Br. J. clin. Pharmac. (1985), **19**, 721–729

Attempts to phenotype human liver samples *in vitro* for debrisoquine 4-hydroxylase activity

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1 Twentyeight samples of human liver have been characterised for cytochrome P-450 content, aldrin epoxidase, debrisoquine 4-hydroxylase and bufuralol 1′-hydroxylase activities.

2 Evidence is presented here and elsewhere that bufuralol 1′-hydroxylase and debrisoquine 4-hydroxylase are activities catalysed by the same form of cytochrome P-450 in man, and that this form is different from that catalysing the epoxidation of aldrin.

3 Attempts to phenotype liver samples *in vitro*, in the absence of any metabolic data *in vivo* for debrisoquine 4-hydroxylation status, met with limited success. A combination of enzyme assays will most probably be required in any such phenotyping of human liver samples.

Keywords human liver bufuralol 1′-hydroxylase debrisoquine 4-hydroxylase phenotype oxidation polymorphism

Introduction

There is increasing interest in the possibility of establishing human tissue banks (FRAME Toxicity Committee, 1983), comprising well-characterised samples, that can be used to provide both small numbers of samples for specific studies and large numbers of samples for population studies. There are at least three human liver banks in existence (von Bahr *et al.*, 1980; Boobis *et al.*, 1980a; Meier *et al.*, 1983) and we, as well as others, have established methods that enable the preparation and storage of liver samples for long periods of time, with only minimal loss of activity over several years (von Bahr *et al.*, 1980; Boobis *et al.*, 1980b).

Although tissue can be obtained surplus to histological requirements from patients undergoing diagnostic biopsy, either by the percutaneous needle method or by wedge resection at laparotomy, the size of such samples is usually limited to less than 1 g (Boobis *et al.*, 1980b). Larger samples are available from organ transplant donors, subjects who have met sudden accidental deaths and who are maintained on life support systems until such time as the organ required for transplantation is removed (von Bahr *et al.*, 1980; Boobis *et al.*, 1980a; Kremers *et al.*, 1981). At this time a liver sample may be taken for research purposes (this may require both Local Research Ethics Committee permission and coroner's approval). Although this source of tissue enables samples of several grams, or more, to be obtained, virtually nothing will be known about the subjects from whom the samples are obtained. For example, unlike the protocol used for a previous study on patients undergoing diagnostic liver biopsy (Davies *et al.*, 1981), it would not be possible to phenotype transplant donors for their debrisoquine hydroxylation status *in vivo* prior to obtaining the liver sample.

With the increasing importance of oxidation polymorphisms becoming apparent (see Boobis

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& Davies, 1984) it is necessary to type liver samples in a tissue bank in some way with respect to known oxidation polymorphisms. Such typing will, of necessity, almost always have to be performed *in vitro*. This paper presents results of attempts to develop methods enabling such typing to be performed. One of the major difficulties is that any disturbance of the normal morphology of the liver, such as by hypoxia (Montgomery & Rubin, 1973) or altered hormonal balance (Wilson & Spelsberg, 1976) is often associated with a widespread decrease in monooxygenase activity. Thus, determining only debrisoquine 4-hydroxylase activity, for example, may well not be sufficient to enable phenotyping of the sample unequivocally. We have measured cytochrome P-450 content and the activity of three monooxygenases in a group of 28 liver samples. The data have been subjected to frequency distribution and cross-correlation analysis.

Methods

Liver samples

Liver samples were obtained from organ transplant donors in France as previously described (Kremers *et al.*, 1981). The permission of the appropriate Ethics Committees was obtained to use such samples in these studies. Each sample was cut into small (< 10 g) pieces, frozen on solid carbon dioxide and stored at −80°C. The samples were transferred to London, UK, on solid carbon dioxide and stored at −80°C for 2–4 weeks. Not more than 5 g from each sample was placed in a beaker containing 0.25 M potassium phosphate buffer, pH 7.25, 0.15 M potassium chloride, 1 mM EDTA on ice and kept until thawed out. The samples were then chopped coarsely with scissors and drained of as much buffer as possible. The chopped samples were rinsed in two further changes of buffer, drained as completely as possible and homogenized in 4 vol of the above buffer using a Polytron tissue homogenizer (Kinematica GmbH, model PT-10) as previously described (Boobis *et al.*, 1980b). Subcellular fractionation of the samples by ultracentrifugation was performed as reported previously (Boobis *et al.*, 1980b), the microsomal pellet finally being resuspended in 1 vol of 0.25 M potassium phosphate buffer, pH 7.25 containing 30% (v/v) glycerol to each g of liver used in the preparation.

The microsomal suspensions were stored as aliquots at −80°C until required. Only vials not previously thawed were used in the enzyme assays or in the determination of cytochrome P-450 content.

Enzyme assays

Microsomal protein content, with bovine serum albumin, fraction V, as standard (Lowry *et al.*, 1951; Boobis *et al.*, 1980b), debrisoquine 4-hydroxylase activity (Kahn *et al.*, 1982; Boobis *et al.*, 1983), at a substrate concentration of 0.2 mM, bufuralol 1′-hydroxylase activity (Boobis *et al.*, 1985) at a substrate concentration of 0.05 mM, and cytochrome P-450 content (Boobis *et al.*, 1980b) were all determined as previously described. Aldrin epoxidase activity was assayed by a modification (Boobis & Davies, 1984) of the method of Wolff *et al.* (1979) at a substrate concentration of 0.05 mM.

All enzyme assays were conducted under conditions that were linear with respect to both protein content and duration of the incubation. Substrate concentrations used were selected as at least twice the K_m of the respective enzyme (Boobis & Davies, 1984).

Analysis of the data

The data for each metabolic variable were analysed by cumulative frequency distribution and probit transformation, to test for normality of distribution, and to enable samples at the extremes of the range to be identified. Each pair of activities was compared by least squares linear regression analysis. All data analysis was performed using the HP statistical software package, revision 1, on a HP 9836 microcomputer with 0.5 MBytes of RAM (Hewlett-Packard Ltd, St Albans, Herts).

Results

The mean and median values for the four metabolic variables studied, together with details of the distribution of values, are shown in Table 1. Coefficients of variation for cytochrome P-450 content and aldrin epoxidase activity were similar, at approximately 30%, whereas debrisoquine and bufuralol hydroxylase activities showed more scatter, with coefficients of variation of 56 and 68% respectively. All samples had detectable activity with the three substrates investigated. The ranges of cytochrome P-450 content and aldrin epoxidase activity were 3–4 fold whereas the range for debrisoquine 4-hydroxylase activity was 13.5–fold and for bufuralol 1′-hydroxylase activity it was 47-fold. There was very close agreement between median and mean for all four variables, although there was a slight difference, of 14%, between them for bufuralol 1′-hydroxylase activity.

Frequency distribution histograms, overlayed with the predicted normal probability curves,

Table 1 Summary of parameters of drug metabolising activity determined in 28 samples of human liver

Parameter	*Cytochrome P-450 content (nmol/mg)*	*Aldrin epoxidase activity (pmol mg^{-1} min^{-1})*	*Debrisoquine 4-hydroxylase activity (pmol mg^{-1} min^{-1})*	*Bufuralol 1'-hydroxylase activity (pmol mg^{-1} min^{-1})*
Mean	0.37	219	30.2	89.6
s. e. mean	0.02	13	3.2	11.7
s.d.	0.11	71	16.9	61.7
Variance	0.01	5000	286	3810
Coefficient of variation	29.0	32.0	56.0	68.9
Median	0.38	217	31.5	77.0
Maximum	0.61	422	62.5	283
Minimum	0.17	95	5.0	6.0
Skewness	0.179	0.725	0.197	1.711*
Kurtosis	–0.166	1.310	–0.847	3.227**

* Distribution of this activity shows significant skew ($P < 0.01$)
** Distribution of this activity shows significant kurtosis ($P < 0.01$)

are shown in Figure 1, together with the corresponding probit plots, for the four metabolic variables. Cytochrome P450 content was normally distributed in the 28 samples ($\chi^2 = 20.7$, $P > 0.05$). None of the other variables was normally distributed. However, the manner in which they varied from normality differed. Aldrin epoxidase activity showed no significant

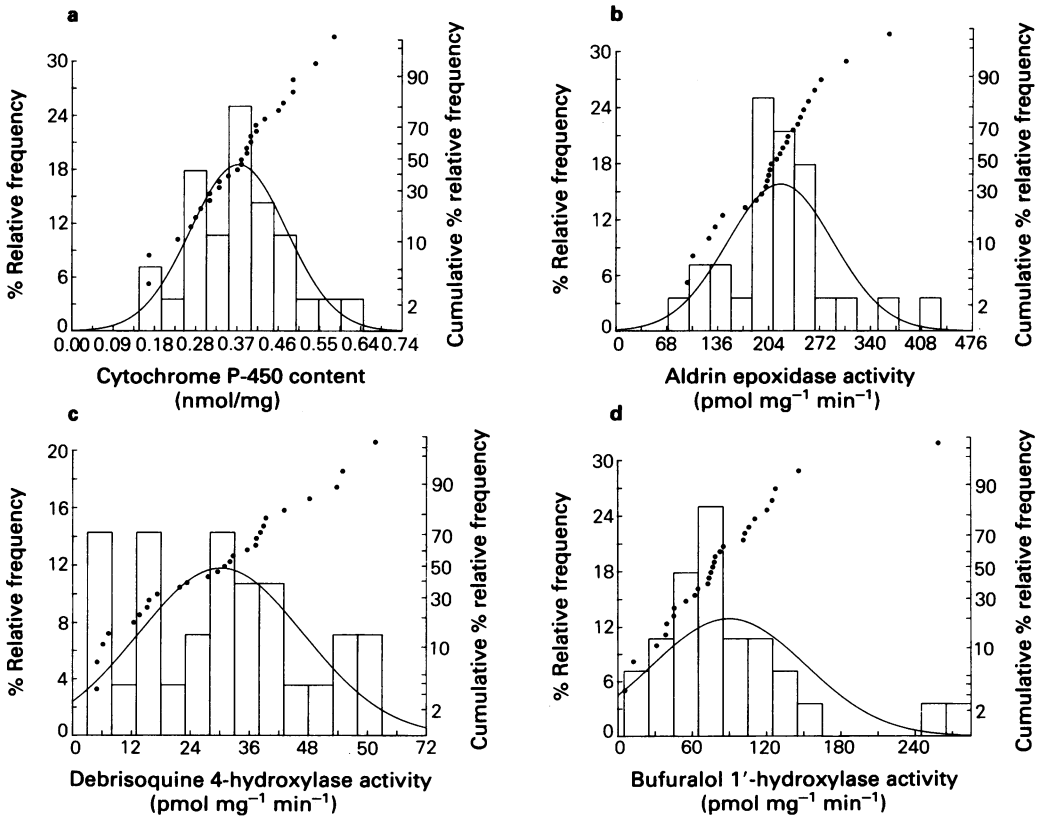

Figure 1 Frequency distribution of metabolic activities of 28 samples of human liver: (a) cytochrome P-450 content; (b) aldrin epoxidase activity; (c) debrisoquine 4-hydroxylase activity; (d) bufuralol 1'-hydroxylase activity. The curved lines on the plots show the computed normal distribution curve for the data and the filled symbols show cumulative percent relative frequency of the observed values.

skew in its distribution, but a small number of samples had higher activity than would be predicted from a normal distribution of the data. Similarly, a few of the samples had higher values of bufuralol 1′-hydroxylase activity than would be predicted from the normal distribution curve. However, the distribution of values for this enzyme activity was significantly skewed to lower values and also showed significant kurtosis. Debrisoquine 4-hydroxylase activity was also not normally distributed. In this instance, despite a lack of significant skew, there were several samples with lower activity than would be predicted from the normal distribution curve. Probit transformation of the data revealed that approximately 10% of values fell into this group.

Figure 2 shows the data for each pair of variables plotted individually to make any relationships between the variables clearer.

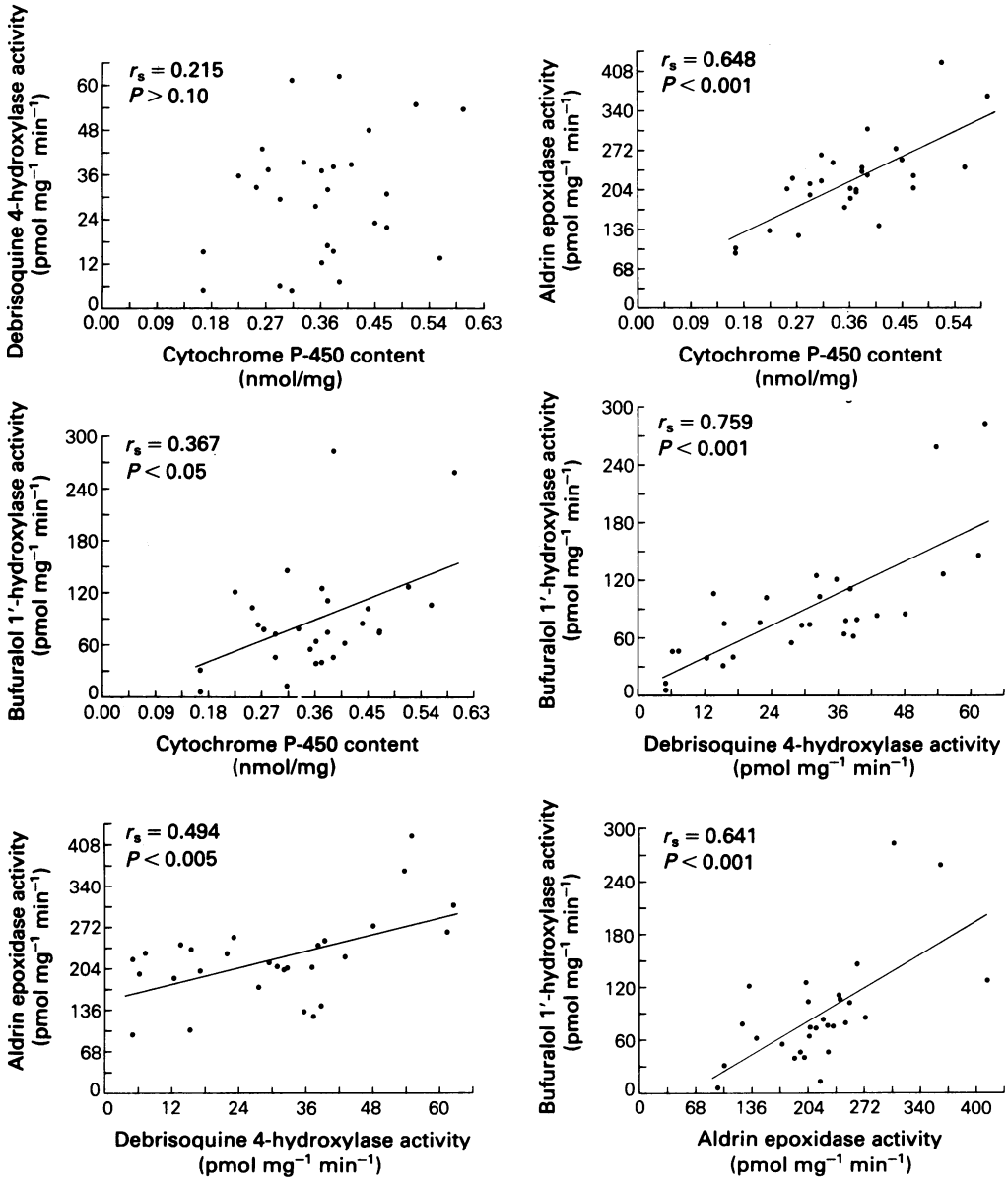

Figure 2 Relationships between four metabolic activities of 28 samples of human liver. The symbols show the observed data and, where drawn, the *solid line* shows the computed regression line. r_s is the Spearman rank correlation coefficient.

Also shown are the Spearman rank correlation coefficients (r_s). There was no significant correlation between debrisoquine 4-hydroxylase activity and cytochrome P-450 content (r_s = 0.215, $P > 0.10$). In contrast, aldrin epoxidase activity correlated well with cytochrome P-450 content (r_s = 0.648, $P > 0.001$). There was a very good correlation between debrisoquine 4-hydroxylase and bufuralol 1′-hydroxylase activities (r_s = 0.759, $P > 0.001$), although the samples with the highest bufuralol 1′-hydroxylase activity were well off the regression line. The two samples with the lowest debrisoquine 4-hydroxylase activity were also the two samples with the lowest bufuralol 1′-hydroxylase activity. The correlation between debrisoquine 4-hydroxylase and aldrin epoxidase activities was relatively weak (r_s = 0.494, $P > 0.01$) and there were no obvious relationships at the extremes of the regression line. Bufuralol 1′-hydroxylase activity correlated moderately well with aldrin epoxidase activity (r_s = 0.641, $P > 0.001$) but this was largely due to the influence of the three samples with the highest values for aldrin epoxidase activity. Omitting them from the regression analysis resulted in a much weaker correlation (r_s = 0.501) although it was still significant ($P > 0.01$).

Discussion

Phenotyping liver samples *in vitro* with respect to their debrisoquine 4-hydroxylation status has proved relatively difficult, even with a group of samples collected with the same protocol, i.e. all from organ transplant donors. Nevertheless, careful analysis of the data does permit certain conclusions to be drawn.

The cytochrome P-450 content of the samples was normally distributed, whereas none of the enzyme activities was. This is perhaps not surprising if one accepts that there are multiple forms of cytochrome P-450 (see Boobis & Davies, 1984) and that cytochrome P-450 content, determined by CO-difference spectroscopy, will represent the sum of all of the forms present (Dannan *et al.*, 1983). In contrast, the substrates studied are probably metabolised by one, or a small number of specific forms of cytochrome P-450 (see Boobis & Davies, 1984). Certain environmental factors, such as cigarette smoking, can increase monooxygenase activity in the absence of any detectable alteration in cytochrome P-450 content (Boobis *et al.*, 1980b). This is due, presumably, to selective induction of one, or a small number of, forms of cytochrome P-450 with a high activity towards such substrates.

There is a tendency for most monooxygenase activities to show a significant correlation with cytochrome P-450 content, this despite any outliers at the extremes of the range (Kapitulnik *et al.*, 1977; Boobis *et al.*, 1980b; von Bahr *et al.*, 1980, Pelkonen *et al.*, 1980; Jakobsson *et al.*, 1982; Meier *et al.*, 1983). Thus, aldrin epoxidase activity correlated very well with cytochrome P-450 content in the present study. In a previous study, with benzo(a)pyrene as substrate, it was found that, in a heterogeneous group of biopsy samples, the major determinant of both cytochrome P-450 content and aryl hydrocarbon hydroxylase activity is the degree of hepatic dysfunction, assessed histologically (Brodie *et al.*, 1981). It seems more likely that both aldrin epoxidase activity and cytochrome P-450 content, in the present study, are merely reflecting the functional state of the liver samples, rather than that a major proportion of the cytochrome P-450 present is necessarily catalysing the epoxidation of aldrin. There is good evidence that aldrin is a substrate for only one, or a very small number of forms of cytochrome P-450 in the rat (Newman & Guzelian, 1983). A similar situation may well obtain in man.

Debrisoquine 4-hydroxylase activity showed no significant correlation with cytochrome P-450 content, and bufuralol 1′-hydroxylase activity showed only a weak correlation ($P < 0.05$). It has been shown that the activity of debrisoquine 4-hydroxylase in liver samples from renal transplant donors is not significantly different from that of histologically normal biopsy samples (Kahn *et al.*, 1982). Both cytochrome P-450 content and aryl hydrocarbon hydroxylase activity are significantly reduced in the liver samples from renal transplant donors (Boobis *et al.*, 1980a). The 4-hydroxylation of debrisoquine is catalysed by a specific form of cytochrome P-450 (Boobis *et al.*, 1983). It thus appears that this form of the cytochrome is more resistant than other monooxygenases to impairment of activity by those factors operating in transplant donors. There is every indication from the present study that bufuralol 1′-hydroxylase activity is similarly resistant.

Thus, unlike the relationship between aldrin epoxidase activity and cytochrome P-450 content which might well reflect the functional status of the liver, the strong correlation between debrisoquine 4-hydroxylase and bufuralol 1′-hydroxylase activities reported here may be of some mechanistic relevance. There does appear to be a common resistance of these activities to impairment by factors that reduce other monooxygenase activities. Both the 4-hydroxylation of debrisoquine (Mahgoub *et al.*, 1977) and the 1′-hydroxylation of

bufuralol (Dayer *et al.*, 1982) are impaired in the PM phenotype *in vivo* and both activities are reduced in biopsy samples from phenotyped PM subjects (Davies *et al.*, 1981; Minder *et al.*, 1983). In addition, debrisoquine and bufuralol are potent competitive inhibitors of each other's metabolism, with K_i values for inhibition very similar to the K_m values for the oxidation of the inhibitor (Boobis *et al.*, 1985). Thus, there is good evidence that the same form of cytochrome P-450 catalyses the two reactions, thereby explaining the good correlation between the activities.

All of the samples in the present study had detectable debrisoquine 4-hydroxylase activity whereas, in a previous study, (Davies *et al.*, 1981) one subject phenotyped as a poor metaboliser *in vivo* had no detectable debrisoquine 4-hydroxylase activity in a biopsy sample. However, in the earlier study the assay was performed with the mass spectrometer operating in electron impact mode, which gave a limit of detection for 4-hydroxydebrisoquine of 5 pmol mg^{-1} min^{-1}, under the conditions of the assay used (Kahn *et al.*, 1982). In the present study the mass spectrometer was operated in the chemical ionization mode, and this improved the sensitivity of the assay to 1 pmol mg^{-1} min^{-1} (Murray *et al.*, 1983). Thus, samples were very low, but detectable activity in the present study, would have been classified as having no activity using the earlier assay.

Examination of the frequency distribution curve for debrisoquine 4-hydroxylase activity revealed a group of 4 samples, from a total of 28, with very low activity, representing a discrete mode. It is not possible to discern any intermediate group, either from the frequency histogram or the probit plot. Monooxygenase activities of the four samples are shown in Table 2. Two of the samples with the lowest debrisoquine 4-hydroxylase activity were also the two with the lowest bufuralol 1'-hydroxylase activity. However, only one of these samples, P60, appears clearly to come from a PM subject. Both debrisoquine and bufuralol oxidation by this sample are at the extremes of the range, and cytochrome P-450 content and aldrin epoxidase activity are both well within the range for the rest of the group.

Although sample P49 has extremely low activity for both debrisoquine and bufuralol oxidation, both cytochrome P-450 content and aldrin epoxidase activity are also at the lowest extremes of their respective ranges. It seems likely that this sample has a generalized impairment in monooxygenase activity due to the handling of the sample prior to processing in our laboratory. Obviously, if only debrisoquine

Table 2 Rank order and metabolic activities of human liver samples with lowest debrisoquine 4-hydroxylase activity

Sample	*Cytochrome P-450 content* (*nmol/mg*)	*Rank**	*Debrisoquine 4-hydroxylase activity* (*pmol mg^{-1} min^{-1}*)	*Rank*	*Bufuralol 1'-hydroxylase activity* (*pmol mg^{-1} min^{-1}*)	*Rank*	*Aldrin epoxidase activity* (*pmol mg^{-1} min^{-1}*)	*Rank*
P49	0.17	1	5.0	1	6.0	1	95.0	1
P60	0.32	9	5.0	1	13.0	2	219	15
P61	0.30	7	6.2	3	46.0	6	195	8
P63	0.40	19	7.3	4	46.0	6	229	18

* Samples are ranked from 1, lowest activity, to 28, highest. Samples with the same activity have been given the same rank.

4-hydroxylase activity had been determined this sample would have been classified as from a PM subject, illustrating the difficulties of such assignment.

Samples P61 and P63 are more difficult to categorise. Both cytochrome P-450 content and aldrin epoxidase activity are well within the range of the group, but whereas debrisoquine 4-hydroxylase activity is extremely low, bufuralol 1′-hydroxylase activity is well above the lowest values, although still within the lowest quartile. However, Minder *et al.* (1983) have reported that a liver sample from a subject phenotyped with debrisoquine *in vivo* as PM, 1′-hydroxylated bufuralol *in vitro* at approximately half the rate of samples from EM subjects.

From the frequency distribution curves and the correlation plots it is apparent that a small number of samples have higher monooxygenase activities than would be predicted from the probability plots. This is particularly true for aldrin epoxidase and bufuralol 1′-hydroxylase activities, for which the probit plots indicate a separate small population with high activity. Activities for the four samples involved are shown in Table 3. Examination of these data reveals that all activities are elevated. These samples have both the highest debrisoquine 4-hydroxylase and bufuralol 1′-hydroxylase activities and are four of the five samples with highest aldrin epoxidase activity. Cytochrome P-450 content is not at the extreme of the range for these samples.

The explanation for the elevations of monooxygenase activities in these samples is not known. The subjects may well have been receiving enzyme inducing drugs, such as anticonvulsants, or perhaps they represent a discrete group with genetically determined elevations of monooxygenase activity. It is obviously not possible to determine the true explanation from the available information.

The present study was conducted to determine the feasibility of phenotyping liver samples for their debrisoquine 4-hydroxylation status, in the absence of any prior information on the subjects from whom the samples were obtained. It appears that the most reliable method might involve determining debrisoquine 4-hydroxylase activity, aldrin epoxidase activity, or perhaps some other monooxygenase activity not impaired in the debrisoquine oxidation polymorphism, and cytochrome P-450 content. In view of the results reported here and by Minder *et al.* (1983), the use of bufuralol 1′-hydroxylase activity as a phenotypic marker *in vitro* must, at present, await further validation.

Table 3 Rank order and metabolic activities of human liver samples with the highest debrisoquine 4-hydroxylase activity

Sample	*Cytochrome P-450 content* (*nmol/mg*)	*Rank**	*Debrisoquine 4-hydroxylase activity* (*pmol mg^{-1} min^{-1}*)	*Rank*	*Bufuralol 1′-hydroxylase activity* (*pmol mg^{-1} min^{-1}*)	*Rank*	*Aldrin epoxidase activity* (*pmol mg^{-1} min^{-1}*)	*Rank*
P41	0.61	28	53.7	25	259	27	365	27
P67	0.53	26	55.0	26	127	25	422	28
P58	0.32	9	61.4	27	146	26	264	24
P70	0.40	19	62.5	28	283	28	308	26

* Samples are ranked from 1, lowest activity, to 28, highest activity.

We are indebted to Dr Frantz, Dr Moussin and Dr Kreis for the provision of the human liver samples used in this study. We are extremely grateful to Roche Products Limited (Welwyn Garden City) for gifts of bufuralol, debrisoquine and their metabolites and to Dr D. Hutson (Shell Research Centre, Sittingbourne) for providing samples of aldrin and dieldrin. This work was supported by grants from the Medical Research Council and the Wellcome Trust.

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(*Received October 30, 1984, accepted January 22, 1985*)