METABOLIC CHARACTERIZATION OF THE MAJOR HUMAN SMALL INTESTINAL CYTOCHROME P450S

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ABSTRACT:

Human small intestine epithelial cells (enterocytes) provide the first site for cytochrome P450 (CYP)-catalyzed metabolism of orally ingested xenobiotics. CYP3A4 is the major form of CYP expressed in enterocytes and CYP2C is also expressed at a significant level. In this study, we further characterized the expression of CYP3A4 and CYP2C in human enterocytes and their interindividual variations by examining the metabolic activities from 10 individuals. CYP3A4 in human jejunum microsomes, as determined by 6β-testosterone hydroxylase activity, varied from 0.36 to 2.46 nmol/min/mg. The apparent average $K_m$ and $V_{max}$ values from two representative individuals were 54 $\mu$M and 3.2 nmol/min/mg, respectively. CYP2C9 and CYP2C19 in human jejunum microsomes, as determined by diclofenac 4'-hydroxylase and mephénytoin 4'-hydroxylase activities, varied over an 18-fold range (7.3–129 pmol/min/mg) and 17-fold range (0.8–13.1 pmol/min/mg), respectively. The mean apparent $K_m$ for diclofenac 4’-hydroxylase was 9.9 $\mu$M, whereas the apparent mean $K_m$ for S-mephénytoin 4’-hydroxylase was 79.3 $\mu$M. The mean intrinsic clearance ($V_{max}/K_m$) was approximately 130-fold greater for diclofenac 4’-hydroxylase than for mephénytoin 4’-hydroxylase. The metabolic activities of CYP2C9 and CYP2C19 were confirmed by inhibition with sulfaphenazole for CYP2C9 and ticlopidine for CYP2C19. In addition, CYP2C9 activities did not correlate with CYP3A4 activities, while CYP2C19 activities had a significant but poor correlation with those of CYP3A4. Thus the major CYP activities in human enterocytes have large interindividual variabilities that are not strongly related.

There is a growing recognition that human small intestinal metabolism of orally administered xenobiotics, including therapeutic drugs, can play a significant role in overall first-pass metabolism (Hebert, 1997). This recognition has fostered a number of investigations to determine which phase I and phase II metabolic enzymes are expressed in the human small intestine, with emphasis on cytochrome P450s (CYPs). This emphasis reflects the overall predominant role of CYPs in xenobiotic metabolism.

The importance of the small intestinal CYPs arises from their location in the epithelial cells (enterocytes) of this organ, which provide the first site of metabolism for orally administered drugs. Additionally, the enterocytes perform presystemic metabolism, which can result in diminished systemic uptake of drugs (Watkins, 1997). Several studies have added support for the importance of the enterocyte CYPs in pharmacology. Thus, midazolam is metabolized to a major extent in the small intestine as assessed by varying its route of administration from oral to i.v. and inhibition of its metabolism by ketoconazole, a preferential inhibitor of small intestinal CYP metabolism (Gibbs et al., 1999). The opioid, fentanyl, also undergoes substantial small intestinal metabolism relative to that in the liver, while 50% of orally administered cyclosporin A is metabolized in the small intestine (Hebert, 1997). Reports that grapefruit juice inhibits the function of the major small intestine CYP but not the hepatic enzyme (Lown et al., 1997) provide a powerful tool for assessing the extent of CYP3A4 substrate drug metabolism in the small intestine (Bailey et al., 1998). A substantial increase in blood levels of a drug when administered after or with intake of grapefruit juice implies that the drug is substantially metabolized in the small intestine; examples are dihydropyridines, terfenadine, saquinavir, cyclosporin, midazolam, triazolam, and verapamil, and also possibly lovastatin, cisapride, and astemizole (Bailey et al., 1998).

Recent studies have identified several of the complement of human small intestinal CYPs. The predominant form is clearly CYP3A4, which is inducible by rifampin (Kolars et al., 1992). We reported that CYP2C was the second most highly expressed CYP protein subfamily in enterocytes but did not resolve which forms were expressed (Zhang et al., 1999). In contrast, mRNAs of other CYP2 family members, CYP2A6, CYP2A7, and CYP2A13, were not detected by reverse transcriptase-polymerase chain reaction in human duodenum (Koskela et al., 1999). Thus it is clear that the complement of CYP proteins expressed in the human small intestine is limited relative to that expressed in the liver.

To gain a better understanding of the metabolic capability of the human small intestine and the capacity of presystemic metabolism to affect the fate of orally administered drugs, we have metabolically characterized the major CYPs expressed in the human small intestine and assessed the interindividual variability in the CYP activities.
Materials. Anti-rat CYP2C6 serum, S-mephenytoin, 4'-hydroxymephentoin, and sulfaphenazole were purchased from GENTEST Corp. (Woburn, MA). Peroxidase-conjugated rabbit anti-goat IgG, testosterone, 6β-hydroxytestosterone, 11β-hydroxytestosterone, diclofenac, ticlopidine, and NADPH were from Sigma Chemical Co. (St. Louis, MO). 4'-Hydroxycyclofenac was synthesized according to a published method (Moser et al., 1990). The bichinoninic acid protein assay kit was from Pierce Chemical Co. (Rockford, IL), and the enhanced chemiluminescence kit was from Amersham Pharmacia Biotech (Arlington Heights, IL). Solvents and other reagents were obtained from common commercial sources.

Human Enterocyte Microsomes. Human small intestines were obtained from the International Institute for the Advancement of Medicine (Scranton, PA) through the Organ Procurement Organization. Details of intestine collection, intestine treatment, isolation of human intestinal epithelial cells, and preparation of microsomes were as described previously (Zhang et al., 1999). The details of the donors are provided in Table 1.

Immuno blot Analysis. Microsomal proteins were separated by SDS-polyacrylamide gel electrophoresis as previously described (Laemmli, 1970) in 10% polyacrylamide gels. Proteins were electrophoretically transferred to nitrocellulose sheets (Towbin et al., 1979), which were then treated with 5% nonfat dry milk in TBST for 1 h at room temperature, incubated with a polyclonal antibody to rat CYP2C6 in TBST containing 2.5% milk for an overnight incubation. Enzyme kinetics were determined over a diclofenac substrate concentration of 1.0 to 100 μM. All other activity measurements were conducted at a substrate concentration of 5.0 μM. In some studies the inhibitor sulfaphenazole was added to the reaction together with the substrate.

Dioclofenac 4'-Hydroxylase Assay. Diclofenac 4'-hydroxylase activity was measured using a previously described HPLC-mass spectrometry method (Obach, 2000) adapted for human intestinal microsomes. After a preliminary assessment of linearity of product formation over time, a microsomal protein concentration and incubation time of 0.6 mg/ml and 20 min were used, respectively, for all subsequent incubations. Enzyme kinetics were determined over a diclofenac substrate concentration range of 1.0 to 100 μM. All other activity measurements were conducted at a substrate concentration of 50 μM. In some studies the inhibitor ticlopidine was added to the reaction together with the substrate.

S-Mephenytoin 4'-Hydroxylase Assay. S-Mephenytoin 4'-hydroxylase activity was measured using a previously described HPLC-mass spectrometry method (Obach, 2000) adapted for human intestinal microsomes. A preliminary assessment of linearity of product formation over time established the suitability of a microsomal protein concentration and incubation time of 3.0 mg/ml and 20 min, respectively, for all subsequent incubations. Enzyme kinetics were determined over an S-mephenytoin substrate concentration range of 5.0 to 500 μM. All other activity measurements were conducted at a substrate concentration of 50 μM. In some studies the inhibitor ticlopidine was added to the reaction together with the substrate.

Testosterone 6β-Hydroxylase Assay. Testosterone 6β-hydroxylase activity in human small intestinal microsomes and HPLC analysis for its metabolites were performed essentially as described previously (Ding and Coon, 1994; Baltes et al., 1998), with some modifications. In brief, testosterone (100 μM) was incubated for up to 30 min at 37°C in a total reaction volume of 500 μl containing 50 mM potassium phosphate buffer, pH 7.4, 1 mM NADPH, and 50 μg of microsomal protein. The reaction was initiated by the addition of NADPH and terminated by the addition of 2 ml of ethyl acetate. The internal standard was 1 nmol of 11β-hydroxytestosterone. Control experiments were performed in which NADPH was omitted. The sample was vortex-mixed for 1 min and centrifuged at 1500g for 5 min. After removal of the organic layer, the remaining aqueous solution was extracted with an additional 2 ml of ethyl acetate. The pooled extract was evaporated under nitrogen. The residue was reconstituted with 100 μl of methanol, and 50-μl aliquots were analyzed by HPLC. The metabolite formation was linear over the incubation time.

For the HPLC analysis of testosterone metabolites, a Waters Nova-Pak C18 column (8 × 100 mm), preceded by a C18 precolumn cartridge, was used for all assays, in conjunction with a Waters HPLC system consisting of a model 600E controlling unit, a model 710 solvent delivery system, a model 712 satellite sample injector, a model 996 photodiode array detector, and the Millennium 2010 software package. Eluting solvents comprised solvent A (50% acetonitrile) and solvent B (50% acetonitrile, 50% water) and solvent B (90% methanol, 10% acetonitrile). A 15-min linear gradient from 100% A to 65% A was applied at a flow rate of 2 ml/min followed by an additional 5 min at 65% A. Testosterone metabolites were identified by comigration with authentic standards and quantified using peak areas at 236 nm.

Other Methods and Materials. Spectral determination of total CYP was performed according to published procedures (Omura and Sato, 1964). Protein concentrations were determined using bichinoninic acid reagent (Pierce) with bovine serum albumin as the standard.

Data Analysis. Enzyme kinetic analysis was performed using SigmaPlot software (SPSS Inc., Chicago, IL). Data were initially transformed and plotted on Eadie-Hofstee plots to assess the potential for atypical versus typical Michaelis-Menten kinetics. Enzyme kinetic parameters were subsequently determined by fitting the reaction velocity versus substrate concentration data to the Michaelis-Menten equation. Correlation coefficients were determined by Pearson Moment and Spearman rank order correlations using SigmaStat Software (SPSS, Inc.).

Results

The testosterone 6β-hydroxylation rates for 10 representative human jejunal microsomal preparations are shown in Table 2. They varied over approximately a 7-fold (0.36-2.46 nmol/min/mg) range with a mean ± S.D. of 1.40 ± 0.73 nmol/min/mg. The kinetics of testosterone 6β-hydroxylation were also examined for two human jejunal microsomal preparations with concentrations of testosterone varying between 10 and 100 μM (Fig. 1). The apparent Km values were 53 and 55 μM, respectively, and Vmax values were 3.7 and 2.7 nmol/min/mg, respectively (Table 3).

The CYP2C subfamily is the second most highly expressed CYP subfamily in human small intestine, presumably constitutively. Figure 2 shows the CYP2C immunoblot analysis data for the same 10

### Table 1

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<tr>
<th>Subject</th>
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<th>Age</th>
<th>Ethnic Status</th>
<th>Cause of Death</th>
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<td>47</td>
<td>C</td>
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<td>27</td>
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<td>4</td>
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<td>Head trauma</td>
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<td>Cerebral vascular accident</td>
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<tr>
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<td>37</td>
<td>A</td>
<td>Intracranial hemorrhage</td>
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C. Caucasian, A. Asian.

### Table 2

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<tr>
<th>Subject</th>
<th>Diclofenac 4'-Hydroxylase</th>
<th>Mephenytoin 4'-Hydroxylase</th>
<th>Testosterone 6β-Hydroxylase</th>
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<td>pmol/min/mg</td>
<td>pmol/min/mg</td>
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</tr>
<tr>
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<td>2.27 ± 0.06</td>
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<tr>
<td>7</td>
<td>53.5 ± 2.8</td>
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<tr>
<td>8</td>
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<td>1.22 ± 0.13</td>
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<tr>
<td>9</td>
<td>56.5 ± 1.8</td>
<td>5.66 ± 0.11</td>
<td>0.79 ± 0.12</td>
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<tr>
<td>10</td>
<td>66.7 ± 1.5</td>
<td>1.81 ± 0.13</td>
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individuals as those featured in Table 2. The expression of CYP2C protein was detected in all samples examined but at varying levels. Since the antibody used cannot distinguish members of the CYP2C subfamily, specific substrate probes were used to characterize small intestinal CYP2C9 and CYP2C19 in the same 10 individuals. Table 2 shows the rates for diclofenac 4'-hydroxylase (a specific substrate for CYP2C9), and mephenytoin 4'-hydroxylase (a specific substrate for CYP2C19). CYP2C9 and -2C19 activities varied over an 18-fold

Fig. 1. Substrate saturation and Eadie-Hofstee plots of diclofenac 4'-hydroxylase, S-mephenytoin 4'-hydroxylase, and testosterone 6β-hydroxylase activities of representative human small intestinal microsomal preparations.

Data points represent the mean and standard deviations of triplicate analyses. Analytical methods are described under Experimental Procedures. Diclofenac concentrations were varied from 1.00 to 100 μM, S-mephenytoin concentrations from 5.0 to 500 μM, and testosterone concentrations from 10 to 100 μM.
mephenytoin 4'-hydroxylase activities were markedly higher, 48 to 96-fold (Bio-Rad, Hercules, CA) are indicated.

Enzyme kinetics for diclofenac 4'-hydroxylase and S-mephenytoin 4'-hydroxylase activities were determined in human intestinal microsome samples from four individual donors. In all cases, the linearity of the Eadie-Hofstee plots (Fig. 1) suggested simple Michaelis-Menten kinetic behavior. The kinetic parameters are listed in Table 3. Diclofenac 4'-hydroxylase activity, Michaelis-Menten constants ranged from 5.7 to 13.9 μM, and the corresponding values for mephenytoin 4'-hydroxylase activities were markedly higher, 48 to 107 μM. Compared with diclofenac 4'-hydroxylase activity, S-mephenytoin 4'-hydroxylase activities were substantially lower, as evidenced by both a greater Km and a lower Vmax. Overall mean intrinsic clearance (Vmax/Km) was approximately 130-fold greater for diclofenac 4'-hydroxylase than mephenytoin 4'-hydroxylase activities.

CYP enzyme-specific inhibitors were used to confirm that diclofenac 4'-hydroxylase and S-mephenytoin 4'-hydroxylase activities in human small intestine are attributable to CYP2C9 and CYP2C19, respectively. Diclofenac 4'-hydroxylase activity was inhibited by the CYP2C9-specific inhibitor sulfaphenazole (Miners et al., 1988; Hickman et al., 1998), with an IC50 value of 0.52 μM (Fig. 3A). Mephenytoin 4'-hydroxylase activity was inhibited by ticlopidine, an inhibitor of CYP2C19 and CYP2D6 (Donahue et al., 1997; Mankowski, 1999), with an IC50 value of 2.3 μM (Fig. 3B). The inhibition curves projected values for the maximal inhibition of these activities of 98 and 94% for sulfaphenazole and ticlopidine, respectively.

To determine whether the interindividual variability in CYP2C9, -2C19, and -3A4 activities was related, a series of correlations were derived. Diclofenac and S-mephenytoin hydroxylase activities did not correlate with each other in this panel of intestinal microsomes (r = 0.57; Fig. 4C), supporting the conclusion that at least two different enzymes were responsible for catalysis of these reactions and that the interindividual variations in CYP2C19 and CYP2C9 were independent. Interestingly, CYP2C9 activities did not correlate with those of CYP3A4 (r = 0.46, p = 0.18; Fig. 4B), while CYP2C19 activities had a significant correlation with CYP3A4 activities (r = 0.65, p < 0.05; Fig. 4A). Nonparametric correlations yielded essentially the same results.

**Discussion**

Previous studies from one of our laboratories (Zhang et al., 1999) revealed that CYP3A4 and the CYP2C subfamily are the members of the CYP superfamily most prominently expressed in enterocytes of the human small intestine. This determination was based on quantitative immunoblot analyses, which also revealed considerable interindividual differences in the expression of these enzymes.
though the bands were not clearly resolved and did not migrate identically with that of cDNA-expressed CYP3A5 (Gibbs et al., 1999). In our study only CYP1A1, CYP2C, and CYP3A4 protein were detected by immunoblot analysis in enterocytes, whereas CYP1B1, CYP2D6, CYP2E1, and CYP3A5 were not detected (Zhang et al., 1999). Enterocyte CYP1A1 was previously detected and was inducible by omeprazole and smoking (Buchthal et al., 1995). Very low levels of CYP2D6 have been reported in human jejunum, but the associated first pass metabolite metabolism was estimated to be only 1.8% that of the liver (Madani et al., 1999). The current studies extend our previous results and were undertaken to determine in human enterocytes which forms of the CYP2C subfamily are expressed as functional enzymes, the interindividual variability of these forms, the interindividual variability of functional CYP3A4, and the relationships of the various enterocyte CYP activities to one another.

Identification of the specific forms of CYP2C expressed in human enterocytes is essential for evaluating the roles of enterocyte CYP in pharmacogenetics, since some of the CYP2C forms are known to be polymorphic; of the four identified human CYP2Cs only CYP2C8 has no reported polymorphic forms. Thus CYP2C9*2 and CYP2C9*3 exhibit diminished activity relative to the wild-type CYP2C9*1 (Cre-spi and Miller, 1997; Takanashi et al., 2000), and nine gene products of polymorphic forms of CYP2C19 are without activity (Ferguson et al., 1998; Ibeanu et al., 1999). Two linked mutations in the 5'-flanking region of CYP2C18 were first reported in 1996 (Iwahashi et al., 1996), and subsequently a T204 → A substitution in exon 2 was reported (Kubota et al., 1998).

We have concentrated our efforts in characterizing the functional CYP2C forms in enterocytes on two of the four known forms, CYP2C9 and CYP2C19. CYP2C9 has been identified as being of major importance in drug metabolism (Miners and Birkett, 1998), while CYP2C9 and CYP2C19 are the major forms of CYP2C expressed in human liver (Venkatakrishnan et al., 1998). In contrast CYP2C18 has very few known substrates (Minolletti et al., 1999).

The choices of diclofenac 4'-hydroxylase to identify CYP2C9 activity, S-mephentoin 4'-hydroxylase to identify CYP2C19 activity, and testosterone 6β-hydroxylase to identify CYP3A4A activity were based on published reports of their specificities (Yamazaki and Shimada, 1997; Lasker et al., 1998; Tang et al., 1999). The linearity of the Eadie-Hofstee plots of these activities in the enterocyte microsomal preparations suggests that primarily single enzymes contribute to each of the activities. Our mean $K_m$ and $V_{max}$ values for enterocyte microsomal S-mephyton 4'-hydroxylase activity of 79.3 μM and 10.9 pmol/min/mg show differences from published values in human liver microsomes of 51 μM ($K_m$) and 85 pmol/min/mg ($V_{max}$) (Coller et al., 1999), 42 μM and 159 pmol/min/mg (Venkatakrishnan et al., 1998), and 31.4 μM and 150 pmol/min/mg (Schmider et al., 1996). While our enterocyte $K_m$ values are similar to published values in hepatic microsomes, the $V_{max}$ values are markedly lower. Similarly, our $K_m$ value for enterocyte diclofenac 4'-hydroxylase of 9.9 μM is comparable with published values for human liver microsomes of 3.4 μM (Transon et al., 1996) and 9 μM (Bort et al., 1999), while our $V_{max}$ value of 179 pmol/min/mg is lower than the published values in hepatic microsomes of 750 pmol/min/mg (Transon et al., 1996) and 432 pmol/min/mg (Bort et al., 1999). Finally, our values for enterocyte testosterone 6β-hydroxylase activity of 54 μM ($K_m$) and 3.2 nmol/min/mg ($V_{max}$) are comparable with the corresponding published values for hepatic microsomes of 50 to 60 μM ($K_m$) and 4.5 to 4.9 nmol/min/mg ($V_{max}$) (Draper et al., 1998). These kinetic data add further support to our previous conclusion that CYP3A4 and not CYP3A5, which has a corresponding $K_m > 400$ μM (Waxman et al., 1991), is expressed in human small intestine. The low values reported

![Graph A](image1.png)

![Graph B](image2.png)

![Graph C](image3.png)

**Fig. 4.** Correlations of diclofenac 4'-hydroxylase, S-mephyton 4'-hydroxylase, and testosterone 6β-hydroxylase activities in human small intestinal microsomal preparations from a panel of 10 human donors.

Data points represent mean and standard deviations of triplicate analyses. Other conditions are as in Fig. 3. A, $r = 0.65, p < 0.05$; B, $r = 0.46, p = 0.18$; C, $r = 0.57, p = 0.09$.

Variability in expressed protein levels of these CYPs. As part of our comprehensive assessment of CYP protein and mRNA expression in human enterocytes, CYP3A4 but not CYP3A5 protein was detected by immunoblot assays in a sample of 10 human small intestines (Zhang et al., 1999). This was confirmed in a sample of 33 small intestines (unpublished data). CYP3A5 was, however, reported to be expressed in two of three intestinal preparations examined by immunoblot analysis, al-
here for enterocyte microsomal CYP2C 
Vmax values relative to hepatic microsomal values are consistent with previously reported discrepancies with other CYPs. Thus a comparison of the kinetics of human liver and small intestinal CYP2D6-catalyzed microsomal metabolism of metoprolol revealed that the Vmax in the small intestine was less than 7% that of the liver despite similar Km values of 44 and 26 μM, respectively (Madani et al., 1999).

There is no obvious reason for the lower Vmax values of CYP2C and CYP2D6 activities in small intestine versus liver. One possibility is a lower concentration of the CYP and reductase proteins in the membranes of the small intestine relative to those of the liver. In light of these Vmax differences for CYP2C19 and CYP2C9 activities, we confirmed that the enterocyte 5-mephentoin 4-hydroxylase and diclofenac 4-hydroxylase activities were reflective of CYP2C19 and CYP2C9 activities, respectively, by use of the specific inhibitors sulfaphenazole (Hickman et al., 1998) and ticlopidine (Ko et al., 2000).

The use of 10 intestinal microsomal preparations in this study provides insight into the interindividual variability of CYP3A4, CYP2C9, and CYP2C19 activities in the small intestine. All of the activities exhibited considerable interindividual variability, but the ranges reported are only reflective of the populations selected and are considered considerably broader in a larger population for CYP3A4 (unpublished results) and probably for the CYP2Cs. In this study we did not genotype the donor population and are thus unable to assess the role of polymorphisms in the variability of CYP3A4, CYP2C9, and CYP2C19 expression in the small intestine. In the case of CYP3A4, however, the only polymorphism known to influence the activity of the gene product (Sata et al., 2000) could not explain the enterocyte interindividual variability of this enzyme’s activity.

The absence of any significant correlation between the individual variabilities of CYP2C9 and CYP2C19 and between CYP2C9 and CYP3A4 suggests that these activities varied independently. While the correlation between CYP3A4 and CYP2C19 activities was in fact statistically significant, the extent of the correlation was not high. It is thus apparent that the interindividual variabilities of the enterocyte CYP activities are not a consequence of the tissue isolation and microsomal preparation, which would be expected to influence all CYP activities to comparable extents. Regulatory variations, and possibly dietary or therapeutic inducers, must be major contributors to the interindividual activity variations.

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References


