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ASSAY AND CHARACTERISATION OF DEBRISOQUINE 4-HYDROXYLASE ACTIVITY OF MICROSOMAL FRACTIONS OF HUMAN LIVER

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1 A method for the assay of debrisoquine 4-hydroxylase activity *in vitro* by microsomal fractions of human liver is described. The assay utilises gas chromatography-mass spectrometry with d_9-4-hydroxydebrisoquine as internal standard.

2 The limit of detection of 4-hydroxydebrisoquine was 2 ng ml^{-1} and the coefficient of variation was 4.4%.

3 Debrisoquine 4-hydroxylase activity was linear with protein to concentrations above 2.1 mg ml^{-1} and with incubation times of at least 15 min.

4 Debrisoquine 4-hydroxylase is a microsomal enzyme with a requirement for NADPH. Activity was inhibited by carbon monoxide. It is concluded that the activity is catalysed by cytochrome P-450.

5 In three samples of human liver the mean value for V_{max} of debrisoquine 4-hydroxylase activity was 69.9 ± 14.3 pmol mg^{-1} min^{-1} and for Km it was 130 ± 24 μM.

6 The only variable from smoking status, alcohol ingestion, sex of the patients, source of liver sample and presence of liver disease that had a significant effect on 4-hydroxylation of debrisoquine was the presence of liver disease. This was associated with a decrease in enzyme activity.

Introduction

Debrisoquine (3,4-dihydro-2(1H)-isoquinolinecarboxamidine) is a post-ganglionic sympathetic blocking agent that has been in use in the treatment of hypertension for about 15 years. Although its clinical use is now declining debrisoquine has become the focus of considerable attention over the last 4 years with the discovery that its oxidation in man exhibits a genetic polymorphism (Mahgoub *et al.*, 1977; Tucker *et al.*, 1977). The major route of debrisoquine metabolism is alicyclic hydroxylation at the carbon-4 position with lesser amounts of aromatic hydroxylation and traces of ring scission (Allen *et al.*, 1975, 1976; Angelo *et al.*, 1976). Initial reports (Mahgoub *et al.*, 1977; Tucker *et al.*, 1977) showed that the 4-hydroxylation of debrisoquine in man is under monogenic control and that the reduced ability to 4-hydroxylate debrisoquine is inherited as an autosomal recessive trait (Mahgoub *et al.*, 1977). Later studies have shown that reduced phenolic hydroxylation may co-segregate with the defect in 4-hydroxylase activity (Woolhouse *et al.*, 1979). In the Caucasian population 9% of subjects are so-called poor metabolisers (PM phenotype) with respect to 4-hydroxylation (Price Evans *et al.*, 1980). This is determined by measuring the ratio of debrisoquine to 4-hydroxydebrisoquine excreted in urine (metabolic ratio) over the 8 h following ingestion of an oral dose of debrisoquine (Mahgoub *et al.*, 1977). Subjects with a ratio of greater than 20 are designated PM phenotype (Mahgoub *et al.*, 1977). The oxidation of several other drugs appears to be under the control of the same pair of alleles as those regulating the alicyclic hydroxylation of debrisoquine. These include sparteine (Inaba *et al.*, 1980; Bertilsson *et al.*, 1980a), guanoxan (Sloan *et al.*, 1978), phenytoin (Sloan *et al.*, 1981), metiamide (Idle *et al.*, 1979b), 4-methoxyamphetamine (Kitchen *et al.*, 1979), phenformin (Shah *et al.*, 1980), nortriptyline (Bertilsson *et al.*, 1980b), encainide (J.A. Oates, personal communication) carbocisteine (Mucodyne) (Waring *et al.*, 1981) and phenacetin *O*-dealkylation (Sloan *et al.*, 1978). In some instances, such as sparteine (Eichelbaum *et al.*, 1979), and phenformin (Shah *et al.*, 1980), there is apparently an absolute inability of the PM phenotype to generate any of the affected metabolite whereas in other instances the PM phenotype can undoubtedly

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produce some of the affected metabolite e.g. phenacetin (Sloan *et al.*, 1978) and phenytoin (Sloan *et al.*, 1981). In the case of phenacetin the defect is manifest in a reduced ability to *O*-deethylate the drug whilst its 2-hydroxylation is unaffected (Ritchie *et al.*, 1980). Thus, the defect does not represent an alteration in all routes of oxidation. The oxidation of drugs such as amylobarbitone (Inaba *et al.*, 1980), antipyrine (Tucker *et al.*, 1977; Inaba *et al.*, 1980; Davies *et al.*, 1981) tolbutamide (Idle *et al.*, 1979c) and acetanilide (Wakile *et al.*, 1979) is totally unaffected by this locus.

The specificity of the hepatic mixed function oxidase system resides in the terminal electron acceptor, the haemoprotein cytochrome P-450 of which multiple forms are known to exist both in animals and in man (reviewed in Lu & West, 1980). It thus seemed quite possible that the defect observed in the 4-hydroxylation of debrisoquine represents an alteration in or absence of a particular form of cytochrome P-450. It was thus desirable to investigate the oxidation of debrisoquine *in vitro* by microsomal fractions of human liver. Preliminary results of such studies showed that within a group of patients phenotyped *in vivo* the only liver biopsy sample with no detectable debrisoquine 4-hydroxylase activity was from the only patient phenotyped PM (Davies *et al.*, 1981). Although our earlier work suggested that the activity was cytochrome P-450 mediated the data were not conclusive. We have now modified our assay for debrisoquine 4-hydroxylase activity to include a deuterated internal standard and have characterised the enzyme in microsomal fractions of human liver.

Methods

Materials

Debrisoquine sulphate, 4-hydroxydebrisoquine sulphate, decadeutero-debrisoquine (d_{10}-debrisoquine) and [^{14}C]-debrisoquine sulphate (3,4-dihydro-2-(1H)-isoquinoline [^{14}C]-carboxamidine sulphate) with a specific activity of 2.45 μCi mg^{-1} were generously provided by Roche Products Ltd, Welwyn Garden City, England. Aqueous solutions of these compounds were kept refrigerated at 4°C. Hexafluoroacetylacetone (HFAA) was purchased from Fluorochem Ltd, Glossop, Derbyshire and trifluoroacetic anhydride (TFAA) was from Sigma Chemical Co., Poole, Dorset. This company also supplied reduced β-nicotinamide adenine dinucleotide phosphate (tetrasodium salt, type 1) (NADPH) and bovine serum albumin fraction V. All other chemicals were of Analar grade. Toluene was redistilled before use and ethyl acetate was redistilled and stored over calcium hydride to exclude water. The glassware used in the derivatisation procedure was washed in concentrated nitric acid and rinsed extensively in deionised water.

Tissue samples

Human liver samples were obtained by wedge biopsy at laparotomy for diagnostic reasons. Tissue surplus to histological requirement was made available for these studies. Samples were also obtained from renal transplant donors who had met traumatic deaths and were maintained on life support systems until removal of their kidneys. Local Research Ethics Committee and, where appropriate, Coroner's permission were obtained to use such tissue in these studies. Microsomal fractions were isolated and stored as a suspension in 0.25 M potassium phosphate buffer pH 7.25, containing 30% (v/v) glycerol at −80°C as previously described (Boobis *et al.*, 1980). Biopsies used in characterising the enzyme were histologically normal or showed minor non-specific abnormalities only. Protein was assayed by a modification of the method of Lowry *et al.* (1951) with crystalline bovine serum albumin (fraction V) as standard.

Preparation of nonadeutero-4-hydroxydebrisoquine (d_9-4-hydroxydebrisoquine)

In view of the sensitivity and specificity of the assay required to determine the small amounts of 4-hydroxydebrisoquine produced *in vitro* by microsomal fractions of human liver a combined gas chromatographic-mass spectrometric assay was used. The ideal internal standard for such an assay is a stable isotope labelled analogue of the compound being measured. As no such analogue was available we prepared d_9-4-hydroxydebrisoquine by administering 7.4 mg of d_{10}-debrisoquine sulphate orally in aqueous solution to a subject previously phenotyped as a homozygous extensive metaboliser. All urine was collected for 24 h and analysed for both d_{10}-debrisoquine and d_9-4-hydroxydebrisoquine. The metabolic ratio, which was less than 0.1 when unlabelled debrisoquine was administered, increased to greater than 1 following dosing with d_{10}-debrisoquine. This more than 10-fold increase presumably represents a profound isotope effect altering the balance for alicyclic oxidation to renal excretion of the parent compound.

The full mass spectrum of d_9-4-hydroxydebrisoquine (as the TFA pyrimidino derivative) is shown in Figure 1. The spectrum of the corresponding derivative of d_0-4-hydroxydebrisoquine is shown for comparison. The base peak of the unlabelled compound is at m/z 344 (M-115) and the corresponding peak for the d_9-compound is at m/z 350. There was no discernible ion at m/z 344 in the d_9-4-hydroxydebriso-

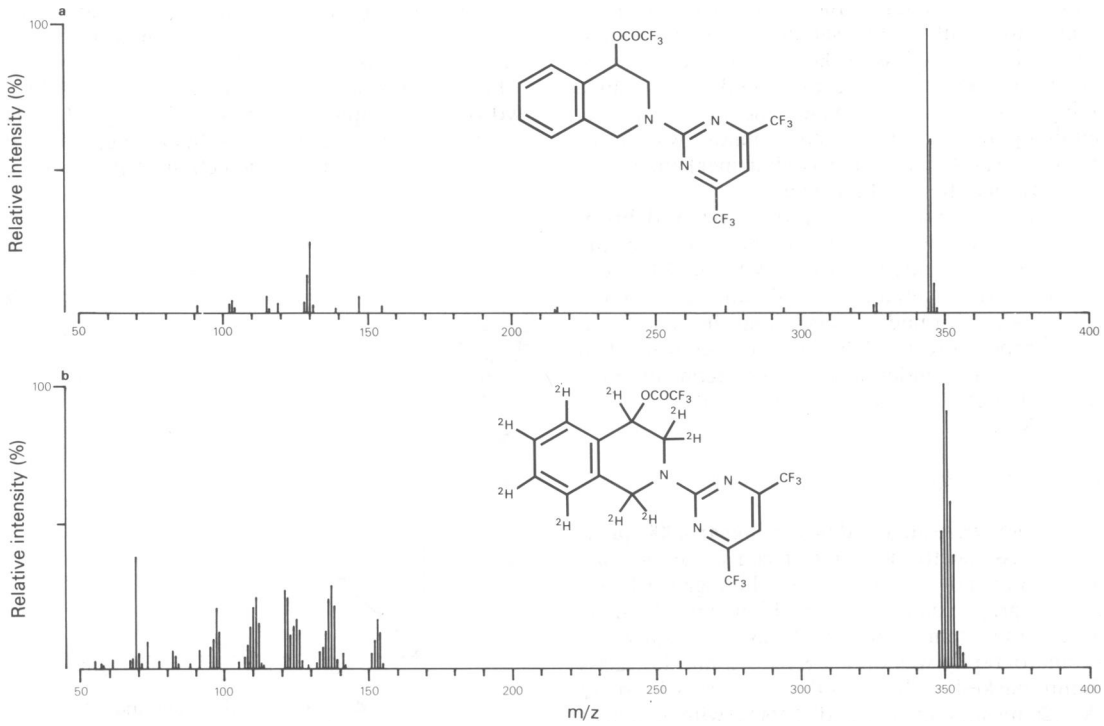

Figure 1 Full mass spectra of the TFA pyrimidino derivatives of (a) d_0-4-hydroxydebrisoquine and (b) d_9-4-hydroxydebrisoquine. GC-MS conditions were as described under **Methods**.

quine spectrum, nor at m/z 350 in the d_0-4-hydroxydebrisoquine spectrum.

Incubation conditions

Incubation mixtures comprised the substrate debrisoquine (1 mM in maximum velocity studies, 0.02–1.0 mM in full kinetic studies), magnesium chloride (6 mM), Tris-hydrochloride buffer pH 7.4 (50 mM) and between 0.5 and 1.0 mg microsomal protein in a final volume of 1.0 ml. The reaction was started by the addition of NADPH to a final concentration of 1.2 mM to samples previously kept at 4°C on ice. In blank incubations NADPH solution was replaced by a corresponding volume of distilled water. In preliminary studies, additional blanks were used to check for possible interference on the chromatographic trace from microsomal material or from the substrate debrisoquine itself. Incubations were performed in air at 37°C in a shaking water bath for 12 min, and were terminated by the addition of 200 μl 1 M NaOH which altered the pH to approximately 13.5. Immediately, 100 μl human urine containing approximately 100 ng d_9-4-hydroxydebrisoquine were added to each sample and to a series of aqueous standards (1 ml volume) of 4-hydroxydebrisoquine with a range of concentrations from 0 to 500 ng ml^{-1}.

Extraction of debrisoquine

Samples and standards were extracted at pH 13.5, as suggested by Idle *et al.* (1979a), with chloroform (3 × 3 ml) and the organic phases, containing most of the debrisoquine, were discarded. After each extraction as much of the aqueous phase as possible was transferred cleanly to a fresh tube. The volume was adjusted to 1.0 ml with NaOH on each occasion. The aqueous phase, containing the metabolite 4-hydroxydebrisoquine together with its internal standard d_9-4-hydroxydebrisoquine, was taken to pH 8.5 by the addition of 200 μl 1 M HCl followed by 200 μl aqueous saturated solution of sodium bicarbonate.

Derivatisation of debrisoquine and 4-hydroxydebrisoquine

The amidino group of the two compounds was derivatised essentially as described by Malcolm & Marten (1976), the only modification being an increase in the volume of the aqueous phase. The derivatisation pro-

cedure involved adding 100 μl HFAA and 1 ml toluene to 1 ml of the sample in 10 ml round-bottomed ground-glass necked tubes fitted with air condensers. Samples were incubated for 2 h in a boiling water bath after which the toluene phase, containing the derivatised compounds, was transferred to a reactivial (Pearce & Warriner) and blown to dryness in a stream of nitrogen.

The alicyclic hydroxyl group of 4-hydroxydebrisoquine was then derivatised. To the residue in the reactivials were added 50 μl TFAA and 50 μl dry redistilled ethyl acetate. The vials were capped and the derivative formed by incubating the samples at room temperature for 1 h. The samples were then blown to dryness under nitrogen and reconstituted in dry distilled ethyl acetate (20 μl) for injection on to the GC-MS.

Gas chromatography-mass spectrometry

The samples were analysed on a Finnigan 4000 quadrupole mass spectrometer interfaced via an all glass transfer line and jet separator to a Finnigan 9610 gas chromatograph combined with a Finnigan 6115 interactive data system running with revision I software. Chromatography was on a 1.8 m × 2 mm i.d. glass column packed with 3% OV1 on Gas Chrom Q (100–120 mesh) and operated at 160°C with helium as carrier gas at a flow rate of 30 ml min^{-1}. The injection port temperature was maintained at 250°C. The mass spectrometer was tuned to monitor ions at m/z 344 and m/z 350 (see Figure 1). The retention time of debrisoquine was 5.2 min, of d_0-4-hydroxydebrisoquine it was 5.65 min and of d_9-4-hydroxydebrisoquine it was 5.60 min. Data acquisition and reduction were performed by the interactive data system.

Results

Efficiency of extraction procedures

The retention times of debrisoquine and 4-hydroxydebrisoquine differed by only 0.45 min. Examination of the mass spectrum of debrisoquine revealed a small fragment ion at m/z 344, the ion channel used to monitor 4-hydroxydebrisoquine. At high concentrations of debrisoquine this ion would interfere with quantitation of 4-hydroxydebrisoquine. It was thus necessary to extract as much debrisoquine from the sample as possible. Efficiency of debrisoquine extraction was checked using [^{14}C]-debrisoquine. On successive extractions with chloroform approximately 90% of debrisoquine present was extracted. Thus a double extraction enabled 99% of the substrate to be removed. However a third extraction was used to ensure maximum removal of the debrisoquine.

Comparing 4-hydroxydebrisoquine recovered in extracted standards with that in unextracted standards revealed that the recovery of 4-hydroxydebrisoquine was approximately 60%.

Figure 2 shows a standard curve for extracted 4-hydroxydebrisoquine from 0 to 250 ng ml^{-1}. The correlation coefficient for the linear regression line was 0.9997. The limit of detection of the assay was 2 ng ml^{-1}.

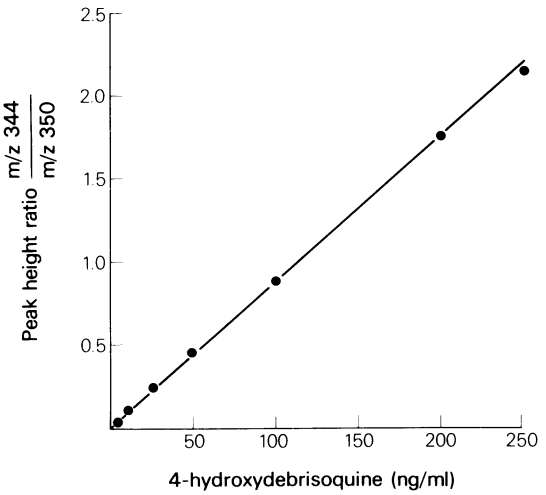

Figure 2 Standard curve for the estimation of 4-hydroxydebrisoquine, produced in microsomal incubations, using d_9-4-hydroxydebrisoquine as internal standard. The equation of the line is y = 0.00854x + 0.01516 and $r = 0.9997$ ($P < 0.001$).

Optimum incubation conditions

Linearity of the formation of 4-hydroxydebrisoquine by microsomal fractions of human liver with respect to time was investigated at 0.5 mM debrisoquine concentration. Protein concentration was 0.56 mg ml^{-1}. The reaction was linear up to 15 min and even at 21 min deviation from linearity was only slight (Figure 3). The effects of protein concentration on 4-hydroxydebrisoquine production by microsomal fractions of human liver are shown in Figure 4. The concentration of debrisoquine was again 0.5 mM. Incubation time was 10 min. Activity increased linearly with protein concentration up to 2.1 mg ml^{-1}, the highest concentration studied.

Cofactor requirements

The effect of NADPH on debrisoquine 4-hydroxylase activity is shown in Table 1. Activity was determined in duplicate with a pool of microsomal fractions from six separate liver samples with histologically normal hepatic architecture. In the absence of debrisoquine

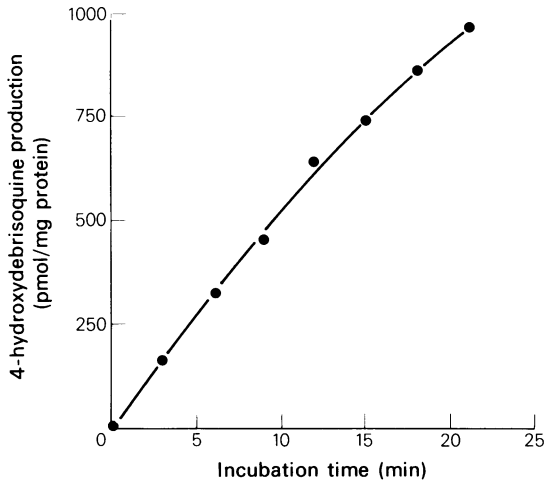

Figure 3 Effect of time of incubation on hepatic microsomal debrisoquine 4-hydroxylase activity. Protein concentration was 0.56 mg ml $^{-1}$. Debrisoquine concentration was 0.5 mM.

there were no extraneous peaks in the selected ion record. This was also true when the microsomal fraction was omitted from the incubation. When NADPH was omitted there was a small amount of activity detected, due presumably to residual NADPH in the microsomal fraction, which was not washed. However, this activity was only 7.4% of that observed in the presence of 1.2 mM NADPH. Table 1 also shows the effect of replacing the nitrogen in air with an equal percentage (by flow rate) of carbon monoxide. Activity was reduced by 76.5%.

Reproducibility of the assay

The reproducibility of the assay for 4-hydroxydebrisoquine production by microsomal fractions of human liver was investigated and the results are shown in Table 2. Activity was determined in five replicate samples from a pool of microsomal fractions of human liver. The coefficient of variation of the complete assay was 4.4%.

Kinetics of debrisoquine-4-hydroxylase activity

The effects of varying debrisoquine concentration from 0.02 to 1.0 mM on 4-hydroxylase activity are shown in Figure 5 for microsomal fractions from three separate human liver samples with normal histology.

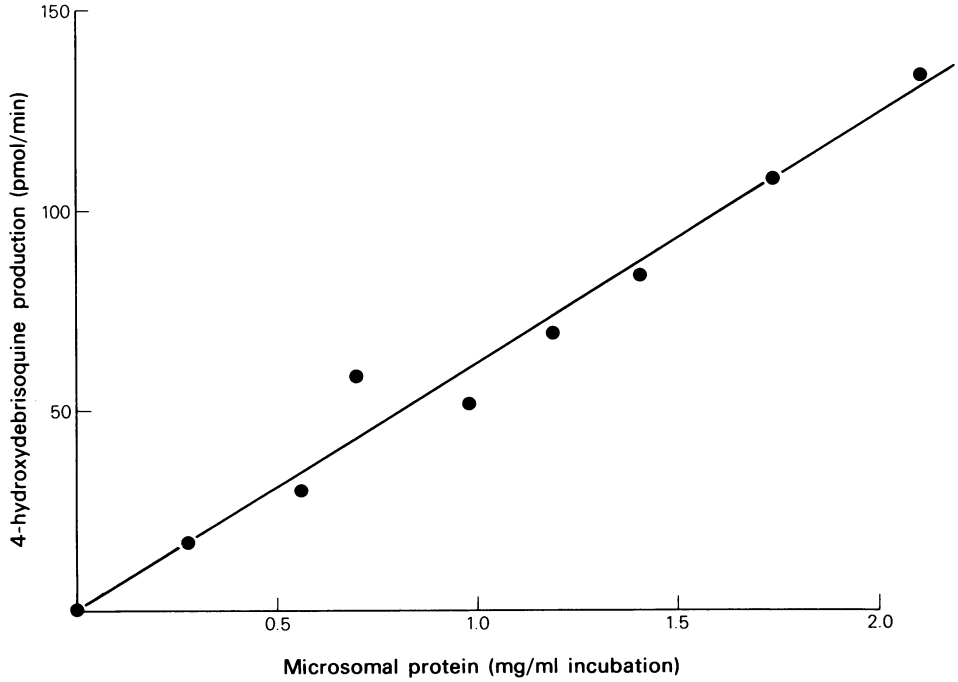

Figure 4 Effect of protein concentration on hepatic microsomal debrisoquine 4-hydroxylase activity. Time of incubation was 10 min. Debrisoquine concentration was 0.5 mM.

Table 1 Cofactor requirements for debrisoquine 4-hydroxylase activity of microsomal fractions of human liver. Activity was determined using a pool of six separate liver samples.

Debrisoquine concentration (mM)	*NADPH concentration (mM)*	*Debrisoquine 4-hydroxylase activity (pmol mg^{-1} min^{-1})*
0	1.2	<2
1.0	0	5.6
1.0	1.2	75.8
Atmosphere		
Air		75.8
80% CO:20% O_2		17.9

Values are mean of two determinations which varied by less than 10% from each other.

Table 2 Reproducibility of 4-hydroxydebrisoquine production by microsomal fractions of human liver. Replicate samples from the same pool of microsomal fractions of human liver were incubated for 13.5 min with 1 mM debrisoquine. Protein concentration was 0.5 mg ml^{-1}

Sample replicate	*Debrisoquine 4-hydroxylase activity (pmol mg^{-1} min^{-1})*
1	57.4
2	57.4
3	54.4
4	55.9
5	51.4
Mean ± s.d.	54.4 ± 2.4

Estimates of Vmax and Km were obtained by iterative non-linear least squares regression analysis. The mean value for Vmax for the three samples was 69.9 ± 14.3 pmol mg^{-1} min^{-1} (mean ± s.e. mean) and the mean value for Km was 130 ± 24 μM.

Variability in Vmax for debrisoquine 4-hydroxylase activity of biopsy samples of human liver

Microsomal debrisoquine 4-hydroxylase activity was assayed at a saturating substrate concentration of 1 mM in 18 biopsy samples of human liver and 6 samples of liver from different renal transplant donors who had met traumatic deaths. The activities found in these samples varied from 28.4 to 156.9 pmol mg^{-1} min^{-1}. Activity with renal donor samples fell within this range.

Debrisoquine 4-hydroxylase activity was analysed for the effects of environmental factors and disease and the results are shown in Table 3. Cigarette smoking, alcohol ingestion and the sex of the patient had no discernible effects on 4-hydroxylase activity. Liver disease, diagnosed histologically, was associated with a significant reduction in 4-hydroxylase activity together with reduced cytochrome P-450 content. There was no difference between the activities of wedge biopsy samples and those of renal transplant donor samples. In addition, the activities of samples stored at −80°C for up to 3 years were not significantly lower than those of samples stored for only a few weeks (data not shown).

Discussion

The method of Malcolm & Marten (1976) was used to measure 4-hydroxydebrisoquine generated by microsomal fractions of human liver. However, it was necessary to make two major modifications. (1) In the microsomal incubations there is greater than 1000-fold excess of debrisoquine over 4-hydroxydebrisoquine and this would interfere with the quantitation of the metabolite even with the specificity of a GC-MS assay. Differential extraction of the debrisoquine at pH 13.5 with chloroform solved the first problem, and enabled greater than 99% of the debrisoquine to be removed. (2) In the original method (Malcolm & Marten, 1976) d_{10}-debrisoquine was used as a common internal standard in the quantitation of debrisoquine and its metabolite. Adoption of the differential extraction procedure described above precludes the use of d_{10}-debrisoquine as an internal standard. Synthesis of d_9-4-hydroxydebrisoquine would have proven extremely time consuming. However, the biological preparation proved satisfactory. The urine containing d_9-4-hydroxydebrisoquine used as internal standard solution did not interfere in the assay.

Although there have now been numerous reports on the oxidation of debrisoquine in man (e.g. Mahgoub *et al.*, 1977; Tucker *et al.*, 1977; Bertilsson *et al.*, 1980a; Inaba *et al.*, 1980), these have all been *in vivo* studies, and the nature of the enzyme involved in the oxidation had not been determined, although it

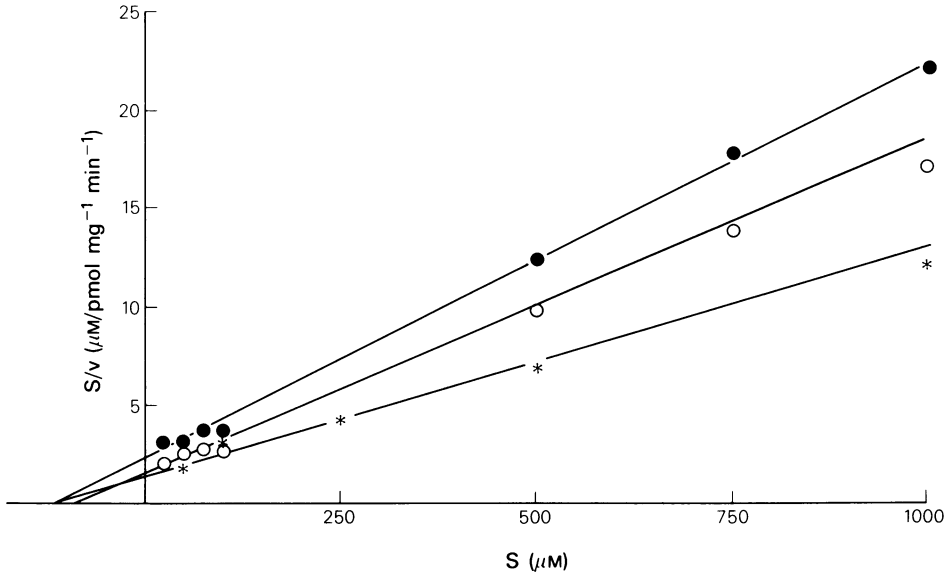

Figure 5 Hanes plots of the form S/v against S for debrisoquine 4-hydroxylase activity of microsomal fractions from three separate liver samples.

Table 3 Debrisoquine 4-hydroxylase activity of 24 human liver samples analysed for the effects of environmental factors and disease

Variable	*Group*	n	*Debrisoquine 4-hydroxylase activity*[1] *(pmol mg^{-1} min^{-1})*
Cigarette smoking	Non-smokers	9	74.2 ± 14.4
	Smokers	7	72.5 ± 12.9
Alcohol ingestion[2]	⩽ 20 units/week	12	69.7 ± 9.7
	> 20 units/week	4	84.8 ± 27.3
Liver disease	Histologically normal	11	82.4 ± 9.6
	Histological evidence of liver disease	7	51.7 ± 14.7*
Sex	Male	8	79.8 ± 13.8
	Female	9	64.2 ± 12.5
Source of liver sample	Wedge biopsies	18	70.5 ± 8.7
	Renal transplant donors	6	74.4 ± 8.6

[1] Values are mean ± s.e. mean
[2] Alcohol ingestion was based on 1 unit = 1 glass of wine = 0.5 pt beer = 1 measure of spirits (20 units = 125 g ethanol)
* Significantly lower ($P < 0.05$) than histologically normal group.

has been shown that it is not dopamine β-hydroxylase (Tucker *et al.*, 1977). Mitchell *et al.* (1980) had shown that the metabolism of debrisoquine by rat liver was catalysed by a microsomal enzyme with a requirement for NADPH. The current studies, using a specific assay for 4-hydroxydebrisoquine, have demonstrated that in human liver the formation of this metabolite is catalysed by a microsomal enzyme which requires NADPH and is inhibited by carbon monoxide. This provides good evidence that human hepatic debrisoquine 4-hydroxylase activity is cytochrome P-450 dependent.

The 4-hydroxylase activity of human liver has been partially characterised. The Michaelis-Menten constant (Km) was intermediate in value at 100–200 μM, between that of high affinity 7-ethoxycoumarin *O*-deethylase activity (Km 2 μM) (Boobis *et al.*, 1981b) at the one extreme and antipyrine oxidative reactions (Km 5–8 mM) (Boobis *et al.*, 1981a) at the other. Maximum velocity (Vmax) was quite low at 70 pmol mg^{-1} min^{-1} which could be due to a low turnover number of the form of cytochrome P-450 involved or to a low concentration of that enzyme in the microsomal fraction.

Of 25 human liver samples screened for debrisoquine 4-hydroxylase activity one produced no detectable product. This biopsy was from a subject previously phenotyped as a poor metaboliser *in vivo* and this result has already been published (Davies *et al.*, 1981). Taken together with the data in the present paper it seems likely that the 4-hydroxylation of debrisoquine is catalysed by one form of cytochrome P-450 and that this is the form that is either missing or defective in poor metabolisers.

A number of other drugs are oxidised polymorphically in man, apparently by the same enzyme system that oxidises debrisoquine. These drugs include sparteine (Inaba *et al.*, 1980; Bertilsson *et al.*, 1980a), phenacetin (Sloan *et al.*, 1978), guanoxan (Sloan *et al.*, 1980), phenformin (Shah *et al.*, 1980), nortriptyline (Bertilsson *et al.*, 1980b) and probably encainide (J.A. Oates, personal communication). The polymorphism in debrisoquine 4-hydroxylation may thus help in elucidating the structural specificity of a single form of cytochrome P-450 in man. *In vitro* studies of debrisoquine oxidation could help in phenotyping liver samples from subjects who could not otherwise be phenotyped. The use of such tissue samples in metabolic and toxicity testing of new compounds is thus potentially of considerable value.

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 their labelled analogues. The work was supported in part by grants from the Medical Research Council and the Wellcome Trust.

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