbance at 230 nm. Unknown concentrations of hydroxy-TB were determined by comparison of peak height ratios with those of a calibration curve prepared using hydroxy-TB concentrations in the range 0.1–5.0 μM. The mobile phase was acetate buffer (10 mM, pH 4.3)-acetonitrile (78:22) at a flow rate of 2.0 ml.min^{-1}. Under these conditions the retention times for hydroxy-TB and chlorpropamide were 3.3 min and 11.3 min, respectively. Representative chromatograms are shown in Fig. 1. Using this procedure the mean recovery of hydroxytolbutamide, calculated by

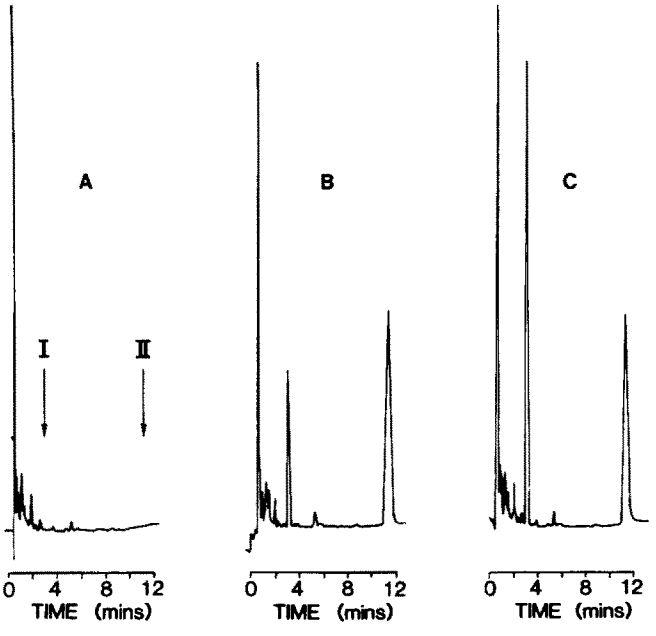

Fig. 1. Representative chromatograms of extracts: (A) blank chromatogram: microsomal incubation performed without tolbutamide and without internal standard added during extraction; (B) standard containing hydroxytolbutamide, 1μM; (C) microsomal incubation performed with tolbutamide, 1 mM. I and II in blank chromatogram show retention times of hydroxytolbutamide and internal standard, respectively.

comparing the peak height for extracted compound with that of an equal amount injected directly into the chromatograph, was 87.0 ± 3.8% for six samples in the concentration range 0.1–5.0 μM. The within-day coefficient of variation of the assay, determined by measuring hydroxy-TB formation in twelve separate incubations of the same batch of hepatic microsomes, was 3.6%.

In experiments performed to determine the Michaelis–Menten parameters for hydroxy-TB formation, activity was measured for ten TB concentrations over the range 25–2000 μM using microsomes from five human livers (F5–F9). The extent of inhibition of hydroxy-TB formation by a range of compounds was screened in microsomes from three livers (F5, F8, F9). Compounds screened for inhibitory effects are listed in Table 2. The effects of two inhibitor concentrations (generally 50 μM and 500 μM; Table 2) on hydroxy-TB formation for a single TB concentration (100 μM) were assessed in the screening studies. Where >15% inhibition was observed in the screening experiments, full kinetic studies were performed using microsomes from liver F8 to characterise the nature of the inhibition.

Other assays. Microsomal protein concentration was measured by the procedure of Lowry *et al.* [16] using crystalline bovine serum albumin as standard.

Analysis of results. All results are presented as mean ± SD. Initial estimates of the Michaelis–Menten parameters, apparent K_m and V_{max}, were obtained from graphical analysis of Eadie–Hofstee plots. These values were then used as first estimates for MKMODEL, an extended least squares modelling programme [17]. The method of Dixon [18] was used for the calculation of apparent K_i values.

RESULTS

Microsomes from all five livers F5–F9 converted TB to hydroxy-TB. In the absence of NADPH generating system no metabolism occurred. Inhibition of hydroxy-TB formation by anti-human liver NADPH-cytochrome P-450 reductase IgG tended to plateau at antibody to microsomal protein concentration ratios above 5:1 (Fig. 2). At an antibody to microsomal protein concentration ratio of 20:1 hydroxy-TB formation was reduced to 45.1 ± 7.7% of control values in microsomes from livers F5–F9.

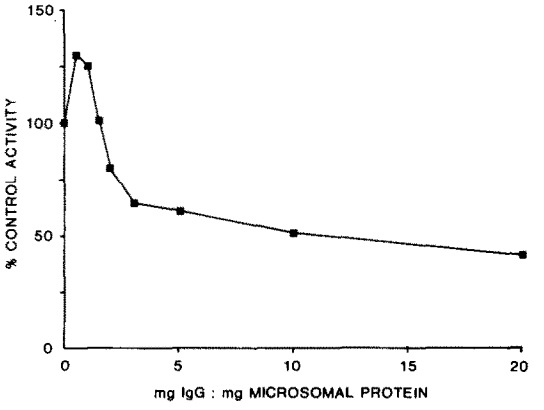

Fig. 2. Effect of increasing concentration of antibody to human liver NADPH–cytochrome P-450 reductase on hydroxytolbutamide formation in pooled microsomes from livers F5–F9.

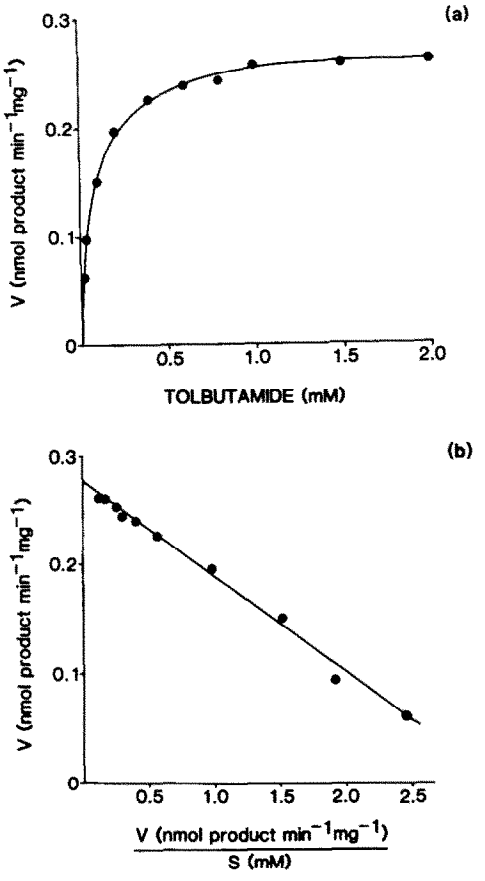

Fig. 3. Representative velocity versus substrate concentration plot (panel a) and Eadie–Hofstee plot (panel b) for hydroxytolbutamide formation in microsomes from liver F8. Points are experimentally determined values while solid lines are the computer-generated curves of best fit.

Hydroxy-TB formation followed Michaelis–Menten kinetics in the five livers studied. Representative velocity versus substrate concentration and Eadie–Hofstee plots for hydroxy-TB formation are shown in Fig. 3. Computer derived Michaelis–Menten parameters for hydroxy-TB formation are summarised in Table 1. The mean apparent K_m and V_{max} values

Table 1. Computer derived Michaelis–Menten parameters for hydroxytolbutamide formation in human liver microsomes

Liver	Apparent K_m (μM)	V_{max} (nmol.min^{-1}.mg^{-1})
F5	120	0.224
F6	176	0.213
F7	62	0.393
F8	88	0.274
F9	123	0.292
Mean	120	0.273
±SD	±41	±0.066

Table 2. Inhibitory effects of various compounds on hydroxytolbutamide formation in human liver microsomes

Compound (inhibitor)	Percent control activity	
	"Low" inhibitor* concentration	"High" inhibitor concentration
Aminopyrine	99.6 ± 5.8	91.8 ± 0.8
Aniline	75.9 ± 3.0	28.6 ± 1.3
Benzo(*a*)pyrene	96.1 ± 1.6	88.8 ± 6.5
Caffeine	97.0 ± 1.5	97.7 ± 3.9
Cimetidine	76.5 ± 3.9	31.7 ± 2.9
Debrisoquine	99.4 ± 5.0	99.2 ± 4.7
Dextropropoxyphene	82.6 ± 2.4	49.3 ± 4.4
Erythromycin	98.3 ± 4.7	91.9 ± 2.9
7-Ethoxycoumarin	101.1 ± 0.4	99.2 ± 7.5
Mephenytoin	92.3 ± 0.9	75.8 ± 2.8
Nifedipine	34.8 ± 3.0	10.4 ± 3.7
Paraxanthine	98.7 ± 3.6	90.7 ± 0.4
Phenacetin	96.0 ± 3.4	87.9 ± 1.2
Phenylbutazone	37.5 ± 7.5	8.6 ± 3.1
Propranolol	98.3 ± 1.0	86.4 ± 0.9
Sulphaphenazole*	41.0 ± 1.3	7.4 ± 3.1
Sulphinpyrazone	71.7 ± 9.8	31.5 ± 4.2
Theobromine	99.1 ± 3.9	96.9 ± 1.7
Theophylline	102.5 ± 8.0	101.1 ± 3.9
Verapamil	61.0 ± 3.3	32.0 ± 2.1

Values are mean ± SD from 3 livers (F5, F8, F9).

* The concentration of tolbutamide was 100 μM. The "low" and "high" inhibitor concentrations were 50 μM and 500 μM, respectively, except for sulphaphenazole where the "low" and "high" inhibitor concentrations were 0.5 μM and 50 μM, respectively. Concentrations of sulphaphenazole above 100 μM abolished hydroxytolbutamide formation.

for hydroxy-TB formation in microsomes from livers F5–F9 were 120 ± 41 μM and 0.273 ± 0.066 nmol.min^{-1}.mg^{-1}, respectively. There was 2.8-fold range in apparent K_m and a 1.8-fold range in V_{max}.

The effects of two added concentrations of a range of compounds on hydroxy-TB formation in microsomes from livers F5, F8 and F9 are summarised in Table 2. The highest concentration of the compounds used in the inhibitor screening studies was generally five times the TB concentration (500 μM vs 100 μM); compounds causing less than a 15% reduction in hydroxy-TB formation at the highest concentration were designated non-inhibitors of TB metabolism. (Under the experimental conditions followed in the inhibitor screening studies, compounds with apparent K_i values <1.25 mM would produce >15% inhibition of hydroxy-TB formation [19]). Of the compounds screened aniline, cimetidine, dextropropoxyphene, mephenytoin, nifedipine, phenylbutazone, sulphaphenazole, sulphinpyrazone and verapamil caused >15% inhibition of hydroxy-TB formation. Aminopyrine, benzo(*a*)pyrene, caffeine, debrisoquine, erythromycin, 7-ethoxycoumarin, paraxanthine, phenacetin, propranolol, theobromine and theophylline were non-inhibitors of hydroxy-TB formation. Full kinetic studies with the compounds shown to inhibit hydroxy-TB formation by >15% were performed using microsomes from liver F8 to determine apparent K_i values. Dixon plots (Fig. 4) and replots of slopes (from Dixon plots) versus reciprocal substrate concentration indicated that the inhibition of hydroxy-TB formation by aniline, cimetidine, dextropropoxyphene, mephenytoin, nifedipine, phenylbutazone, sulphaphenazole and verapamil was competitive in nature; apparent K_i values for these compounds were 182 μM, 140 μM, 225 μM, 375 μM, 15 μM, 11 μM, 0.12 μM and 118 μM, respectively. The inhibition of hydroxy-TB formation by sulphinpyrazone followed atypical kinetics (Fig. 4).

DISCUSSION

Data presented here provide direct biochemical evidence for the involvement of cytochrome P-450 in the conversion of TB to hydroxy-TB in man. For example, anti-human liver NADPH-cytochrome P-450 reductase IgG inhibited hydroxy-TB formation and hydroxy-TB was not formed when the NADPH generating system was omitted from microsomal incubations. It is interesting to note that the maximal extent of inhibition of TB hydroxylation by anti-NADPH-cytochrome P-450 reductase IgG in microsomes from the five livers studied ranged from 48% to 68%, whereas the same antibody almost completely abolished theophylline [20] and 2-acetylaminofluorene [14] metabolism in human liver microsomes. Guengerich *et al.* have reported [21] that an antibody to the rat NADPH–cytochrome P-450 reductase differentially inhibited benzo(*a*)-pyrene hydroxylation and *d*-benzphetamine *N*-demethylation in microsomes from certain human

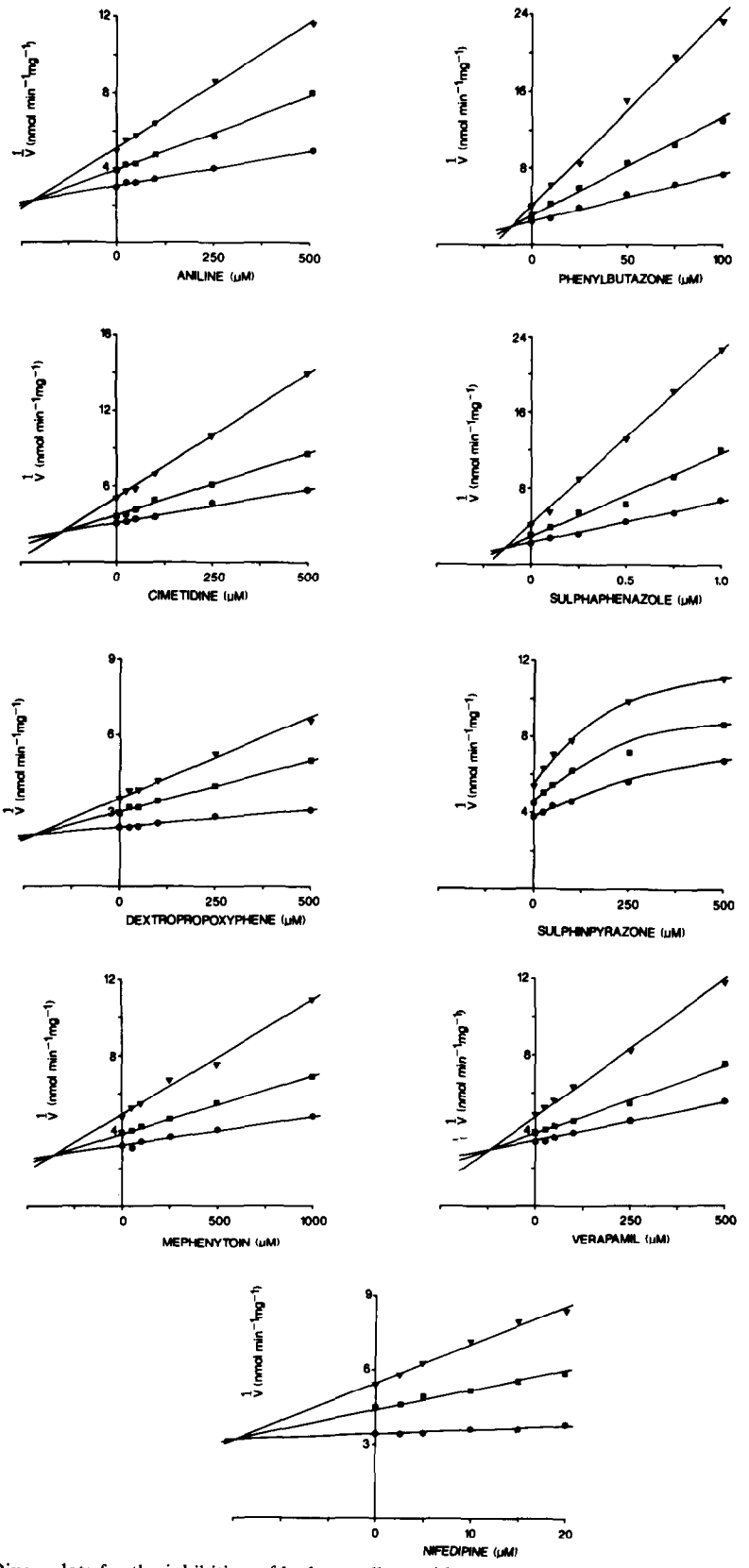

Fig. 4. Dixon plots for the inhibition of hydroxytolbutamide formation in microsomes from liver F8 by aniline, cimetidine, dextropropoxyphene, mephenytoin, nifedipine, phenylbutazone, sulphaphenazole, sulphinpyrazone and verapamil. Concentrations of tolbutamide were 50 μM (▼), 100 μM (■) and 200 μM (●).

livers. An explanation put forward for this observation was that different forms of cytochrome P-450 may bind to the reductase at different sites, which are not blocked by the antibody in the same manner. If this is the case, then results obtained in the present study are consistent with the hypothesis that different forms of cytochrome P-450 are involved in TB and theophylline metabolism (see below) and the data also suggest that distinct isozymes of cytochrome P-450 may be involved in TB and 2-acetylaminofluorene metabolism.

In all five livers studied TB hydroxylation followed Michaelis–Menten kinetics. The involvement of a single enzyme in TB metabolism is consistent with the apparent monogenic control of TB metabolism in man [4, 5], although it is acknowledged that the existence of two or more forms of cytochrome P-450 with similar affinities for TB would not be distinguished in the kinetic studies. There were 2.8-fold, 1.8-fold and 5.2-fold ranges in the respective apparent K_m, V_{max} and intrinsic clearance (V_{max}/K_m) values for hydroxy-TB formation in the five livers (Table 1). Michaelis–Menten parameters for the metabolism of [ureiyl-^{14}C]TB in microsomes from human liver samples obtained at post-mortem have previously been published in abstract form [22]. The K_m (60–200 μM) and V_{max} (0.15–0.29 nmol.min^{-1}.mg^{-1}) values obtained in the *post-mortem* livers are similar to the values reported here for renal transplant donor livers.

In addition to TB, genetic polymorphism has been shown for a number of other drugs in man, including debrisoquine [23], mephenytoin [24] and nifedipine [25]. Cytochrome P-450 isozymes metabolising each of these substrates have recently been isolated from human liver samples [26–28]. Separate cytochromes P-450 metabolising phenacetin [29] and erythromycin [30] have also been purified from human liver. Thus, debrisoquine, mephenytoin, nifedipine, phenacetin and erythromycin were screened for inhibitory effects on hydroxy-TB formation. Debrisoquine, phenacetin and erythromycin did not inhibit TB metabolism to any significant extent, suggesting that hydroxy-TB formation is mediated by a form of cytochrome P-450 distinct from the forms involved in debrisoquine, phenacetin and erythromycin metabolism. In interpreting these results it is acknowledged that compounds which have very low affinities for the TB hydroxylating enzyme may not have been identified in the inhibitor screening studies. As indicated in Results, however, compounds with apparent K_i values as high as 1.0–1.25 mM would still have produced detectable inhibition and so all but the weakest of inhibitors of TB hydroxylation would have been characterised in the present study.

Mephenytoin was a relatively weak inhibitor of TB hydroxylation, with an apparent K_i value of 375 μM. It is now accepted that mephenytoin polymorphism arises from diminished activity of the cytochrome P-450 responsible for the stereoselective 4-hydroxylation of *S*-mephenytoin [31]. Racemic mephenytoin was used in the present study and it is not clear whether inhibition of TB hydroxylation was due to the *S*- or *R*-enantiomer of mephenytoin, or both. Knodell *et al.* [32] have recently reported that TB is a substrate for the purified human liver cytochrome P-450 isozyme responsible for *S*-mephenytoin 4-hydroxylation but phenotypically poor metabolisers of mephenytoin were found not to have impaired hydroxylation of TB *in vivo*. This suggests that the *S*-mephenytoin 4-hydroxylase isozyme is quantitatively unimportant in TB hydroxylation. In contrast to mephenytoin, nifedipine was a relatively potent inhibitor of hydroxy-TB formation, with a K_i value of 11 μM. However, this observation should not be over-interpreted. While the lack of inhibition of one substrate by another is reasonable evidence that different enzyme forms are involved in the metabolism of the two compounds, reciprocal inhibition does not necessarily indicate metabolism by the same isozyme. Indeed, nifedipine also inhibits the *N*-demethylations of theophylline in human liver microsomes [20] and it is apparent that different forms of cytochrome P-450 are involved in TB and theophylline metabolism (see below).

As indicated in the Introduction, TB and theophylline metabolism *in vivo* are differentially affected by cigarette smoking [4, 7] and by pretreatment with propranolol [8, 9], sulphaphenazole [10, 11] or sulphinpyrazone [12, 13]. These observations have led to the suggestion [8, 9, 13] that the forms of cytochrome P-450 involved in theophylline metabolism are distinct to that involved in TB hydroxylation. In the present study the methylxanthines theophylline (1,3-dimethylxanthine), theobromine (3,7-dimethylxanthine), paraxanthine (1,7-dimethylxanthine) and caffeine (1,3,7-trimethylxanthine) were all without effect on TB metabolism *in vitro*. Available evidence [33] suggests that a similarly regulated family of cytochromes P-450 mediate methylxanthine biotransformation in man and data presented here confirm that these methylxanthine-metabolising forms are different from the cytochrome P-450 involved in TB metabolism.

A number of drugs have previously been investigated for inhibitory effects on TB clearance in man *in vivo*; these include cimetidine, dextropropoxyphene, phenylbutazone, propranolol, sulphaphenazole and sulphinpyrazone. The inhibitory effects of each of these compounds on *in vitro* hydroxy-TB formation determined in the present study are in general agreement with the *in vivo* interaction studies. Sulphaphenazole and phenylbutazone are potent inhibitors of TB metabolism *in vivo*; sulphaphenazole may prolong TB half-life by as much as 800% [2, 11] and phenylbutazone prolongs TB half-life 3- to 4-fold [11]. Both sulphaphenazole and phenylbutazone were potent competitive inhibitors of TB metabolism *in vitro*, with K_i values of 0.12 μM and 15 μM, respectively. Sulphaphenazole, even at very high concentrations, does not inhibit theophylline metabolism [20] and may therefore be a selective probe for the cytochrome P-450 isozyme involved in TB hydroxylation. Sulphinpyrazone treatment has been shown [12] to reduce TB clearance by 40% and this drug also inhibited hydroxy-TB formation *in vitro*; the inhibition kinetics were atypical (Fig. 4) and may indicate an allosteric mechanism for the inhibition of TB metabolism by sulphinpyrazone. Although dextropropoxyphene treatment has no effect on TB clearance [34] and cimetidine is only a

weak inhibitor of TB metabolism *in vivo* [35], both compounds inhibited hydroxy-TB formation *in vitro*. However, the apparent K_i values for these compounds were relatively high (dextropropoxyphene, 225 μM; cimetidine, 140 μM) and far exceed plasma concentrations achieved during therapeutic dosing. Propranolol treatment has no effect on TB clearance in man [8] and this compound was also shown in the present study to be without effect on hydroxy-TB formation *in vitro*. The general agreement between the *in vivo* and *in vitro* data indicate that microsomal inhibition studies may be a useful method of screening potential drug interactions. In this regard, the effect of the calcium channel antagonist verapamil on *in vitro* TB metabolism was determined since there is some evidence that verapamil may be an inhibitor of oxidative drug metabolism in man [20, 36, 37]. Verapamil was shown to be a competitive inhibitor of *in vitro* hydroxy-TB formation but the apparent K_i value (118 μM) is at least two orders of magnitude greater than verapamil plasma concentrations achieved during clinical use. A clinically significant interaction between TB and verapamil is therefore unlikely.

In addition to screening inhibition of *in vitro* TB metabolism by clinically used drugs, the present study investigated the effect of aminopyrine, aniline, benzo(*a*)pyrene and 7-ethoxycoumarin on hydroxy-TB formation in human liver microsomes. These compounds have been widely used as probes for cytochrome P-450 activity in laboratory animals. Of these compounds, only aniline inhibited TB metabolism (apparent K_i 182 μM). Again, interpretation of this data is difficult since the specificity of purified human cytochromes P-450 towards aminopyrine, aniline, benzo(*a*)pyrene and 7-ethoxycoumarin is, at present, unknown. Aminopyrine, aniline, benzo(*a*)pyrene and 7-ethoxycoumarin appear to be substrates for multiple forms of cytochrome P-450 in rat [38, 39] and rabbit [40] liver, although aniline is a major substrate for the ethanol inducible form j from rat liver [39] and form 3a from rabbit liver [40]. It is also known that chronic alcohol consumption induces TB metabolism in man [41] but any relationship between the ethanol inducible forms of cytochrome P-450 in animals and human liver TB-hydroxylase must remain speculative.

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