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# Relative expression of cytochrome *P*450 isoenzymes in human liver and association with the metabolism of drugs and xenobiotics

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Cytochrome *P*450s play a central role in the metabolism and disposition of an extremely wide range of drugs and chemical carcinogens. Individual differences in the expression of these enzymes may be an important determinant in susceptibility to adverse drug reactions, chemical toxins and mutagens. In this paper, we have measured the relative levels of expression of cytochrome *P*450 isoenzymes from eight gene families or subfamilies in a panel of twelve human liver samples in order to determine the individuality in their expression and whether any forms are co-regulated. Isoenzymes were identified in most cases on Western blots based on the mobility of authentic recombinant human cytochrome *P*450 standards. The levels of the following *P*450 proteins correlated with each other: CYP2A6, CYP2B6 and a protein from the *CYP2C* gene subfamily, CYP2E1 and a member of the *CYP2A* gene subfamily, CYP2C8, CYP3A3/A4 and total cytochrome *P*450 content. Also, the levels of two proteins in the *CYP4A* gene subfamily were highly correlated. These correlations are consistent with the relative regulation of members of these gene families in rats or mice. In addition, the level of expression of specific isoenzymes has also been compared with the rate of metabolism of a panel of drugs, carcinogens and model *P*450 substrates. These latter studies demonstrate and confirm that the correlations obtained in this manner represent a powerful approach towards the assignment of the metabolism of substrates by specific human *P*450 isoenzymes.

## INTRODUCTION

The hepatic cytochrome *P*450-dependent mono-oxygenases (*P*450s) catalyse a diverse spectrum of reactions, including the metabolism of endogenous compounds such as cholesterol, fatty acids and steroids and exogenous compounds such as drugs and environmental chemicals (Guengerich, 1988*a*). The extraordinarily broad substrate specificity of the *P*450 system results from the multiplicity of distinct molecular forms which have different, but overlapping, substrate specificities (Lu & West, 1980; Nebert & Negishi, 1982). Many different *P*450 isoenzymes have been purified from human liver (Wang *et al.*, 1983; Guengerich, 1987, 1988*a*), their corresponding cDNAs cloned (see Gonzalez, 1990) and classified into families and subfamilies based on their DNA and amino acid sequence similarity (Nebert *et al.*, 1991).

In rodents, endogenous, exogenous and genetic factors are involved in the regulation of expression of *P*450 isoenzymes (Adesnik & Atchison, 1985; Wolf, 1986). Hormones and steroids, for example, have been implicated in the endogenous control of *P*450s (Skett, 1987; Gulati & Skett, 1989; Zaphiropoulos *et al.*, 1989), and the inducibility of many *P*450 isoenzymes by exogenous compounds has been known for almost 30 years (see Okey, 1990). Different compounds can exhibit a selectivity in the *P*450 forms which they induce. For example, members of the CYP1A subfamily are inducible by polycyclic hydrocarbons, CYP2E1 isoenzymes are induced by ethanol and acetone, and the CYP2B and CYP2C subfamilies contain members which are induced to varying degrees by phenobarbital.

Although animal studies have identified many factors which may be important in the endogenous regulation of human *P*450s, the role of hormones in their regulation remains unclear. However, there is now a great deal of evidence demonstrating that genetic and environmental factors play a role in the regulation of expression of the human isoenzymes (Guengerich, 1987, 1988*a*; Wolf *et al.*, 1987, 1990). Studies into the consequences of the genetic polymorphism of debrisoquine 4-hydroxylation, associated with mutations in the *CYP2D6* locus, exemplify the pharmacological consequences of individuality in *P*450 expression (Eichelbaum & Gross, 1990), where the absence of the isoenzyme CYP2D6 results in an inability to metabolize debrisoquine and at least 25 other drugs.

In view of the diverse functions of cytochrome *P*450s, individuality in isoenzyme expression can have a variety of consequences. It can cause altered drug pharmacokinetics, resulting in reduced drug efficacy or enhanced drug side-effects. It can also alter susceptibility to toxins and carcinogens which are either activated or detoxified by *P*450 enzymes. As a consequence of the important role certain *P*450s play in the metabolism of steroid hormones, it can also influence circulating hormone levels and therefore interfere with the biological effects which they mediate.

It is therefore of central importance to establish the degree of individuality in cytochrome *P*450 isoenzyme expression, and the reasons for this individuality. In the current study, we have identified and immunoquantified the relative levels of thirteen *P*450 isoenzymes, *P*450 reductase, cytochrome $b_5$ and Alpha and

Abbreviations used: 2-AF, 2-aminofluorene; $R_s$, Spearman rank correlation coefficient; GST, glutathione *S*-transferase; the cytochrome *P*450 nomenclature used in this paper is based on that in Nebert *et al.* (1991).

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Mu class glutathione *S*-transferases (GSTs) in a panel of human liver microsomes, in order to show whether any common factors regulate the levels of different isoenzymes. We have also correlated the levels of specific isoenzymes with the metabolism of a range of drugs, carcinogens and model *P*450 substrates.

## MATERIALS AND METHODS

### Materials

Resorufin ethers and resorufin were purchased from Molecular Probes Inc., Junction City, OR, U.S.A. Other reagents were of high purity and were obtained from the usual commercial sources.

### Microsomal samples

Human livers were obtained from kidney transplant donors. Livers were frozen in liquid nitrogen and stored at −70 °C within 1 h of removal. The available information about patient case history is given in Table 1. Microsomal and cytosolic protein fractions were prepared by differential centrifugation as previously described (Forrester *et al.*, 1990), and protein content was determined by the method of Lowry *et al.* (1951) using BSA as standard. A panel consisting of twelve different livers was used in this study, and cytochrome *P*450 levels ranged from 0.2 to 0.47 nmol/mg of microsomal protein. There was no evidence of any *P*450 degradation to cytochrome *P*420. Human liver microsomes were sterilized for mutation assays by irradiation (40000 rad) using a caesium-137 source at 4 °C. This treatment did not affect the metabolic capacity of the microsomal samples.

### Immunoblotting

Microsomal or cytosolic proteins (7.5 $\mu$g) were separated on 9% or 12% gels respectively SDS/PAGE (Laemmli, 1970), transferred to nitrocellulose (Towbin *et al.*, 1979) and probed with anti-*P*450 or anti-GST antibodies as previously described (Lewis *et al.*, 1988; Forrester *et al.*, 1990). Bound antibody was visualized by the addition of $^{125}$I-Protein A (Lewis *et al.*, 1988). In order to determine *P*450 or GST content the appropriate exposures of autoradiographs were scanned using a Joyce–Loebl scanning densitometer. The relative cytochrome *P*450 or GST content was established by preparing standard curves from the microsomal samples loaded in a series of concentrations.

Antibodies were raised against purified rat *P*450 isoenzymes (CYP1A2, CYP2A1, CYP2B1, CYP2C6, CYP3A1 and CYP4A1), rat *P*450 reductase and cytochrome $b_5$ (Wolf & Oesch, 1983; Adams *et al.*, 1985; Wolf *et al.*, 1988). The rat anti-CYP2E1 antibody was a gift from Dr. C. S. Yang, Department of Chemical Biology and Pharmacology, Rutgers University, Piscataway, NJ, U.S.A. GST antibodies raised against GST-Pi, -Mu and -Alpha isoenzymes were a gift from Dr. J. D. Hayes (University of Edinburgh) (Hussey *et al.*, 1986; Hayes *et al.*, 1989). The monoclonal antibody to CYP2D6 was supplied by Dr. U. A. Meyer, Department of Pharmacology, Biocenter of the University, Basel, Switzerland (Zanger *et al.*, 1988).

### Northern blotting

RNA was isolated from 9 out of 12 of the human liver samples by the method of Cox (1968). RNA concentration and purity were estimated spectrophotometrically, and the RNA was tested for integrity by ethidium bromide staining following separation on a denaturing formaldehyde gel. After electrophoresis, RNA was transferred to Hybond membranes, and *P*450 or GST mRNA content was determined using the conditions described previously (Meehan *et al.*, 1984). Blots were washed at 65 °C with 0.3 M-NaCl/0.03 M-trisodium citrate (pH 7.4). cDNA probes (see below) were labelled by the random-priming method (Feinberg & Vogelstein, 1983, 1984).

The human cDNA probes used were CYP1A1, CYP2A6, CYP2B6, CYP2C8, CYP2D6, CYP2E1, CYP3A3 and GST B1, and with the exception of CYP2E1 these were as previously described (Lewis *et al.*, 1988; Meehan *et al.*, 1988*a*,*b*; Stevenson *et al.*, 1989; Miles *et al.*, 1990; Gough *et al.*, 1990). The CYP2E1 cDNA was a gift from Dr. F. J. Gonzalez, Laboratory of Molecular Carcinogenesis, National Cancer Institute, NIH, Bethesda, MD, U.S.A.

### Enzyme assays

Total cytochrome *P*450 content was determined by the method of Omura & Sato (1964), with *P*450 concentrations calculated from the absorbance difference between the 450 nm peak and 490 nm peak using a molar extinction coefficient of 91 mM$^{-1}$·cm$^{-1}$. *P*450 reductase was determined as previously described (Adams *et al.*, 1985). The *O*-dealkylation of resorufin analogues was determined at 37 °C by the method of Burke *et al.* (1985), and the *O*-dealkylation of coumarin analogues was measured as reported by Kitteringham *et al.* (1988), using the method of Greenlee & Poland (1978) to assay the product 7-hydroxycoumarin. Testosterone metabolism was carried out by a modification of the method of Gustafsson & Sternberg (1974). Oestradiol and ethinyloestradiol 2-hydroxylase activities were determined as previously described (Ball *et al.*, 1990). 4-Hydroxylation and *N*-demethylation of both *R*- and *S*-mephenytoin were carried out as previously described (Meier *et al.*, 1985).

Diazepam hydroxylase and *N*-demethylase activities were determined by incubation of diazepam (25 $\mu$M) with microsomal protein (2 mg) and NADPH (1 mM) in 0.67 M-phosphate buffer (total volume 2.5 ml) for 6 min. Following extraction with diethyl ether, diazepam, temazepam and nordiazepam were quantified by h.p.l.c. Separations were performed using a $\mu$-Bondapak $C_{18}$ radial compression cartridge and a mobile phase of ammonium phosphate buffer (pH 3, 0.05%)/methanol/acetonitrile (9:9:2, by vol.); flow rate 1.8 ml·min$^{-1}$. Under these conditions the

**Table 1. Patient information**

Shown is the relevant medical history of people whose livers formed the panel used in this study. F, female, M, male.

| Patient | Sex | Age (years) | Case history | Drug history |
|---|---|---|---|---|
| 4 | F | 66 | Brain haemorrhage | Anaesthetic |
| 5 | M | 60 | Brain haemorrhage | None |
| 7 | M | 29 | Head injuries | None |
| 8 | F | 46 | Brain haemorrhage | Phenobarbital, phenytoin frusamide, dopamine, tolbutamide |
| 9 | M | 27 | Head injuries | None |
| 10* | M | 54 | Heart failure/asphyxia | None |
| 11 | M | 68 | Head injuries | None |
| 12 | M | 8 | Head injuries | Phenytoin |
| B1 | M | 27 | Head injuries | None |
| W1* | M | 18 | Head injuries | None |
| E6 | M | 42 | Road accident | Dexamethasone |
| E8* | F | 49 | Brain haemorrhage | None |

* Denotes smoker

retention times for diazepam, temazepam and nordiazepam were 11, 7.5 and 9 min respectively.

Mianserin hydroxylase, *N*-demethylase and *N*-oxidase activities were determined from incubations of [$^{14}$C]mianserin (10 $\mu$M, 0.5 $\mu$Ci) with microsomal protein (1 mg) and NADPH (1 mM) in 0.67 M-phosphate buffer (final volume 4 ml). The reaction was terminated after 15 min by the addition of dichloromethane (5 ml) to extract unchanged mianserin and its metabolites, which were assayed using radiometric h.p.l.c. as previously described (Kitteringham *et al.*, 1988).

Tolbutamide 4-hydroxylase activity was determined as previously described (Back *et al.*, 1988). Cyclosporin hydroxylase (to metabolite 17) and *N*-demethylase (to metabolite 21) activities were also determined by a previously published method (Tjia *et al.*, 1989). Ciamexon hydroxylase activity was determined from incubations of [$^{14}$C]ciamexon (10 $\mu$M, 0.5 $\mu$Ci) with microsomal protein (1 mg) and NADPH (1 mM) in 0.67 M-phosphate buffer (final volume 2 ml). The reaction was terminated after 15 min by the addition of methanol (5 ml) to extract unchanged ciamexon and ciamexon alcohol, which were assayed using radiometric h.p.l.c. Separations were performed on a $C_{18}$ reversed-phase column (Spherisorb ODS, 5 $\mu$m), fitted with an on-line guard column. Compounds were eluted with a mobile phase of 30% methanol in ammonium phosphate buffer (pH 3.0; 0.043 M) for 2.5 min followed by a linear increase in the proportion of methanol to 55% over 15 min (flow rate 1 ml·min$^{-1}$).

### Mutagenicity assays

The metabolic activation of 2-aminofluorene (2-AF) was assessed using the standard bilayer plate incorporation mutation test developed by Ames *et al.* (1973, 1975), with the modifications described by Forrester *et al.* (1990). Microsomal protein (0.5 mg) was added to a cofactor mixture containing $MgCl_2$ (8 mM), KCl (33 mM), glucose 6-phosphate (5 mM) and NADP$^+$ (4 mM) in 50 mM-phosphate buffer (pH 7.4). The mixture was poured on to a pre-poured agar plate (Difco Labs, Detroit, MI, U.S.A.) immediately after the addition of 2-AF (1 $\mu$g), 0.1 ml of *Salmonella typhimurium* strain TA98 (overnight culture) and 2 ml of 0.6% agar containing NaCl (0.6%), L-histidine/HCl (50 $\mu$M), biotin (50 $\mu$M) and ampicillin (500 $\mu$g/ml). Plates were incubated for 48 h at 37 °C before counting the number of revertant colonies.

### Statistical analyses

Relative cytochrome *P*450 content and mono-oxygenase catalytic activities were compared with the relative level of expression of the *P*450 isoenzymes using the Spearman rank ($R_s$) correlation test.

## RESULTS

### Antibody characterization

The specificity of the anti-(rat cytochrome *P*450) antibodies for the human *P*450 isoenzymes was tested using recombinant human proteins produced from cDNAs expressed in HepG2 cells using a vaccinia virus vector system (Fig. 1). Although evidence for cross-reactivity was limited by the recombinant human *P*450s available, our data are consistent with other reports in the literature where comparisons can be made. All of the antibodies used showed high specificity for the recombinant *P*450s derived from the same gene subfamily except for the antibody to CYP2A1, which appeared to react weakly with recombinant *P*450 CYP2C9, and anti-CYP2E1, which reacted very weakly with CYP3A3 and CYP3A4

### *P*450 assays

Table 2 shows the results of total *P*450 estimations, *P*450 reductase assays and the metabolism of several *P*450 model substrates and drugs in the panel of human liver microsomal samples. Values given are means (±S.D.), with the minimum and maximum values and fold variation calculated for each assay. The mean value for *P*450 content was calculated to be 0.29 nmol/mg of microsomal protein, which is consistent with reported values for human liver microsomal samples (Distelrath & Guengerich, 1988). Although the total cytochrome *P*450 content varied only 2.3-fold, the rate of metabolism of some substrates was subject to a very much larger variation.

### Immunoblotting of microsomal samples

Fig. 2(*a*) shows immunoblots of twelve human liver samples probed with the antibodies described above. One protein band of $M_r$ 53000 was detected using an anti-CYP1A2 antibody, and the variation between individuals was approx. 10-fold. Anti-CYP2A1 detected two protein bands, of $M_r$ 51000 and 54500, that varied 13- and 22-fold respectively. The $M_r$-51500 protein has previously been shown to be CYP2A6 (Miles *et al.*, 1990). The immunoblot obtained using this antibody was identical to a blot probed with an anti-[mouse Coh (Cyp2a-5)] antibody (Jovonen *et al.*, 1988; Raunio *et al.*, 1988), and there was a high correlation between bands recognized by the two different antibodies [$R_s = 0.71$ ($M_r$ 51500) and $R_s = 0.85$ ($M_r$ 54500); results not shown]. A major protein band was detected by the anti-CYP2B1 antiserum of $M_r$ 51500 (CYP2B6), although a higher-$M_r$ band (54500) was present in some samples at trace levels. Three CYP2C-related proteins ($M_r$ 51000, 52500 and 54500) were detected in human

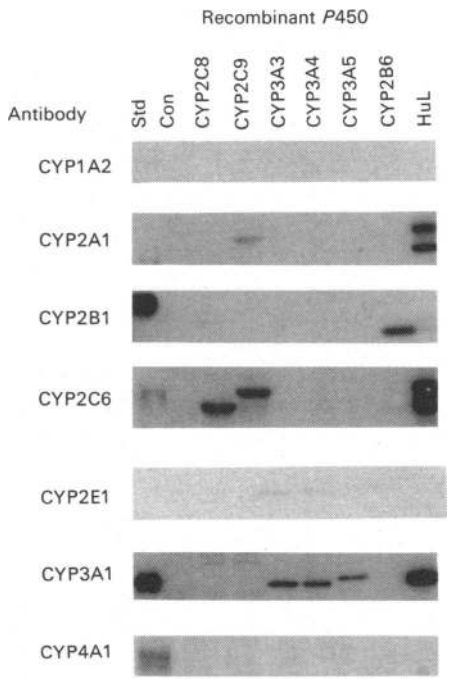


**Fig. 1. Immunoblotting of recombinant human *P*450 proteins with anti-(rat *P*450) antisera**

HepG2 cell lysates (12 $\mu$g) containing expressed human *P*450 cDNAs (CYP2C8, CYP2C9, CYP3A3, CYP3A4, CYP3A5 and CYP2B6) were separated on SDS/9% PAGE, transferred to nitrocellulose and probed with various anti-*P*450 antisera as detailed in the Materials and methods section. Std, rat *P*450 standard (0.8 pmol); Con, control; HuL, human liver microsomal sample. This sample only expressed some of the *P*450 forms recognized by the rat *P*450 antisera.

**Table 2. Cytochrome mono-oxygenase activities in a panel of human livers**

All enzyme activities are pmol·min$^{-1}$ mg of protein$^{-1}$, unless otherwise indicated.

| Enzyme activity | Mean ± S.D. | Min. | Max. | Fold variation |
|---|---|---|---|---|
| Cytochrome *P*450 (nmol·mg of protein$^{-1}$) | 0.29 ± 0.10 | 0.2 | 0.47 | 2.3 |
| *P*450 reductase (units·mg of protein$^{-1}$) | 234 ± 88 | 164 | 413 | 2.5 |
| Coumarin 7-hydroxylase | 30.2 ± 18 | 13 | 69 | 3 |
| Ethoxyresorufin *O*-de-ethylase | 95 ± 47 | 27 | 182 | 6.7 |
| Benzyloxyresorufin *O*-de-ethylase | 45 ± 47 | 8 | 144 | 18 |
| Methoxyresorufin *O*-de-ethylase | 224 ± 153 | 17 | 488 | 29 |
| Ethoxycoumarin *O*-de-ethylase | 170 ± 64 | 46 | 340 | 7.4 |
| Methoxycoumarin *O*-demethylase | 430 ± 190 | 52 | 750 | 14 |
| Testosterone β-hydroxylase | 372 ± 202 | 92 | 624 | 6.8 |
| 17-Hydroxysteroid dehydrogenase | 650 ± 404 | 360 | 1860 | 5.2 |
| Oestradiol 2-hydroxylase | 670 ± 260 | 150 | 1500 | 7.7 |
| Ethinyloestradiol 2-hydroxylase | 250 ± 92 | 120 | 450 | 3.8 |
| *R*-Mephenytoin 4-hydroxylase | 18.2 ± 19.5 | 4 | 71 | 17 |
| *R*-Mephenytoin *N*-demethylase | 52 ± 41 | 14 | 123 | 8.8 |
| *S*-Mephenytoin 4-hydroxylase | 38 ± 22 | 5 | 78 | 17 |
| *S*-Mephenytoin *N*-demethylase | 105 ± 97 | 3 | 355 | 112 |
| Diazepam 3-hydroxylase | 150 ± 100 | 50 | 350 | 7 |
| Diazepam *N*-demethylase | 50 ± 30 | 15 | 120 | 8 |
| Tolbutamide hydroxylase | 180 ± 90 | 80 | 340 | 4.3 |
| Cyclosporin hydroxylase | 330 ± 330 | 50 | 1320 | 26 |
| Cyclosporin *N*-demethylase | 4.2 ± 4.9 | 0.7 | 19 | 27 |
| Mianserin hydroxylase | 96 ± 33 | 42 | 190 | 4.5 |
| Mianserin *N*-demethylase | 58 ± 27 | 13 | 140 | 11 |
| Ciamexon hydroxylase | 300 ± 160 | 34 | 580 | 17 |

liver samples with an anti-CYP2C6 antiserum. The proteins of $M_r$ 52500 and 54500 had the same mobilities as recombinant CYP2C8 and CYP2C9 respectively. The level of expression of CYP2C9 was reasonably constant between individuals, with only a 3-fold variation, whereas CYP2C8 varied 60-fold. The $M_r$-51000 band detected by the anti-CYP2C6 antibody was absent in liver from patient W1 but was present at highly variable levels in all of the other individuals (115-fold variation). The anti-CYP2D6 antibody detected a single $M_r$-51500 protein in eleven out of the twelve individuals. Using a DNA-based assay, this individual (liver from patient 7) has been shown to be nulled at the *CYP2D6* locus (Gough *et al.*, 1990). The level of this protein in other samples varied approx. 16-fold. The anti-CYP2E1 antibody detected two protein bands ($M_r$ 54500 and 51000) in human liver. The reason for this was unclear, particularly in view of the evidence that there is only one *CYP2E* gene in man. Neither of the bands identified had the same mobility as CYP3A3/A4, ruling out the possibility that one of the observed bands was due to cross-reactivity with these proteins. The level of expression of the $M_r$-54500 protein varied approx. 4-fold. The lower-$M_r$ band was undetectable in several liver samples. Anti-CYP3A1 antibody recognized one protein band of $M_r$ 52500, which was detected in all livers. The mobility of this protein was the same as that of recombinant CYP3A3 and CYP3A4. The level of the band detected was subject to a 60-fold variation. It is interesting to note that the antibody used also reacted with recombinant CYP3A5, but this protein, which has a different mobility in SDS/acrylamide gels to CYP3A3 and CYP3A4 (Fig. 1), was not detected in any of the liver samples studied. Indeed, we have not been able to detect the protein in at least 25 liver samples studied to date. Wrighton *et al.* (1989) reported the polymorphic expression of Hlp3, a member of the human CYP3A subfamily, and found expression in only 11 out of 46 liver samples tested. Whether this is the same protein is unclear. Antibodies to CYP4A1 detected three proteins, including a very prominent band of approx. $M_r$ 60000, which was subject to 15-fold variation. The less prominent bands, of $M_r$ 51500 and 50000, had mobilities similar to that of the rat standard and were subject to 30- and 40-fold variation respectively.

The level of expression of cytochrome $b_5$ was very consistent between individuals and served as an indicator for the integrity of the microsomal samples. *P*450 reductase levels, however, did appear to be subject to considerable (14-fold) variation, and it was interesting that this was not reflected in variability in reductase activity.

### Correlations between the expression of *P*450 isoenzymes and total cytochrome *P*450 content

Highly significant correlations were observed between the levels of CYP2A6 and CYP2B6 ($R_s = 0.83$, $P < 0.001$); the concentration of these proteins was also weakly correlated with CYP2C8 levels, and also appeared to correlate with the protein of $M_r$ 51000 recognized by the anti-CYP2E1 antibody. This latter correlation introduces the possibility that the protein of $M_r$ 51000 recognized by this antibody is CYP2B6 (Table 3). The levels of the two lower-$M_r$ bands recognized by the anti-CYP4A antiserum were also highly correlated ($R_s = 0.86$, $P < 0.001$). A weak correlation was observed between the expression of the protein tentatively identified as CYP2E1 and the level of the CYP2A-related protein ($M_r$ 54500). Of particular interest was the finding that CYP3A3/3A4 was highly correlated with total *P*450 levels ($R_s = 0.92$, $P < 0.001$); a correlation of total *P*450 levels with CYP2C8 expression was also observed.

### Immunoblotting of cytosolic fractions

A high and relatively consistent level of Alpha-class GST subunits was found in all samples (Fig. 2*b*). Two proteins were detected using the anti-GST-Mu antibody, a lower-$M_r$ protein which was present in all livers, and a higher-$M_r$ protein that was absent from eight out of twelve livers shown. This is the polymorphic GST-Mu protein described by Seidegard *et al.* (1986) and Hussey *et al.* (1987). Relative to the *P*450s, the lower-

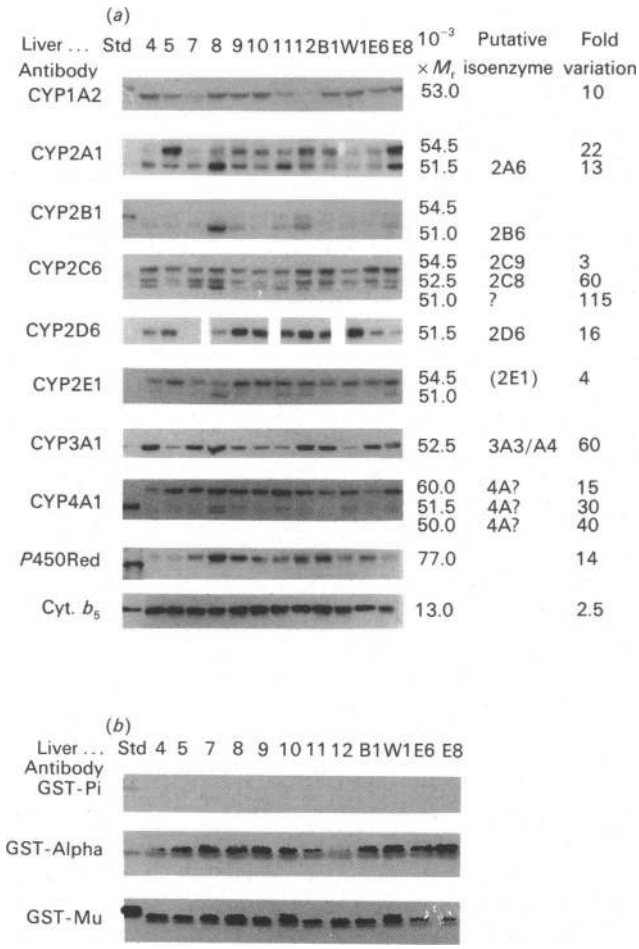


**Fig. 2. Characterization of cytochrome *P*450 and GST expression in human livers**

Microsomal (*a*) and cytosolic (*b*) fractions (7.5 $\mu$g) from the panel of human livers were separated on SDS/PAGE (9% and 12% gels respectively), transferred to nitrocellulose and probed with anti-*P*450 and anti-GST antisera, as detailed in the Materials and methods section. $M_r$ values were calculated as described by Forrester *et al.* (1990). Std, standard; *P*450 Red, *P*450 reductase; cyt., cytochrome.

$M_r$ Mu subunit was also not subject to significant variation in its level of expression. Pi class GST was not detected in any of the liver samples used in this study.

### Cytochrome *P*450 mRNA levels

The levels of mRNA encoding *P*450 and GST proteins shown in Fig. 3 were assessed by Northern blot hybridization. Extremely large variations in the levels of mRNA between samples was observed, but this did not appear to bear any relation to the level of expressed *P*450 protein (Figs. 2*a* and 2*b*). It is possible that in some cases this could be accounted for by hybridization of the cDNA probes to more than one mRNA species of the same size from a specific gene family; however, for certain enzymes (e.g. CYP2E1) this was clearly not the case. This discrepancy was also not related to the integrity of the mRNA, as in samples where the mRNA levels encoding a certain *P*450 were low, the levels of other *P*450 mRNAs were high. Although actin mRNA levels were also subject to variability, based on absorbance at 260 nm and ethidium bromide staining of the 18S and 28S RNA bands on agarose gels, this did not appear to be an artifact. It is

**Table 3. Correlations between the expression of human hepatic *P*450 isoenzymes and total *P*450 content**

The Table shows correlation of *P*450 protein bands, as detected by Western blotting, with each other and with total *P*450 levels as detailed in the Materials and methods section. Values are Spearman rank correlation coefficients. Significance of correlations: $^*P < 0.05$, $^{**}P < 0.005$, $^{***}P < 0.001$.

| | CYP2A? | CYP2A3 | CYP2B6 | CYP2C9 | CYP2C8 | CYP2C? | CYP2D6 | CYP2E1 | CYP3A3/4 | CYP4A(1) | CYP4A(2) | Reductase | Total *P*450 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| CYP1A2 | −0.02 | 0.25 | 0.36 | −0.04 | 0.06 | 0.11 | −0.09 | −0.11 | 0.13 | 0.1 | −0.2 | −0.2 | 0.14 |
| CYP2A? | | 0.1 | 0.18 | 0.32 | 0.04 | −0.27 | 0.09 | 0.59* | −0.15 | −0.02 | 0.25 | 0.25 | −0.25 |
| CYP2A6 | | | 0.83*** | 0.22 | 0.18 | 0.76** | −0.36 | 0.15 | 0.45 | 0.09 | 0.04 | 0.34 | 0.23 |
| CYP2B6 | | | | 0.23 | 0.26 | 0.65* | −0.34 | 0.05 | 0.48 | 0.16 | 0.24 | 0.07 | 0.34 |
| CYP2C9 | | | | | 0.39 | 0.13 | −0.22 | −0.17 | 0.53 | −0.17 | 0.02 | 0.04 | 0.49 |
| CYP2C8 | | | | | | 0.41 | −0.07 | −0.33 | 0.68* | −0.01 | 0.29 | 0.27 | 0.70* |
| CYP2C? | | | | | | | −0.45 | −0.33 | 0.50 | 0.17 | 0.04 | 0.15 | 0.40 |
| CYP2D6 | | | | | | | | 0.59* | −0.41 | 0.39 | 0.40 | 0.21 | −0.27 |
| CYP2E1 | | | | | | | | | −0.44 | −0.26 | 0.23 | 0.32 | −0.53 |
| CYP3A3/4 | | | | | | | | | | −0.26 | −0.07 | 0.30 | 0.92*** |
| CYP4A (1) | | | | | | | | | | | 0.86*** | 0.34 | −0.06 |
| (2) | | | | | | | | | | | | 0.38 | 0.09 |
| Reductase | | | | | | | | | | | | | 0.24 |

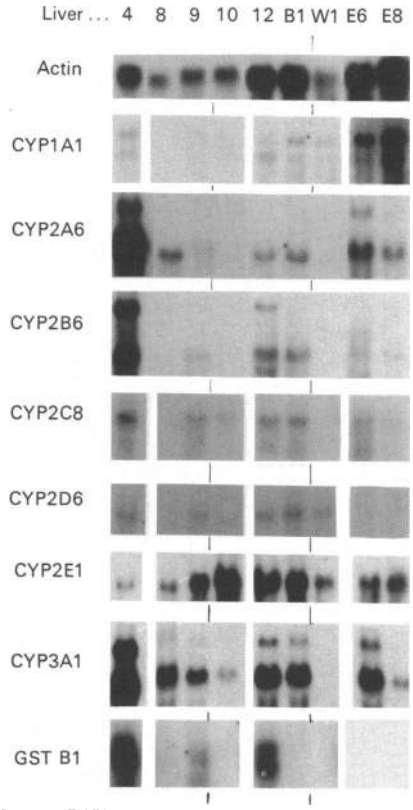


**Fig. 3. Cytochrome *P*450 mRNA levels in a panel of human liver samples**

RNA (10 $\mu$g) from 9 of the 12 human liver samples used in this study was separated on denaturing formaldehyde agarose gels, transferred to Hybond N and probed with *P*450 and GST cDNA probes as outlined in the Materials and methods section.

interesting to note that the lack of correlation between protein and mRNA levels was also observed for the Alpha-class GST (Fig. 3).

### Correlation of *P*450 content with substrate metabolism

The level of expression of the different *P*450 isoenzymes in the liver panel was compared with the rate of metabolism of various known *P*450 substrates (Tables 4 and 5). Correlations were considered significant when the probability value was less than 0.05. The level of expression of CYP1A2 protein correlated with the dealkylation of 7-ethoxyresorufin, 7-methoxyresorufin, 7-ethoxycoumarin and 7-methoxycoumarin, the 2-hydroxylation of oestradiol and the activation of the carcinogen 2-AF. CYP3A3/CYP3A4 expression was highly correlated with total *P*450 levels, as well as with the metabolism of 7-benzyloxyresorufin, testosterone 6$\beta$-hydroxylation, ethinyloestradiol 2-hydroxylation, *R*-mephenytoin 4-hydroxylation and *N*-demethylation, diazepam 3-hydroxylation, and cyclosporin *N*-demethylation. None of the protein bands identified with the CYP2C6 antibody correlated with the rate of *S*-mephenytoin 4-hydroxylation, in spite of the fact that this antibody was an effective inhibitor of this activity in human liver microsomes (Fig. 4). Testosterone 6$\beta$-hydroxylase, oestradiol 2-hydroxylase, *R*-mephenytoin 4-hydroxylase, diazepam 3-hydroxylase and *N*-demethylase, and cyclosporin hydroxylase and *N*-demethylase activities were weakly correlated with the level of CYP2C8 protein. However, ethinyloestradiol 2-hydroxylase activity was highly correlated with this protein. Somewhat surprisingly, the level of expression of CYP2C8 protein also correlated with total *P*450 and *P*450 reductase levels. The level of expression of *P*450

**Table 4. Spearman rank correlations of *P*450 activities with the metabolism of various *P*450 model substrates and steroids**

The Table shows correlation of *P*450 protein bands, as detected by Western blotting, with total *P*450, *P*450 reductase and various *P*450 substrate assays, as detailed in the Materials and methods section. Values are Spearman rank correlation coefficients. Significance of correlations: $^{*}P < 0.05$, $^{**}P < 0.005$. Abbreviations: E-, B- and M-ROD, ethoxy- benzyloxy- and methoxy-resorufin *O*-dealkylation; E- and M-COD, ethoxy- and methoxy-coumarin *O*-dealkylation; 6$\beta$-OH-T, 6$\beta$-hydroxytestosterone; A4-dione, androst-4-ene-3,17-dione; $E_2$, oestradiol; $EE_2$ ethinyloestradiol; n.d., not determined.

| *P*450 | $10^{-3} \times M_r$ | Total *P*450 | EROD | BROD | MROD | ECOD | MCOD | Testosterone 6$\beta$-OH-T | Testosterone A4dione | $E_2$-OH | AF $EE_2$-OH | AF mutagenicity | Reductase |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| CYP1A2 | 53.0 | 0.14 | 0.76** | −0.1 | 0.75** | 0.57* | 0.59* | −0.11 | −0.22 | 0.62* | 0.21 | 0.70* | 0.00 |
| CYP2A? | 54.5 | −0.26 | 0.08 | −0.31 | −0.24 | 0.27 | −0.05 | −0.14 | 0.01 | 0.21 | 0.29 | −0.18 | 0.17 |
| CYP2A6 | 51.5 | 0.23 | 0.18 | 0.16 | −0.18 | 0.21 | 0.04 | 0.29 | −0.16 | 0.54 | 0.48 | −0.33 | 0.16 |
| CYP2B6 | 51.0 | 0.34 | 0.13 | 0.47 | 0.00 | 0.23 | 0.04 | 0.32 | 0.19 | 0.50 | 0.52 | −0.12 | 0.37 |
| CYP2C9 | 54.5 | 0.49 | 0.15 | 0.18 | 0.11 | −0.04 | −0.31 | 0.37 | −0.19 | 0.37 | 0.43 | 0.26 | 0.23 |
| CYP2C8 | 52.5 | 0.70* | 0.26 | 0.55 | 0.12 | 0.11 | 0.36 | 0.69* | 0.26 | 0.58* | 0.75** | 0.42 | 0.83** |
| CYP2C? | 51.0 | 0.40 | 0.01 | 0.47 | −0.10 | 0.33 | 0.13 | 0.36 | −0.13 | 0.61* | 0.49 | −0.02 | 0.41 |
| CYP2D6 | 51.5 | −0.27 | 0.04 | −0.3 | 0.12 | −0.12 | 0.23 | 0.29 | 0.04 | −0.11 | −0.05 | −0.16 | 0.14 |
| CYP2E1 | 54.5 | −0.53 | 0.45 | −0.55 | −0.34 | 0.23 | 0.31 | −0.35 | −0.05 | −0.18 | −0.10 | −0.55 | 0.31 |
| CYP3A3/3A4 | 52.5 | 0.92** | 0.22 | 0.69* | 0.15 | 0.09 | 0.14 | 0.92** | 0.27 | 0.50 | 0.78* | 0.25 | 0.64* |
| CYP4A (1) | 51.5 | −0.06 | 0.07 | 0.15 | 0.12 | 0.27 | 0.27 | −0.38 | −0.17 | −0.09 | 0.10 | 0.02 | 0.11 |
| (2) | 50.0 | 0.09 | 0.04 | 0.31 | 0.01 | 0.09 | 0.06 | −0.09 | 0.19 | 0.31 | 0.37 | 0.06 | 0.39 |
| Reductase | nd | 0.24 | 0.24 | 0.04 | −0.18 | | | 0.30 | −0.06 | | | 0.02 | 0.36 |

**Table 5. Spearman rank correlations of *P*450 activities with the metabolism of various *P*450 drug substrates**

The Table shows correlation of *P*450 protein bands, as detected by Western blotting, with various *P*450 drug substrate assays as detailed in the Materials and methods section. Values are Spearman rank correlation coefficients. Significance of correlations: $*P < 0.05$, $**P < 0.005$. Abbreviations: *R*(4-OH) and *S*(4-OH), R- and S-mephenytoin 4-hydroxylation; *R*(*N*-deM) and *S*(*N*-deM), *R*- and *S*-mephenytoin *N*-demethylation; Tolbut., tolbutamide; *N*-ox, *N*-oxidation; nd, not determined.

| *P*450 | $10^{-3} \times M_r$ | Total *P*450 | Mephenytoin | | | | Diazepam | | Cyclosporine | | Mianserin | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | *R*(4-OH) | *R*(*N*-deM) | *S*(4-OH) | *S*(*N*-deM) | (3-OH) | (*N*-deM) | Tolbut. | (OH) | (*N*-deM) | (OH) | (*N*-deM) | (*N*-ox) | Ciamexon |
| CYP1A2 | 53.0 | 0.14 | −0.01 | −0.19 | −0.22 | 0.00 | −0.05 | 0.01 | 0.14 | 0.18 | 0.03 | 0.27 | 0.18 | 0.19 | −0.21 |
| CYP2A? | 54.5 | −0.26 | −0.03 | −0.13 | −0.25 | −0.29 | 0.07 | 0.23 | 0.40 | 0.29 | 0.18 | 0.06 | 0.19 | −0.19 | −0.03 |
| CYP2A6 | 51.5 | 0.23 | −0.21 | 0.23 | −0.41 | 0.47 | 0.42 | 0.48 | 0.40 | 0.26 | 0.41 | 0.07 | −0.46 | −0.04 | 0.46 |
| CYP2B6 | 51.0 | 0.34 | 0.12 | 0.26 | −0.69 | 0.48 | 0.56 | 0.64 | 0.39 | 0.38 | 0.48 | 0.02 | 0.40 | −0.09 | −0.14 |
| CYP2C9 | 54.5 | 0.49 | 0.58 | 0.54 | −0.07 | 0.36 | 0.25 | 0.18 | 0.19 | 0.49 | 0.46 | 0.08 | 0.05 | −0.04 | 0.04 |
| CYP2C8 | 52.5 | 0.70* | 0.68* | 0.41 | 0.05 | 0.53 | 0.60* | 0.67* | 0.38 | 0.68* | 0.68* | 0.26 | 0.42 | −0.02 | 0.25 |
| CYP2C? | 51.0 | 0.40 | 0.24 | 0.32 | −0.04 | 0.49 | 0.39 | nd | 0.54 | 0.28 | 0.38 | 0.05 | 0.44 | −0.08 | 0.24 |
| CYP2D6 | 51.5 | −0.27 | 0.19 | −0.34 | −0.13 | −0.50 | −0.11 | 0.05 | 0.02 | 0.04 | 0.02 | 0.44 | 0.04 | −0.06 | 0.12 |
| CYP2E1 | 54.5 | −0.53 | −0.35 | −0.28 | −0.40 | −0.45 | 0.16 | 0.03 | 0.15 | 0.00 | 0.12 | 0.74* | 0.30 | 0.48 | 0.30 |
| CYP3A3/A4 | 52.5 | 0.92** | 0.87** | 0.77** | −0.27 | 0.62* | 0.76** | 0.64* | 0.28 | 0.67* | 0.79** | −0.04 | 0.32 | −0.05 | 0.09 |
| CYP4A (1) | 51.5 | −0.06 | −0.15 | −0.49 | −0.2 | −0.05 | 0.03 | 0.22 | 0.49 | 0.00 | 0.05 | 0.13 | 0.12 | 0.41 | 0.00 |
| (2) | 50.0 | 0.09 | 0.04 | −0.33 | −0.34 | 0.13 | 0.55 | 0.44 | 0.06 | 0.47 | 0.47 | 0.10 | 0.33 | 0.36 | 0.10 |
| Reductase | nd | 0.24 | 0.56 | −0.02 | −0.13 | 0.04 | | | | | | | | | |

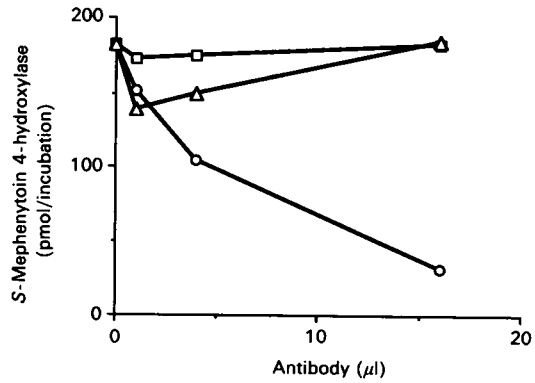


**Fig. 4. Inhibition of human liver microsomal *S*-mephenytoin 4-hydroxylation by an anti-CYP2C6 antiserum**

The 4-hydroxylation of mephenytoin was measured as detailed in the Materials and methods section. Pre-immune ((□) control), anti-CYP3A1 (△) or anti-CYP2C6 (○) serum was added in increasing amounts to a final reaction volume of 250 μl.

reductase, as assayed by immunoblotting, did not correlate with measured *P*450 reductase activity. None of the activities measured correlated with CYP2A, CYP2B6, CYP2D6, CYP4A or *P*450 reductase proteins. Mianserin hydroxylase activity correlated with CYP2E1 protein. There was no significant association between GST isoenzyme and *P*450 isoenzyme expression (results not shown).

## DISCUSSION

Individuality in cytochrome *P*450 expression will have significant pharmacological and toxicological consequences. In this study, and in agreement with our previous studies and those of others (Wang *et al.*, 1983; see Gonzalez *et al.*, 1988), we demonstrate the profound variability in the expression of *P*450 isoenzymes in human liver.

Although genetic polymorphisms play a role in determining the level of expression of specific *P*450 isoenzymes, it appears that environmental and/or hormonal factors play a predominant role in determining cellular *P*450 levels (Guengerich, 1987; Shaw *et al.*, 1989; Wolf *et al.*, 1990). Several approaches have been used to measure cytochrome *P*450 expression in human tissues. In the present study the use of two techniques, Western blotting and Northern blotting, were compared. It is interesting that in almost all cases there was no correlation between the mRNA levels and the level of protein detected by Western blot analysis. This was not due to problems with the integrity of the mRNA, and indicates that the rate of translation and protein turnover may be important in determining human hepatic *P*450 levels. There are precedents for such mechanisms, particularly for the regulation of cytochrome CYP2B1 and CYP2E1 proteins in the rat (Simmons & Kasper, 1983; Meehan *et al.*, 1988*a*; Song *et al.*, 1989).

There are now many examples in rodents where the levels of cytochrome *P*450 isoenzymes from distinct gene families or subfamilies are regulated by the same foreign compound inducing agents. This phenomenon, which to a degree represents coordinate regulation, appears to be subject to some species and strain variation, and it is therefore of central importance to identify which enzymes are regulated by the same compounds in man. It was therefore interesting that many of the cytochrome *P*450 isoenzymes in human liver which appear to be regulated together are regulated by the same inducers in animal models.

Two proteins regulated by the same inducers in animals are

CYP1A1 and CYP1A2. On Western blot analysis only one CYP1A protein could be identified, and the evidence indicates that this is CYP1A2. It has recently been shown that human CYP1A1 has a very similar mobility to CYP1A2 on SDS/acrylamide gels (McManus *et al.*, 1990), and the presence of CYP1A1 cannot be ruled out. In this regard it is interesting that most of the liver samples contained two mRNA species, of 3.3 and 2.8 kb, hybridizing to the CYP1A1 probe. These sizes are consistent with the reported sizes for CYP1A2 and CYP1A1 respectively (McManus *et al.*, 1990), and this indicates that both of these genes are transcribed in human liver. This was particularly clear in sample E8, which was from a heavy smoker where extremely high levels of these two mRNA species were detected. This indicates that the transcription of these genes is regulated by similar agents as in other animal species. It is intriguing that this high level of mRNA was not reflected in a high level of CYP1A1 or CYP1A2 protein. Furthermore, no correlation between the level of CYP1A1/CYP1A2 proteins and smoking history was apparent, although the number of smokers in the panel studied (three out of 12) is too small to draw firm conclusions on this point.

We have previously suggested that CYP2A6 and CYP2B6 may be regulated by the same compounds at a transcriptional level in human liver (Miles *et al.*, 1990). This suggestion is supported here by the close correlation in the levels of these proteins detected by Western blot analysis. In the mouse, CYP2B and CYP2A proteins are known to be both regulated by compounds such as phenobarbital (Lang *et al.*, 1989). The correlation between the level of these proteins and an unidentified member of the *CYP2C* gene subfamily, possibly CYP2C10, is also consistent with the co-regulation of certain CYP2B and CYP2C proteins in the rat by phenobarbital and in the mouse by phenobarbital and also dexamethasone (see Meehan *et al.*, 1988*a*).

A strong correlation between the level of expression of CYP2C8 and CYP3A3/3A4 was observed. The fact that the rat anti-CYP2C6 antibody used to identify CYP2C8 does not react with recombinant CYP3A3/3A4 (Fig. 1) indicates that this correlation is not due to antibody cross-reactivity. The level of CYP3A3/3A4 proteins also correlated with the total cytochrome *P*450 content of the human liver microsomes. This indicates that these proteins (CYP3A3/3A4) may be the major isoenzymes found in human liver, and to a degree may determine the total hepatic *P*450 level. It is important to note, however, that the total *P*450 level varied a great deal less (approx. 2–3 fold) than did the levels of CYP3A3/A4 and CYP2C8 (both approx. 60-fold). Thus it would appear that in many cases these proteins cannot be the predominant *P*450 present.

Another intriguing correlation was in the level of expression of two proteins in the CYP4A subfamily. Such co-ordinate regulation would be consistent with the findings in the rat (Kimura *et al.*, 1989). The correlation between the CYP2A protein ($M_r$ 54000) and CYP2E1 is also of interest, as in the mouse similarities in the regulation of proteins from these gene families have been observed (Lang *et al.*, 1989).

The expression of the cytochrome *P*450 isoenzymes did not correlate with the expression of any of the GSTs detected. Indeed, the variability in the levels of these enzymes was much less than for the *P*450s. This is interesting in view of the finding that in extrahepatic tissues the expression of Alpha and Mu, and to a lesser degree Pi, class GSTs is subject to large individual differences (Carmichael *et al.*, 1988; Moscow *et al.*, 1988; Howie *et al.*, 1990).

Comparisons of the relative levels of cytochrome *P*450 isoenzymes in a panel of human livers with activity towards model substrates and drugs *in vitro* is essential in order to define the therapeutic and toxicological consequences of the inter-individual variation in the expression of these proteins in the liver. This approach has been adopted by a number of groups (Beaune *et al.*, 1986; Shimada *et al.*, 1986; Waxman *et al.*, 1988; Gonzalez *et al.*, 1988; Hall *et al.*, 1989; Ball *et al.*, 1990; Forrester *et al.*, 1990; De Waziers *et al.*, 1990), and the initial observations were investigated further by the use of *P*450 proteins and antibody inhibition. In this study we have found that the metabolism of certain substrates shows a significant correlation with the level of particular cytochrome *P*450 isoenzymes, but that for other substrates no such correlation was observed, despite the fact that these latter substrates were metabolized effectively by all of the individual livers. It is clear from studies both *in vitro* and *in vivo* that particular *P*450 isoenzymes catalyse the metabolism of a range of substrates, and that certain substrates are metabolized by a number of individual isoenzymes. In the present study we have not carried out a detailed kinetic analysis, and it is feasible that the isoenzyme which plays the predominant role in the metabolism of a substrate will depend on relative $K_m$ and $V_{max.}$ values, i.e. on substrate concentration. The *P*450 isoenzyme involved in the metabolism of a particular substrate is exemplified by two extreme situations, i.e. a substrate which is specific for a particular isoenzyme, and a so-called promiscuous substrate, one that is a possible substrate for a number of isoenzymes. The most striking examples of the former are those drugs whose metabolism *in vivo* displays polymorphism in the human population, such as debrisoquine and mephenytoin.

The expression of CYP2D6 has been shown to be genetically determined (Eichelbaum & Gross, 1990). In individuals who express this form there is considerable variation (Gonzalez *et al.*, 1988); in the present study it was 16-fold. However, there was no correlation between the content of this protein and the metabolism of any of the substrates investigated. Furthermore, the one liver deficient in this enzyme (liver 7) was able to metabolize all substrates. This is in agreement with previous work showing that CYP2D6 does not metabolize any of the substrates used in the present study.

In normal extensive metabolizers, *S*-mephenytoin is predominantly metabolized by 4-hydroxylation, before glucuronidation and rapid excretion in urine. The *R*-enantiomer is preferentially *N*-demethylated and more slowly excreted as the pharmacologically active nirvanol. The drug is usually administered as a 1:1 racemic mixture of *R*- and *S*-enantiomers, and in poor metabolizers it appears that the enzyme responsible for *S*-mephenytoin 4-hydroxylation is either absent or defective (Meier *et al.*, 1985; Meier & Meyer, 1987). This polymorphism is associated with the *CYP2C* gene subfamily; however, the isoenzyme in this gene subfamily responsible for the activity remains equivocal (Guengerich *et al.*, 1990; Relling *et al.*, 1990). In agreement with the findings of these workers, using recombinant *P*450s, the rate of *S*-mephenytoin 4-hydroxylation did not correlate with the level of any of three *P*450s in the CYP2C subfamily identified in the present study, in spite of the fact that the antibody used for the Western blot analysis was an inhibitor of *S*-mephenytoin 4-hydroxylation. Interestingly, the *R*- and *S*-mephenytoin *N*-demethylase and *R*-mephenytoin 4-hydroxylase (but not *S*-mephenytoin 4-hydroxylase) activities correlated with CYP3A3/3A4 expression.

Certain model substrates have been proposed as specific markers for particular *P*450 isoenzymes. Consistent with previous studies, the rates of dealkylation of ethoxycoumarin, methoxycoumarin, ethoxyresorufin and methoxyresorufin correlated with levels of CYP1A2, in agreement with the antibody inhibition study of Burke *et al.* (1985). The activation of aromatic amines, such as 2-AF, has also been shown to be catalysed by CYP1A2 (McManus *et al.*, 1990).

The 6$\beta$-hydroxylation of testosterone and the 2-hydroxylation

of ethinyloestradiol have been shown to be catalysed by CYP3A3/3A4 (Kawano *et al.*, 1987; Waxman *et al.*, 1988; Guengerich, 1988*b*), and showed a good correlation with the content of these isoenzymes in the present study. Oestradiol 2-hydroxylation appeared to be catalysed by several isoenzymes, in keeping with studies using individually expressed isoenzymes (Aoyoma *et al.*, 1990).

Finally, turning to the metabolism of therapeutic agents, significant correlations were found between certain biotransformations and certain isoenzymes, but not for others. Thus the hydroxylation and demethylation of diazepam, the hydroxylation and demethylation of cyclosporin and the hydroxylation of mianserin showed correlations with specific isoenzyme levels. The evidence for the involvement of CYP3A3/3A4 in cyclosporine and diazepam metabolism is consistent with previous findings (Kronbach *et al.*, 1988; Reilly *et al.*, 1990). In contrast, for tolbutamide and ciamexone there was no such correlation. The rate of turnover of the latter two substrates suggests that the lack of correlation may reflect variable contributions of more than one enzyme to the overall rate of reaction observed. This is particularly relevant to tolbutamide, which has been shown to be oxidized by recombinant CYP2C proteins (Relling *et al.*, 1990), but is probably also metabolized both *in vivo* and *in vitro* by other *P*450 isoenzymes. This in fact is a major limitation of the correlation approach to the identification of the *P*450s that are actually responsible for a particular biotransformation *in vivo*. Balanced against this is the fact that just because an isolated *P*450 can catalyse a reaction in an *in vitro* system, this does not prove that this is of significance *in vivo*.

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