MOLECULAR PHARMACOLOGY, 23:474–481

Substrate Specificity of the Form of Cytochrome P-450 Catalyzing the 4-Hydroxylation of Debrisoquine in Man

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Received August 16, 1982; Accepted October 28, 1982

SUMMARY

In the present study we have investigated the substrate specificity of the form of cytochrome P-450 catalyzing the 4-hydroxylation of debrisoquine in man by analyzing the kinetics of inhibition of this activity by potential alternative substrates for the enzyme. All three compounds for which there is good *in vivo* evidence for an association between their metabolism and the debrisoquine oxidation polymorphism (viz., sparteine, guanoxan and phenformin) were potent competitive inhibitors of the reaction. The K_i for sparteine was 85 μM, for guanoxan it was 30 μM, and for phenformin it was 205 μM. Two compounds, acetanilide and antipyrine, for which the *in vivo* evidence was against an association between their metabolism and that of debrisoquine, were weak, noncompetitive inhibitors of debrisoquine 4-hydroxylase activity. The K_i values were 1.23 mM and 19.3 mM, respectively. Two additional compounds, tolbutamide and amylobarbitone, for which the *in vivo* evidence was also against an association between their metabolism and the debrisoquine oxidation polymorphism, did not appreciably inhibit the reaction. In fact, amylobarbitone caused a slight stimulation of activity. It is concluded that debrisoquine 4-hydroxylase is a specific form of cytochrome P-450 with a well-defined substrate specificity. Furthermore, it should be possible to identify compounds that might be subject to an oxidation polymorphism prior to the exposure of any subjects to the compound.

INTRODUCTION

Five years ago it was independently reported that debrisoquine (1) and sparteine (2) are oxidized polymorphically in man. Approximately 10% of the Caucasian population are poor metabolizers of either drug (3, 4). The similarity of the frequency of phenotypes for the two drugs and the rarity of oxidation polymorphisms in man soon led to speculation that the two defects were manifestations of the same polymorphism (5). Subsequent studies have indicated that either this is indeed so or the two polymorphisms are very closely linked (6). These *in vivo* studies still left open the question of the nature of the defect, whether a common enzyme is affected or whether some regulatory gene is altered.

Since the first reports in 1977, an ever-increasing number of drugs have been implicated in the polymorphism. In many instances this has been inferred from studies in small groups of subjects previously phenotyped for their debrisoquine oxidation status (see ref. 7).

The defect in debrisoquine oxidation has now been shown to be due to a deficiency or absence of a form of hepatic cytochrome P-450 (8). Thus, the decreased metabolism of other substrates is due either to abnormalities of different forms of cytochrome P-450, controlled by a single regulator gene or that are under very closely linked regulation, or to an abnormality of a single form of the enzyme. Such questions cannot be answered from *in vivo* studies. We have therefore investigated the nature of inhibition of debrisoquine 4-hydroxylase activity of human liver *in vitro* by drugs reportedly associated with, and by drugs reportedly not associated with, the debrisoquine oxidation polymorphism from *in vivo* studies.

EXPERIMENTAL PROCEDURES

Materials. Debrisoquine hemisulfate and 4-hydroxydebrisoquine hemisulfate were generously provided by Roche Products Ltd. (Welwyn Garden City, England). [$^2H_9$]4-Hydroxydebrisoquine was prepared biologically as described previously (9). Dr. D. D. Breimer (Leiden, Netherlands) very kindly supplied us with 3-hydroxymethylantipyrine. Phenformin was a gift from Sterling Winthrop (Surbiton-upon-Thames, England), and SKF 525-A[3] was a gift from Smith Kline & French (Welwyn

This work was supported largely by a grant from the Medical Research Council of the United Kingdom.

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[3] The abbreviations used are: SKF 525-A, 2-diethylaminoethyl-2,2-diphenylvalerate; HPLC, high-pressure liquid chromatography; PM, poor metabolizer phenotype, and EM, extensive metabolizer phenotype for the 4-hydroxylation of debrisoquine as defined in ref. 1.

0026-895X/83/020474-08$02.00/0

Garden City, England). Guanoxan sulfate was kindly provided by Pfizer Ltd. (Sandwich, England). Amylobarbitone sodium was from Eli Lilly and Company Ltd. (Basingstoke, England). (−)-Sparteine sulfate was purchased from Phase Separations Ltd. (Queensferry, Wales). Sigma Chemical Company (Poole, England) supplied antipyrine, tolbutamide, NADPH (tetrasodium salt, Type I), and bovine serum albumin (Fraction V). Norphenazone was purchased from British Drug Houses Ltd. (Poole, England). [3-^{14}C]Antipyrine was synthesized as previously described (10). 4-Hydroxyantipyrine was obtained from Aldrich (Gillingham, England). Hexafluoroacetylacetone was purchased from Fluorochem Ltd. (Glossop, England). All other chemicals were of analytical grade except acetonitrile, which was of HPLC grade (Rathburn Chemicals, Walkerburn, Scotland). Toluene was redistilled before use in the 4-hydroxydebrisoquine assay.

Human tissue samples. Microsomal fractions of human liver were obtained, as previously described (11), either from wedge biopsy samples taken at laparotomy for diagnostic purposes or from samples of liver from renal transplant donors maintained on life-support systems until such time that the kidneys could be removed. The use of such tissue in these studies had local Research Ethics Committee permission and, where appropriate, Coroner's permission. Samples were stored at −80° until required, during which time there was no loss of activity. Samples from renal transplant donors had activity very similar to that of wedge biopsy samples with normal histology (9), and therefore no further distinction has been made between the two groups of samples in this study.

Assay of debrisoquine 4-hydroxylase activity. Debrisoquine 4-hydroxylase activity was assayed by a modification of our previously published method (9). The standard incubation volume comprised 6 mM $MgCl_2$, 50 mM Tris-HCl buffer (pH 7.4), 1.2 mM NADPH, and approximately 0.75 mg of microsomal protein, in 50 μl of 0.25 M potassium phosphate buffer (pH 7.25) containing 30% (v/v) glycerol in a final volume of 1 ml. The reaction was started by the addition of an aqueous solution of debrisoquine hemisulfate to give a final concentration of 0–2.5 mM, representing a 1:20 dilution of the stock solutions of substrate. These were prepared fresh for each experiment. In fixed concentration studies, the final concentration of debrisoquine was 0.2 mM. When used, the inhibitors were added as an aqueous solution at twice the desired final concentration in 0.5 ml. The final incubation volume was maintained at 1 ml. In inhibitor studies, samples were preincubated for 2 min at 37° in the presence of the inhibitor before the addition of the substrate, debrisoquine. Incubations were performed at 37° in air in a shaking water bath for 12 min, the time of incubation and protein concentration having been shown previously to be in the linear range (9). The reaction was stopped by the addition of 200 μl of ice-cold 1 M sodium hydroxide, and the samples were transferred to ice. Blanks were incubated without substrate, which was added after the addition of sodium hydroxide. The internal standard, [$^2H_9$]4-hydroxydebrisoquine, was added to each sample and to a series of standards (1-ml volume) of 4-hydroxydebrisoquine with a range of concentrations from 0 to 500 ng/ml.

Unmetabolized debrisoquine was removed by extraction at pH 13.5 as previously described (9), and the amidine group of the metabolite and its internal standard was derivatized at pH 8.5 with hexafluoroacetylacetone.

Gas chromatography-mass spectrometry was performed by a modification of our earlier method (9). The 4-hydroxy group was not derivatized, and the mass spectrometer was operated in the negative ion-chemical ionisation mode with methane as the reagent gas. The mass spectrometer was tuned to monitor ions at m/e 363 and 371, which were the molecular ions (M^-) from the pyrimidino derivatives of 4-hydroxydebrisoquine and its deuterated internal standard, respectively.

Assay of antipyrine 3-methylhydroxylase activity. 3-Hydroxymethylantipyrine production was assayed by a modification of our published method (10). [3-^{14}C]Antipyrine was synthesized to a specific activity of 5 μCi/μmole. Radioactive substrate (0.825 μCi) was added to each incubation tube, and the benzene, in which the antipyrine had been dissolved, was evaporated at 40° under a stream of nitrogen. The final incubation volume was 250 μl, which contained, in addition to [3-^{14}C]antipyrine, 50 mM Tris-HCl buffer (pH 7.4), 6 mM $MgCl_2$, 1–2 mg of microsomal protein, and sufficient unlabeled antipyrine to give the desired final concentration. In fixed concentration studies this was 10 mM. The reaction was started by the addition of NADPH to give a final concentration of 1.2 mM. Samples were incubated at 37° in air in a shaking water bath for 12.5 min, and the reaction was terminated by the addition of 250 μl of ice-cold 1 M sodium hydroxide. Blanks were incubated without microsomal protein, which was added after the addition of alkali. Both the protein concentration and the time of incubation were in the linear range for the assay. As recovery standards, 25 μg of each of 3-hydroxymethylantipyrine, 4-hydroxyantipyrine, and norphenazone in 25 μl of methanol were added to the samples. A few grains (5–10 mg) of sodium metabisulfite were added to prevent any possible base-catalyzed degradation.

Unmetabolized antipyrine was removed by extraction into toluene (three times, 5 ml each time). The pH of the aqueous phase was adjusted to 7.0 by the addition of 1 ml of 1 M potassium phosphate buffer (pH 7.0), and the metabolites were extracted into ethyl acetate (three times, 5 ml each time). The organic extracts were pooled for each sample and taken to dryness in tapered ground-glass necked tubes at 40° under a stream of nitrogen.

Unextracted standards, used to construct a calibration curve for estimating recovery of 3-hydroxymethylantipyrine, were prepared by adding 0–50 μg of the metabolite in methanol to tapered tubes and blowing to dryness. Samples and standards were reconstituted in 260 μl of HPLC mobile phase.

Chromatography was performed on a Waters Associates (Milford, Mass.) μBondapak reverse-phase ODS column (25 cm × 4.1 mm) coupled to an Altex dual pump HPLC with a microprocessor solvent programmer and flow cell (Altex Scientific Inc., Berkeley, Calif.) in a Hitachi spectrophotometer (Hitachi Ltd., Tokyo, Japan) set to monitor absorbance at 254 nm. Absorption changes

were recorded on a Tekman dual pen chart recorder (Tekman Electronics Ltd., Bicester, Oxon., England). The mobile phase was 10% acetonitrile in water at a flow rate of 2 ml/min. The injected sample was 250 μl. After elution of 3-hydroxymethylantipyrine, the column was washed by increasing the acetonitrile content of the mobile phase to 100% over 5 min, holding at 100% for 5 min, returning to 10% over 5 min, and holding at 10% for 5 min before injecting the next sample.

Metabolite fractions were identified by the relative mobility of the recovery standards, and the 3-hydroxymethylantipyrine fractions were collected into glass scintillation vials to which were added 10 ml of Instagel (Packard Instrument Corporation, Inc., Downers Grove, Ill.). Corresponding samples from the blank incubations were collected, and collections were made immediately before and after the 3-hydroxymethylantipyrine peak. Radioactivity was determined by β-scintillation spectrometry, and the results were corrected for quenching by the interactive data system. The results were also corrected for background counts interpolated from the pre- and post-peak collections and for recovery. Values for blank incubations, calculated similarly, were subtracted and the results expressed as nanomoles of 3-hydroxymethylantipyrine formed per milligram of protein per minute.

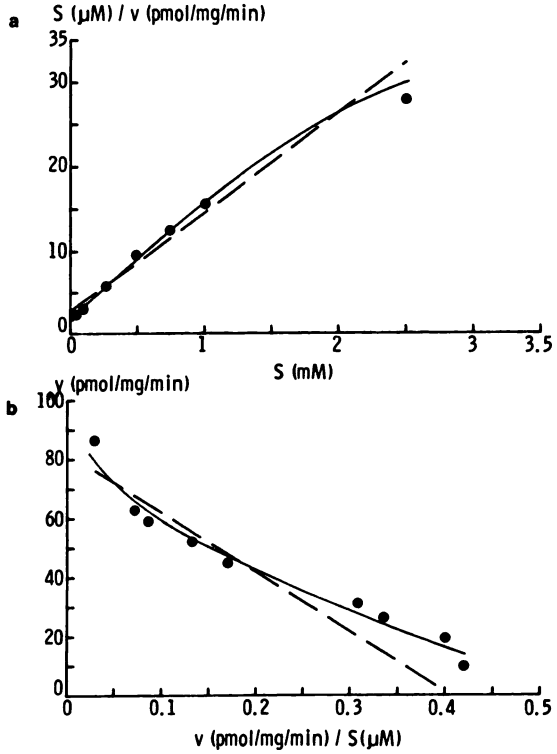

FIG. 1. *Kinetics of debrisoquine 4-hydroxylase activity of microsomal fractions of human liver*

The data from a single liver sample are plotted on a Hanes plot (a) and an Eadie-Hofstee plot (b). Although data from only one sample are shown they are typical of the results obtained (n = 6). The data points (●) show the observed values. The continuous line (——) is the line of best fit to a biphasic enzyme system calculated as described under Experimental Procedures (13). The broken line (– – –) is the line of best fit to a monophasic enzyme system exhibiting classic Michaelis-Menten kinetics. The line was calculated by a computerized nonlinear least-squares iterative procedure.

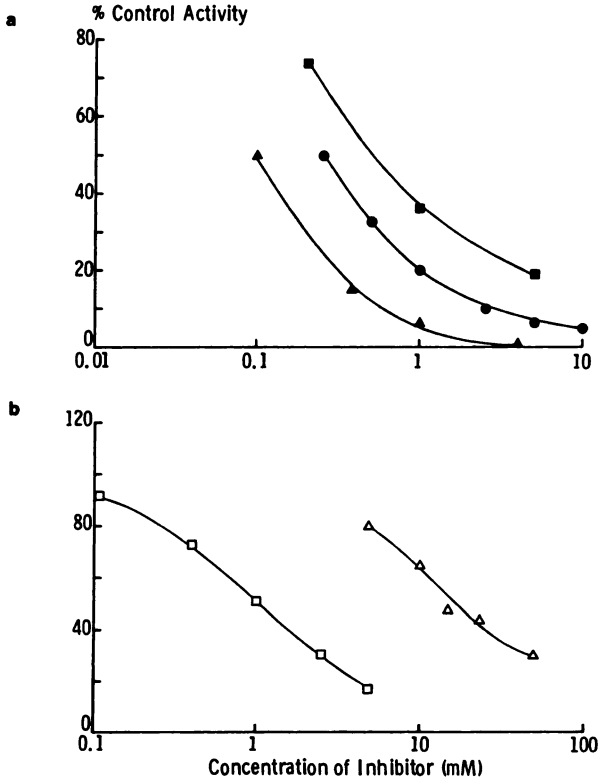

FIG. 2. *Inhibition of debrisoquine 4-hydroxylase activity*

The effects of sparteine (●), guanoxan (▲), and phenformin (■) are shown in a, and the effects of acetanilide (□) and antipyrine (△) are shown in b. The compounds were added, in a range of concentrations, to samples containing microsomal fractions of human liver which were then preincubated at 37° for 2 min. The reaction was started by the addition of substrate, debrisoquine, to give a final concentration of 0.2 mM. Values plotted are activities of debrisoquine 4-hydroxylase in the presence of inhibitor, expressed as a percentage of the corresponding control activity in the absence of inhibitor. Note that the scale of the x-axis changes between a and b.

Protein was assayed by a modification of the method of Lowry *et al.* (11) with crystalline bovine serum albumin, Fraction V, as standard.

Analysis of results. Michaelis-Menten parameters were initially estimated by graphical analysis (12). The values thus obtained were used as first estimates in a computerized nonlinear iterative procedure based on the method of Koeppe and Hamann (13). Inhibitory constants (K_i values) were obtained by substitution of the appropriate values for K_m and V_{max}, determined as above, into the appropriate equation (14).

RESULTS

In a previous paper (9) we reported that debrisoquine was 4-hydroxylated monophasically by microsomal fractions of human liver with substrate concentrations of up to 1 mM. We have since found that at higher substrate concentrations there is evidence of a second component of activity (Fig. 1) characterized by a high K_m and a relatively low V_{max}. Thus, it was necessary to analyze the kinetics of debrisoquine 4-hydroxylase utilizing the procedures described above for a biphasic enzyme system.

TABLE 1

IC_{50} values of inhibitors of debrisoquine 4-hydroxylase activity

Potential inhibitors were added to the samples as an aqueous solution and the samples were preincubated for 2 min at 37° prior to starting the reaction by the addition of the substrate, debrisoquine, to give a final concentration of 0.2 mM. IC_{50} values (concentration of inhibitor causing a 50% reduction in debrisoquine 4-hydroxylase activity) were determined from a semilog plot of inhibitor concentration against the percentage of debrisoquine 4-hydroxylase activity remaining.

Inhibitor	IC_{50}
	mM
Sparteine	0.25
Phenformin	0.55
Guanoxan	0.10
Acetanilide	1.1
Antipyrine	18.0
Tolbutamide	8.3[a]
Amylobarbitone	>10

[a] Extrapolated.

Effects of varying inhibitor concentration on debrisoquine 4-hydroxylase activity determined at a fixed concentration of substrate. A number of compounds were tested for possible inhibitory effects on debrisoquine 4-hydroxylase activity. Phenformin, guanoxan, and sparteine, the oxidation of all of which is reportedly deficient in poor metabolizers of debrisoquine (see ref. 7), were potent inhibitors of 4-hydroxylase activity (Fig. 2). Acetanilide (15) and antipyrine (8, 16), the metabolism of which is reportedly not affected in PM subjects, also inhibited debrisoquine 4-hydroxylase activity (Fig. 2), although these compounds were not as potent as the first group (Table 1). Tolbutamide (17) and amylobarbitone (16), again compounds reportedly not affected in PM subjects, did not inhibit 4-hydroxylase activity (Fig. 3) but rather amylobarbitone caused a modest increase in enzyme activity.

Kinetics of inhibition of debrisoquine 4-hydroxylase activity. Phenformin, guanoxan, and sparteine (Fig. 4) were competitive inhibitors of debrisoquine 4-hydroxylase activity. The K_i for phenformin was 205 μM, for guanoxan it was 30 μM, and for sparteine it was 85 μM (Table 2). Both antipyrine and acetanilide (Fig. 5) were noncompetitive inhibitors of 4-hydroxylase activity. Antipyrine had a K_i of 19.3 mM and for acetanilide it was 1.23 mM (Table 2).

Studies of 3-methylhydroxylation of antipyrine. As stated above, antipyrine was a weak, noncompetitive inhibitor of debrisoquine 4-hydroxylase activity. There have been suggestions that PM subjects have a somewhat reduced ability to generate 3-hydroxymethylantipyrine *in vivo* (18). The effects of debrisoquine on the *in vitro* production of this metabolite were therefore investigated. Debrisoquine, at concentrations of up to 2 mM, was without effect on this activity (Table 3). However, SKF 525-A was an effective inhibitor of 3-hydroxymethylantipyrine formation (IC_{50} = 0.35 mM; antipyrine concentration = 10 mM), and amylobarbitone was a competitive inhibitor (Fig. 6) with a K_i of approximately 0.7 mM.

DISCUSSION

In vivo studies have now established that, if the deficiencies in debrisoquine (1) and sparteine (2) oxidation are due to abnormalities at separate loci, then such loci are extremely closely linked (6). However, it is not possible to determine from such studies whether or not more than one form of cytochrome P-450 is involved. The defect could be of a regulatory gene controlling the synthesis of several different forms of cytochrome P-450. The present studies were undertaken to investigate the possibility that there is only one form of cytochrome P-

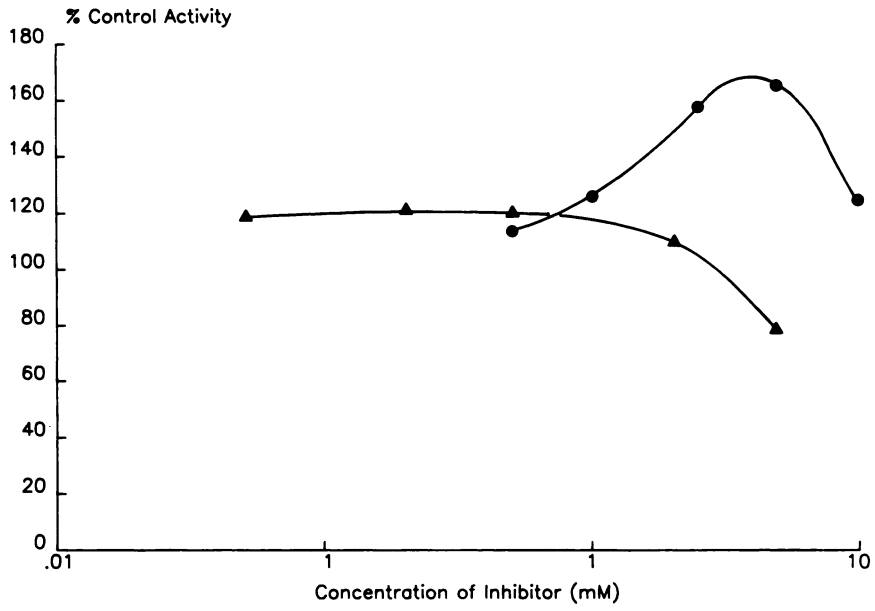

FIG. 3. *Effects of amylobarbitone and tolbutamide on debrisoquine 4-hydroxylase activity*

Amylobarbitone (●) and tolbutamide (▲) were added, in a range of concentrations, to samples containing microsomal fractions of human liver. Details of the procedures are provided in the legend to Fig. 2.

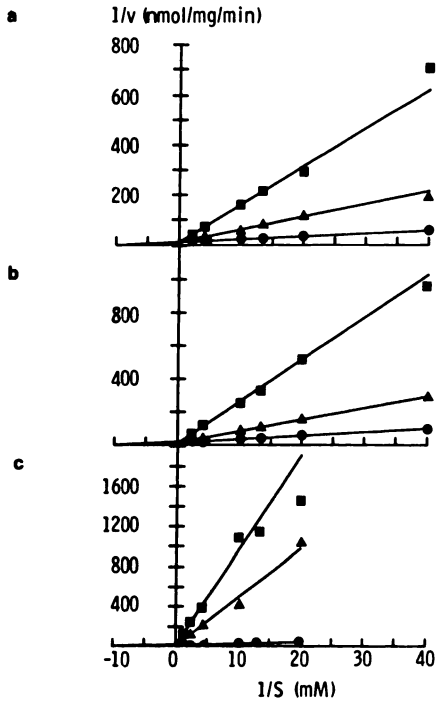

FIG. 4. *Kinetics of inhibition of debrisoquine 4-hydroxylase activity by phenformin, guanoxan, and sparteine*

Debrisoquine 4-hydroxylase activity of microsomal fractions of human liver was determined, over a range of substrate concentrations, in the presence of (a) 0 mM (●), 0.75 mM (▲), and 2.5 mM (■) phenformin; (b) 0 mM (●), 0.1 mM (▲), and 0.3 mM (■) guanoxan; and (c) 0 mM (●), 2.5 mM (▲), and 5 mM (■) sparteine. Samples were preincubated for 2 min at 37° prior to addition of the substrate, debrisoquine. The data were analyzed assuming a biphasic enzyme system (13). The *points* are the observed values corrected for the contribution of the low-affinity component of activity, estimated from the values of K_m and V_{max} determined by computer analysis of the data. The *lines* are those predicted from the computer estimates of K_m and V_{max} for the high-affinity component of activity (see ref. 12 and Experimental Procedures). In the interests of clarity it has not been possible to plot all of the data points at higher substrate concentrations.

450 involved in the oxidation of those substrates, the metabolism of which is reportedly affected in the debrisoquine polymorphism. If this is so, then the substrates should be competitive inhibitors of the metabolism of each other. Furthermore, the K_i for inhibition should be the same as the K_m for metabolism. The possibility that the *in vitro* approach adopted could be used to establish whether or not the metabolism of a given substrate was likely to be affected in PM subjects, without the necessity of large population studies, was also investigated.

As previously reported for phenacetin and 7-ethoxycoumarin (12) we have now found that debrisoquine appears to be biphasically hydroxylated by microsomal fractions of human liver. In this instance the capacities (V_{max}) of the two components are not very different, although there is a 10-fold difference in K_m. The low-affinity component of activity has not been fully characterized in the present study. However, its existence may well explain why some PM subjects produce relatively easily detected amounts of 4-hydroxydebrisoquine whereas others do not (19). The range of activities of the low-affinity component should be normally distributed. At 0.2 mM debrisoquine this component of activity would contribute 0.5–5 pmoles of product per milligram per minute (up to 15%) to total activity *in vitro*.

Guanoxan, phenformin, and sparteine were all potent competitive inhibitors of debrisoquine 4-hydroxylase activity. *In vivo* the oxidation of sparteine is polymorphic (2) and the defect co-segregates with the deficiency in debrisoquine oxidation (6). The evidence that the oxidation of guanoxan is affected in the polymorphism is from a study by Sloan *et al.* (20), who found that the urinary ratio of guanoxan to its hydroxylated metabolites in four subjects previously phenotyped as PM with debrisoquine was on average 140 times that in four subjects previously phenotyped as EM. Shah *et al.* (21) found that whereas four subjects phenotyped as EM excreted 13–18% of a dose of phenformin as *p*-hydroxyphenformin, three subjects phenotyped as PM produced no detectable *p*-hydroxyphenformin. Idle and Islam (22) have shown that the *p*-hydroxylation of phenformin is polymorphic in a population of Saudi women, but crossover studies with debrisoquine have yet to be reported. The *in vivo* data together with the *in vitro* data reported here provide good evidence that all three substrates are metabolized, at least for the most part, by a single form of cytochrome P-450. Recently, it has been shown that debrisoquine is a competitive inhibitor of sparteine oxidation by microsomal fractions of human liver, with a K_i of 60 μM (23). The K_m for debrisoquine oxidation determined in the present study was 120 μM. In addition, the K_i for sparteine inhibition of debrisoquine 4-hydroxylation (85 μM) is very similar to the value found by Otton *et al.* (23) for the K_m for the oxidation of sparteine (75 μM). This similarity in

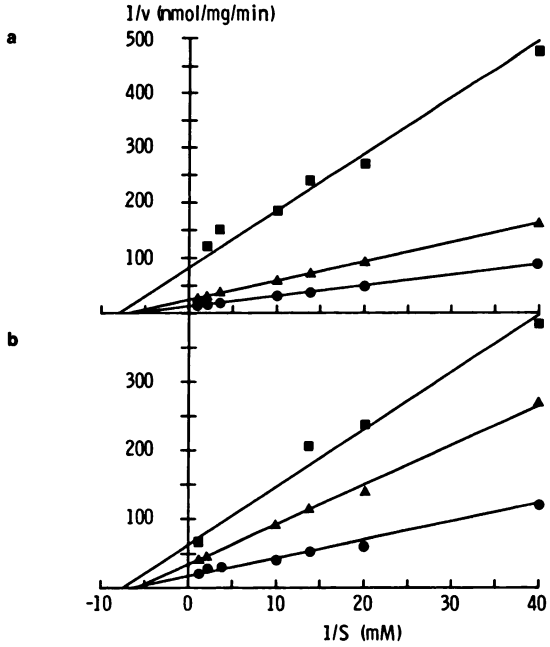

FIG. 5. *Kinetics of inhibition of debrisoquine 4-hydroxylase activity by antipyrine and acetanilide*

Debrisoquine 4-hydroxylase activity of microsomal fractions of human liver was determined, over a range of substrate concentrations, in the presence of (a) 0 mM (●), 20 mM (▲), and 80 mM (■) antipyrine, and (b) 0 mM (●), 1 mM (▲), and 3.5 mM (■) acetanilide. Details of the procedures used are described in the legend to Fig. 4.

TABLE 2

K_i values for inhibitors of debrisoquine 4-hydroxylase activity of human liver

Debrisoquine 4-hydroxylase activity was determined over a range of substrate concentrations from 0 to 5 mM in the presence and absence of inhibitor as described under Experimental Procedures. The data were analyzed assuming a biphasic enzyme system as described in the text. Values in parentheses are the radex errors, defined as the maximal (absolute) amount by which the regression coefficient can be deviated from its "best" value without raising the sum of squares by >100% of its best (smallest) value (13).

Inhibitor	Concentration	Apparent V_{max}	Apparent K_m	K_i	Average K_i
	mM	pmoles/mg/min	μM	μM	μM
Phenformin	0	107	125	—	
		(4.48)	(10.7)		
	0.75	105	550	220	
		(6.04)	(44.3)		205
	2.5	113	1740	190	
		(5.73)	(107)		
Guanoxan	0	61.3	116	—	
		(2.92)	(11.0)		
	0.1	62.8	434	36	
		(3.14)	(31.7)		30
	0.3	61.4	1550	24	
		(3.22)	(98.9)		
Sparteine	0	71.8	110	—	
		(3.02)	(12.0)		
	2.5	67.8	3270	87	
		(5.80)	(354)		85
	5.0	71.0	6730	83	
		(11.2)	(1220)		
Antipyrine	0	68.2	129	—	
		(4.32)	(15.5)		
	20	36.1	120	22,500	
		(1.99)	(12.5)		19,300
	80	11.4	115	16,100	
		(1.41)	(23.9)		
Acetanilide	0	57.4	151	—	
		(6.56)	(29.6)		
	1.0	29.8	171	1,080	
		(4.00)	(40.1)		1,230
	3.5	16.2	137	1,380	
		(5.35)	(66.7)		

K_i and K_m values for the two substrates provides further strong support for the above conclusion.

Acetanilide elimination was virtually identical in four EM and four PM subjects (15). In the present study acetanilide was found to be a noncompetitive inhibitor (K_i = 1.23 mM) of debrisoquine 4-hydroxylase activity. Acetanilide produces a Type II difference spectrum with microsomal fractions of rat liver (data not shown). Type II difference spectra are characteristic of compounds containing a basic nitrogen atom and are the result of complex formation at the sixth ligand of the heme iron of cytochrome P-450 (24). This produces a high- to low-spin transition of the heme iron with consequent reduction in metabolic activity (25). Such a mechanism may well explain why acetanilide can inhibit debrisoquine 4-hydroxylase activity. It seems unlikely that acetanilide is metabolized by the same form of cytochrome P-450 that 4-hydroxylates debrisoquine.

Amylobarbitone did not inhibit debrisoquine oxidation, even at concentrations several times its K_m of 0.5–2.0 mM (26). In fact, some enhancement of activity was observed. Inaba *et al.* (16) reported that one subject phenotyped PM for debrisoquine and two subjects phenotyped as poor metabolizers of sparteine all had "normal" rates of oxidation of amylobarbitone. Thus, the results of the present study confirm the inference from earlier *in vivo* studies (16) that debrisoquine and amylobarbitone do not share a cytochrome P-450 isozyme.

TABLE 3

Effects of debrisoquine on 3-methylhydroxylation of antipyrine by microsomal fractions of human liver

Antipyrine 3-methylhydroxylase activity was assayed as described under Experimental Procedures with a substrate concentration of 10 mM. Values shown are the means of at least two determinations, which varied by less than 10% from each other.

Debrisoquine concentration	Antipyrine 3-methylhydroxylase activity	% Control activity remaining
mM	pmoles/mg/min	
0	0.87	100
0.25	0.85	98
0.5	0.95	110
2.0	0.92	106

Antipyrine was a weak, noncompetitive inhibitor of debrisoquine 4-hydroxylase activity, with a K_i of 19.3 mM. The K_m values for the formation of the oxidative metabolites of antipyrine *in vitro* are 7.3 ± 1.1 mM for 4-hydroxyantipyrine, 5.9 ± 0.6 mM for norphenazone, and 6.2 ± 1.5 mM for 3-hydroxymethylantipyrine (ref. 10 and this work). Thus the K_i for inhibition of debrisoquine 4-hydroxylase is approximately 3-fold the K_m for any pathway of metabolism.

Antipyrine produces a weak Type II difference spectrum with microsomal fractions of rat liver (data not shown), so that the mechanism of inhibition may be similar to that suggested for acetanilide. Otton *et al.* (23) found that concentrations of up to 4 mM antipyrine had no effect on sparteine oxidation, perhaps not surprising in view of the high K_i for debrisoquine oxidation.

Several groups have shown, on small numbers of subjects phenotyped as EM or PM with respect to debrisoquine, that there is no difference between phenotypes either in the over-all clearance of antipyrine or in the rate of formation of any of its oxidative metabolites (16, 27, 28). *In vitro* studies on a liver sample from a subject phenotyped as PM showed normal rates of formation of the three metabolites of antipyrine (8). In one study a small, but significant, reduction in the rate of formation of 3-hydroxymethylantipyrine *in vivo* was reported in PM subjects. Although the *in vitro* production of 3-hydroxymethylantipyrine was readily inhibited by SKF 525-A, debrisoquine at concentrations of up to 2 mM had no effect on this reaction. In addition, amylobarbitone, which does not inhibit debrisoquine 4-hydroxylase, was a competitive inhibitor of 3-hydroxymethylantipyrine formation, with a K_i of 0.5 mM. This compares well with a K_m for the 3-hydroxylation of amylobarbitone of 0.5–2.0 mM (26). Thus, it appears that the same form of cytochrome P-450 can catalyze the 3-hydroxylation of amylobarbitone and the formation of 3-hydroxymeth-

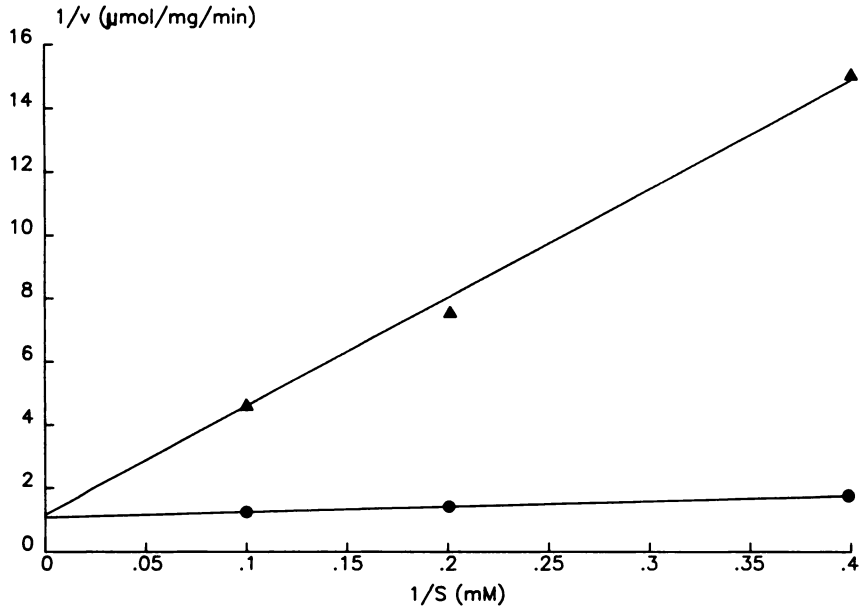

FIG. 6. *Kinetics of inhibition of 3-methylhydroxylation of antipyrine by amylobarbitone*
Antipyrine 3-methylhydroxylase activity of microsomal fractions of human liver was determined, over a range of substrate concentrations, in the presence of 0 mM (●) and 5 mM (▲) amylobarbitone. The conditions of the assay are described under Experimental Procedures.

ylantipyrine but that this is different from the form catalyzing the 4-hydroxylation of debrisoquine.

It has been suggested from *in vivo* studies that tolbutamide might be polymorphically hydroxylated in man, with 24% of the population classified as slow inactivators (29). However, in a limited study on groups of two EM and two PM subjects, Idle *et al.* (17) found no difference in either the half-life of tolbutamide or the rate constant for carboxytolbutamide formation between the groups. In the present study it was found that concentrations of tolbutamide up to 5 mM had virtually no effect on the 4-hydroxylation of debrisoquine *in vitro*. As the K_m for the hydroxylation of tolbutamide is 0.1–0.2 mM (30) it seems unlikely that the two compounds share a common form of cytochrome P-450. Thus, if the oxidation of tolbutamide is polymorphic this may well involve a pair of alleles different from those involved in the debrisoquine polymorphism.

The current investigation has demonstrated that substrates for which there is a known association with the debrisoquine defect are potent competitive inhibitors of debrisoquine 4-hydroxylase activity. Those substrates whose *in vivo* oxidation appears unaffected by the debrisoquine polymorphism were either without effect or were weak noncompetitive inhibitors of debrisoquine 4-hydroxylase activity.

Thus, the data obtained from the *in vitro* approach adopted are entirely consistent with the involvement of a single form of cytochrome P-450 in the oxidation of debrisoquine and a number of other substrates. Even if this form of cytochrome P-450 contributes only a small part to the total metabolism of a substrate, then that substrate would still have to fit the active site of the enzyme. Such a compound would therefore be expected to act as a competitive inhibitor of the 4-hydroxylase reaction when it was present at a high concentration relative to its K_m. In addition, this possibility of determining whether or not the debrisoquine 4-hydroxylase form of cytochrome P-450 contributes to the metabolism of any particular compound can be accomplished without the necessity of large-scale population studies. An early indication of the possible impairment of the oxidation of new compounds in PM subjects may thus be possible.

ACKNOWLEDGMENTS

We wish to thank the professor and staff of the Department of Surgery, Royal Postgraduate Medical School, for their kind cooperation in making human liver samples available to us for use in these studies. We are grateful to Roche Products Ltd. for supplying us with debrisoquine, 4-hydroxydebrisoquine, and the deuterated analogue of the parent compound. Several other drug companies supplied us with compounds essential to this study, and to them we are also grateful.

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