

AUTOMATED DEFINITION OF THE ENZYMOLOGY OF DRUG OXIDATION BY THE MAJOR HUMAN DRUG METABOLIZING CYTOCHROME P450s

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ABSTRACT:

A fully automated assay to determine the enzymology of drug oxidation by the major human hepatic cytochrome P450s (CYPs; CYP1A2, -2C9, -2C19, -2D6, and -3A4) coexpressed functionally in *Escherichia coli* with human NADPH-P450 reductase has been developed and validated. Ten prototypic substrates were chosen for which clearance was primarily CYP-dependent, and the activities of these five major CYPs were represented. A range of intrinsic clearance (CL_{int}) values were obtained for substrates in both pooled human liver microsomes (HLM; $1\text{--}380 \mu\text{l} \cdot \text{min}^{-1}\text{mg}^{-1}$) and recombinant CYPs ($0.03\text{--}7 \mu\text{l} \cdot \text{min}^{-1}\text{pmol}^{-1}$) and thus the percentage contribution of individual CYPs toward their oxidative metabolism could be estimated. All the assignments were consistent with the available literature data. Tolbutamide was metabolized by CYP2C9 (70%) and CYP2C19 (30%), diazepam by CYP2C19 (100%),

ibuprofen by CYP2C9 (90%) and CYP2C19 (10%), and omeprazole by CYP2C19 (68%) and CYP3A4 (32%). Metoprolol and dextromethorphan were primarily CYP2D6 substrates and propranolol was metabolized by CYP2D6 (59%), CYP1A2 (26%), and CYP2C19 (15%). Diltiazem, testosterone, and verapamil were metabolized predominantly by CYP3A4. In addition, the metabolite profile for the CYP-dependent clearance of several markers determined by mass spectroscopy was as predicted from the literature. There was a good correlation between the sum of individual CYP CL_{int} and HLM CL_{int} ($r^2 = 0.8$, $P < .001$) for the substrates indicating that recombinant CYPs may be used to predict HLM CL_{int} data. This report demonstrates that recombinant human CYPs may be useful as an approach for the prediction of the enzymology of human CYP metabolism early in the drug discovery process.

Much interest is currently focused on the early identification of the drug-metabolizing enzymes responsible for the biotransformations commonly encountered in drug development (Becquemont et al., 1998). Such information may help identify the key organs for clearance and explain or even predict the observed variability in pharmacokinetics with some substrates and prioritize drug-drug interaction studies. Because most (~60%) marketed compounds are cleared metabolically by cytochrome P450 (CYP)² enzymes, the major activity in this area has focused on this family of enzymes (Bertz and Granneman, 1997).

Traditionally, human liver microsomes (HLM) have been the *in vitro* tool for these studies and have provided both qualitative, e.g., identifying which CYP isoform(s) metabolize the compound of interest (Pichard et al., 1990; Andersson et al., 1993; Otton et al., 1990; Jacqz-Aigrain et al., 1993; Doecke et al., 1991; Wester et al., 2000; Yasumori et al., 1993; Kroemer et al., 1993) and quantitative information, e.g., predicted CL_{int} (Houston, 1994; Rodrigues, 1994; Carlile et al., 1999). Identifying the enzymology of metabolism by human CYPs has proved somewhat labor- and time-intensive, requiring com-

parative kinetics across a bank of characterized HLM, chemical, and/or antibody inhibition followed by the use of recombinant CYP isoforms (Rodrigues, 1999). The routine access to recombinant CYPs has facilitated direct identification of the isoform(s) responsible for the oxidative metabolism of the drug of interest, although their use *in vitro* has generally been to support HLM data (Aoyama et al., 1990; Tassaneeyakul et al., 1992; Kroemer et al., 1993; Rodrigues et al., 1994; Yamazaki et al., 1997; Von Moltke et al., 1998; Rodrigues, 1999).

With the advent of combinatorial chemistry and parallel synthesis techniques, there is an expectation to achieve both higher throughput and faster turnaround times in many biological assays. There is an increasing emphasis within drug metabolism in the pharmaceutical industry to develop enhanced throughput frontline *in vitro* models, including those to determine both the extent and route of the metabolism of new chemical entities (NCEs) and to screen for inducers and inhibitors of drug-metabolizing enzymes (Ayrton et al., 1998; Moody et al., 1999).

The ability to predict directly the human enzymology using enhanced throughput methods would represent a major breakthrough in this technology (Becquemont et al., 1998; Roy et al., 1999) in a similar manner to that adopted for CYP inhibition assays (Crespi et al., 1998; Moody et al., 1999). This laboratory has demonstrated that the five major human hepatic CYPs expressed in *Escherichia coli* (CYP1A2, -2C9, -2C19, -2D6, and -3A4) are faithful surrogates for their human liver counterparts with respect to their kinetic profiles and inhibition properties (McGinnity et al., 1999; Moody et al., 1999). In this study, the application of recombinant enzymes as a first line

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² Abbreviations used are: CYP, cytochrome P450; HLM, human liver microsomes; CL_{int} , intrinsic clearance; NCE, new chemical entity; RSP, robotic sample processor.

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TABLE 1
CYP isoform characterization of individual HLM pools

Isoform	Probe	Batch 1	Batch 2	Batch 3
			<i>pmol/min/mg</i>	
P450 content	CO difference spectrum	320 ^a	280 ^a	430 ^a
P450 reductase	Cytochrome <i>c</i> reduction	69000	84000	75000
CYP	7-Ethoxycoumarin <i>O</i> -demethylation	255	210	240
CYP1A2	Phenacetin <i>O</i> -deethylation	366	310	193
CYP2A6	Coumarin 7-hydroxylation	17200	81900	58600
CYP2E1	Chlorzoxazone 6-hydroxylation	1010	1911	1441
CYP2C	Mephenytoin 4-hydroxylation	27	233	11
CYP2D6	Dextromethorphan <i>O</i> -demethylation	200	84	106
CYP3A	Testosterone 6 β -hydroxylation	2600	1500	1900
CYP4A	Lauric acid 11-hydroxylation	1000	1600	1600

^a Picomoles of CYP per milligram of microsomal protein.

approach for identifying the CYP(s) responsible for metabolizing NCEs has been proposed. A fully automated assay has been developed using the major drug-metabolizing human hepatic cytochrome P450s (CYP1A2, -2C9, -2C19, -2D6, and -3A4) coexpressed functionally in *E. coli* with human NADPH-P450 reductase, to predict the CYP isoform(s) involved in the oxidative metabolism of NCEs.

Materials and Methods

Chemicals. All chemicals and reagents used were of the highest available commercial grade. Diltiazem, testosterone, dextromethorphan, (\pm)-propranolol, (\pm)-metoprolol, diazepam, tolbutamide, ibuprofen, and β -nicotinamide adenine dinucleotide phosphate, reduced form (β -NADPH) were purchased from Sigma Chemical Co. (Poole, UK). (\pm)-Verapamil was purchased from Aldrich Chemical Co. Ltd. (Gillingham, UK). Omeprazole was synthesized at AstraZeneca R&D Charnwood (Loughborough, UK).

Source of Cytochrome P450. The LINK consortium, a collaboration between UK-based academia and industry, provided stocks of transformed cells with human CYP1A2, CYP2C9, CYP2D6, and CYP3A4 individually coexpressed with human NADPH-P450 reductase in *E. coli* as described previously (McGinnity et al., 1999). All experiments with CYP1A2, CYP2C9, CYP2D6, and CYP3A4 utilized the *E. coli* membrane source. All transformed cells were stored as glycerol stocks at -80°C . Expression of the recombinant proteins and preparation of the respective *E. coli* membranes were carried out as described previously (McGinnity et al., 1999).

Microsomes prepared from insect cells infected with a baculovirus containing the cDNA for human CYP2C19 and rabbit NADPH-P450 reductase were purchased from PanVera Corp. (Madison, WI). All experiments with CYP2C19 utilized this enzyme source. Pooled HLM were purchased from ILM (Leicester, UK) and In Vitro Technologies (Baltimore, MD). Table 1 displays the CYP isoform characterization of the individual HLM pools as determined by the commercial supplier.

Cytochrome P450 contents were estimated spectrally by the method of Omura and Sato (1964). Protein concentrations were measured using the Randox Laboratories Ltd. (Crumlin, UK) protein kit based on pyrogallol red complexing with protein in an acid environment containing molybdate ions (Watanabe et al., 1986), using bovine serum albumin as a standard.

Probe Substrates. Ten commercially available drugs were selected as probe substrates to establish the suitability of this approach. The compounds were selected from the literature as marketed drugs for which the relative CYP-dependent metabolic formation was known and their metabolism by the five CYPs was adequately represented: tolbutamide (Back et al., 1988; Bourrie et al., 1996; Jung et al., 1997; Wester et al., 2000); diazepam (Ono et al., 1996); metoprolol (Otton et al., 1988; Mautz et al., 1995); ibuprofen (Hamman et al., 1997); propranolol (Otton et al., 1990; Yoshimoto et al., 1995); dextromethorphan (Dayer et al., 1989; Broly et al., 1990; Jacqz-Aigrain et al., 1993; Kerry et al., 1994; Von Moltke et al., 1998); omeprazole (Andersson et al., 1993; Kobayashi et al., 1994; Yamazaki et al., 1997); diltiazem (Pichard et al., 1990; Sutton et al., 1997); testosterone (Waxman et al., 1988; Wang et al., 1997); and verapamil (Kroemer et al., 1993; Tracy et al., 1999).

Automated CYP CL_{int} Determination. CYP CL_{int} determination assays were fully automated and performed by a robotic sample processor (RSP)

(Genesis RSP 150; Tecan, Reading, UK). Assays performed by the RSP were done using a program created by the user and not by a default program supplied with the hardware.³ The primary stock of all probe substrates was prepared manually in dimethyl sulfoxide or acetonitrile at 100-fold final incubation concentration. The final concentration of organic solvent in the incubation was 1% v/v. At this concentration dimethyl sulfoxide has been shown to reduce the activities of CYP2C9/19 (Chauret et al., 1998; Hickman et al., 1998), although this effect appears to be substrate-dependent. All substrates were incubated at 3 μM except tolbutamide (CL_{int} calculated by determining V_{max} and K_{m}), ibuprofen (10 μM), and testosterone (10 μM). The RSP was programmed to add chilled quenching solvent (100 μl of acetonitrile) to 96-well refrigerated blocks, and compound stocks were then prediluted in 100 mM potassium phosphate buffer, pH 7.4. *E. coli* membranes and microsomes prepared from baculovirus coexpressing individual CYPs and NADPH-reductase were added to incubation tubes (100 pmol of CYP $\cdot\text{ml}^{-1}$ final concentration) located in a 96-well heated block (37°C). A subaliquot was removed to produce a 0-min time point, and the assay was initiated via addition of NADPH (1 mM final concentration). Aliquots (50 μl) were removed at 5, 10, 15, and 20 min and quenched in acetonitrile. Samples were subsequently removed from the RSP, frozen for 1 h at -20°C , and then centrifuged at 3500 rpm for 20 min. The supernatants were removed and transferred into HPLC vials using the RSP.

Automated Human Liver Microsome CL_{int} Determination. HLM were diluted in 100 mM potassium phosphate buffer, pH 7.4 (1 $\text{mg} \cdot\text{ml}^{-1}$ final). Probe substrates were incubated at identical concentrations as the CYP CL_{int} assay, and incubations were carried out on the RSP as described above. Reactions were again initiated by addition of NADPH (1 mM final concentration), and several aliquots were taken over 45 min.

HPLC Methods. Aliquots (20 μl) were analyzed by HPLC-UV or HPLC-fluorescence for either parent loss or metabolite appearance using a model 1100 Chemstation (Hewlett-Packard, Palo Alto, CA) and a Hewlett-Packard 1046A fluorescence detector. A symmetry shield RP8 3.9- \times 50-mm cartridge (Waters, Watford, UK) and a mobile phase of 0.025% (w/v) ammonium acetate (solvent 1A) and acetonitrile (solvent 1B) was used for the chromatography of most analytes. Testosterone required a mobile phase of 0.025% ammonium acetate:methanol (95:5, v/v) (solvent 2A) and acetonitrile:methanol (95:5, v/v) (solvent 2B). The flow rate for all methods was 1.5 $\text{ml} \cdot\text{min}^{-1}$. Diazepam, metoprolol, propranolol, omeprazole, diltiazem, and verapamil eluted using a 5-min linear gradient from 80% solvent 1A to 20% solvent 1A, tolbutamide 99% to 65% over 5 min, dextromethorphan 80% to 20% over 3.5 min, and ibuprofen 85% to 20% over 5 min. Testosterone was eluted using a linear gradient from 85% solvent 2A to 75% solvent 2A over 12 min, 75% to 20% over 3 min followed by isocratic conditions (20:80) for 2 min. UV detection was performed for omeprazole (302 nm), diltiazem (237 nm), ibuprofen (222 nm), and testosterone (254 nm) and for metabolites of diazepam (229 nm) and tolbutamide (230 nm). Fluorometric detection was performed for metoprolol (Excitation 222 nm and Emission 320 nm), dextromethorphan (270, 312 nm), propranolol (205, 340 nm) and verapamil (280 nm, 310 nm).

Metabolite Identification. HLM or recombinant CYPs were diluted in 100

³ Copies of the program are available from the corresponding author upon request.

TABLE 2
Estimated levels of the five major isoforms in HLM pools

Mean total level = 320 pmol of CYP · mg⁻¹ of microsomal protein.

Isoform	Mean	References
	%	
CYP1A2	13	Shimada et al. (1994), Guengerich and Turvy (1991), Belloc et al. (1996)
CYP2C9	20	Shimada et al. (1994), Becquemont et al. (1998), Wester et al. (2000), Inoue et al. (1997), Lasker et al. (1998)
CYP2C19	4	Wester et al. (2000), Inoue et al. (1997), Lasker et al. (1998)
CYP2D6	2	Shimada et al. (1994), Becquemont et al. (1998), Imaoka et al. (1996)
CYP3A4	30	Shimada et al. (1994), Belloc et al. (1996), Becquemont et al. (1998)

mM potassium phosphate buffer, pH 7.4 (1 mg · ml⁻¹ or 100 pmol · ml⁻¹, respectively). Probe substrates were incubated at 30 μM, reactions were initiated by addition of NADPH (1 mM), and aliquots were quenched in 1:1 (v/v) methanol at 0 and 45 min. Aliquots (20 μl) were analyzed by liquid chromatography-mass spectrometry using the Hewlett-Packard 1100 Chemstation with a symmetry shield RP8 3.9- × 50-mm cartridge and a mobile phase of 0.025% (w/v) ammonium acetate (solvent 3A) and methanol (solvent 3B). Analytes were eluted using a gradient of 95% solvent 3A to 10% solvent 3A over 7 min. Metabolites were detected using a TSQ 7000 mass spectrophotometer (Finnigan MAT, San Diego, CA) with an atmospheric pressure chemical ionization ion source and a triple quadrupole mass analyzer in full scan mode. The molecular ion (either M + H⁺ or M - H⁺ depending on the orifice polarity) was detected for each metabolite.

Data Analysis. Throughout this study, several approaches were adopted for quantifying intrinsic clearance:

Metabolite appearance—low turnover compounds.

$$CL_{int} = V_{max}/k_m \quad (\text{tolbutamide})$$

$$V = \frac{V_{max} \times S}{K_m + S}$$

if $S \ll K_m$ ($\leq 10\%$)

$$V = \frac{V_{max} \times S}{K_m}$$

so

$$\frac{V}{S} = \frac{V_{max}}{K_m} = CL_{int} \quad (\text{diazepam})$$

Parent loss. Because dose/ C_0 gives a term for the volume of the incubation (expressed in ml · pmol of CYP⁻¹) and the elimination rate constant $k = 0.693/T_{1/2}$, an equation expressing CL_{int} in terms of $T_{1/2}$ of parent loss can be derived:

$$CL_{int} = \frac{\text{Volume} \times 0.693}{T_{1/2}} \quad (\text{majority})$$

The contribution of individual CYP to HLM CL_{int} was estimated as follows:

$$\Rightarrow \text{CYP } CL_{int} \times \% \text{ content of CYP isoform in HLM} \\ \times \text{Avg. total CYP in HLM (320 pmol/mg)}$$

Table 2 shows the average percentage content of the five major isoforms in human hepatic microsomes.

All individual data represent means from at least duplicate determinations.

Results

Marker Substrates. CL_{int} values were obtained for each of the prototypic substrates, tolbutamide, diazepam, metoprolol, ibuprofen,

propranolol, dextromethorphan, omeprazole, diltiazem, testosterone, and verapamil in three individual preparations of pooled HLM. Table 3 compares the CL_{int} for the three batches of HLM together with the mean and values obtained from the literature. For substrates with significant CYP3A4 metabolism, diltiazem, testosterone, and verapamil, CL_{int} was significantly higher in batch 1 compared with batches 2 and 3. The coefficient of variation was generally <15% for CL_{int} determined from one pool of HLM.

Substrates were incubated with recombinant CYP1A2, -2C9, -2C19, -2D6, and -3A4, respectively, using the RSP as described under *Materials and Methods*. Figure 1 displays the loss of propranolol against time by the five different CYP isoforms used in the automated assay and shows significant metabolism by CYP1A2, -2C19, and -2D6. Table 4 shows the CYP CL_{int} of individual CYP isoforms to oxidative metabolism for each marker substrate. The range of CYP CL_{int} determined was 0.03 to 7 μl · min⁻¹ pmol of P450⁻¹. The percentage contributions of individual CYPs toward oxidative metabolism of a compound in HLM were estimated, and Table 5 compares our values with common literature assignments. Tolbutamide (CL_{int} determined by V_{max}/K_m) was metabolized by both CYP2C9 (70%) and CYP2C19 (30%), diazepam (10 μM) by CYP2C19 (100%), ibuprofen (10 μM) by CYP2C9 (90%) and CYP2C19 (10%), and omeprazole (3 μM) by CYP2C19 (68%) and CYP3A4 (32%), respectively. Metoprolol (3 μM) and dextromethorphan (3 μM) are primarily CYP2D6 substrates and propranolol (3 μM) was metabolized by CYP2D6 (59%), CYP1A2 (26%), and CYP2C19 (15%). Diltiazem (3 μM), testosterone (10 μM), and verapamil (3 μM) were predominantly metabolized by CYP3A4.

For each compound, the sum of the CL_{int} (μl · min⁻¹mg⁻¹) from the five individual isoforms was compared with the respective CL_{int} derived from the mean of three separate HLM pools (Table 6). Figure 2 shows the correlation ($r^2 = 0.8$, $P < .001$) between the CYP CL_{int} and HLM CL_{int} . Compounds with a HLM CL_{int} of <8 μl · min⁻¹mg⁻¹ may be described as low clearance, 8 to 65 μl · min⁻¹mg⁻¹ as intermediate, and >65 μl · min⁻¹mg⁻¹ as high clearance.

Predictions of HLM CL_{int} from the sum of individual CYP CL_{int} were excellent for tolbutamide (CYP $CL_{int} = 1.3$ μl · min⁻¹mg⁻¹ versus HLM $CL_{int} = 0.7$ μl · min⁻¹mg⁻¹), diazepam (2 ± 1 versus 3 ± 1), and metoprolol (7 ± 0 versus 6 ± 1). All predictions of HLM CL_{int} from the individually summed CYP CL_{int} except for those derived for propranolol (CYP $CL_{int} = 55 \pm 15$ μl · min⁻¹mg⁻¹ versus HLM $CL_{int} = 15 \pm 0$ μl · min⁻¹mg⁻¹) and omeprazole (131 ± 25 versus 34 ± 14) were within 3-fold. The summed CYP CL_{int} of these two compounds significantly overestimated their CL_{int} determined in HLM.

To investigate the relationship between the CL_{int} of propranolol with increasing HLM protein concentration, CL_{int} was determined at 0.4, 1, and 2 mg · ml⁻¹ of HLM. Figure 3 shows the relationship between increasing microsomal protein and decreasing CL_{int} of propranolol.

The metabolite profile for the CYP-dependent clearance of dextromethorphan observed by HPLC-mass spectrometry was as predicted from Von Moltke et al. (1998), where microsomes containing individual CYPs expressed by a human lymphoblastoid expression system were used (Fig. 4). Dextromethorphan was chosen for this detailed analysis, because four of the five major human CYPs (CYP2C9, -2C19, -2D6, and -3A4) have been implicated in its metabolism. Dextromethorphan was incubated at 30 μM with all five isoforms, and as expected, two metabolites, dextropran and 3-methoxymorphinan, were observed, based on their m/z values and distinguished using standards. Based on UV response, 88% of the metabolites formed

TABLE 3
 CL_{int} of marker substrates in different HLM pools and literature values

Compound	CL_{int}				Literature	References
	Batch 1	Batch 2	Batch 3	Mean		
	$\mu\text{l} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$					
Tolbutamide				0.7 ^a	0.5–2.5; 1 ± 0.2	Iwatsubo et al. (1997), Doecke et al. (1991), Back et al. (1988), Bourrie et al. (1996), Obach (1999)
Diazepam	3 ± 1	2	2	3 ± 1	2.6 ± 0.8	Obach (1999)
Metoprolol	7 ± 1	5	5	6 ± 1	14, 19	Mautz et al. (1995)
Ibuprofen	14	5	5	8 ± 2	9.6 ± 1.1	Obach (1999)
Propranolol	16 ± 3	16	15	15 ± 0	11–60	Lave et al. (1997), Obach (1997), Otton et al. (1990)
Dextromethorphan	29 ± 3	30	33	29 ± 3	25–103	Kerry et al. (1994), Broly et al. (1990)
Omeprazole	43 ± 7	11	15	34 ± 14	18, 26	Andersson et al. (1993), Kobayashi et al. (1994)
Diltiazem	56 ± 7	22	22	45 ± 16	17 ± 2	Obach (1999)
Testosterone	81 ± 12	23	35	60 ± 18		
Verapamil	380 ± 44	111	114	256 ± 100	139 ± 3	Obach (1999)

^a Determined from V_{max}/K_m . Where determined, results are presented as mean ± S.D.

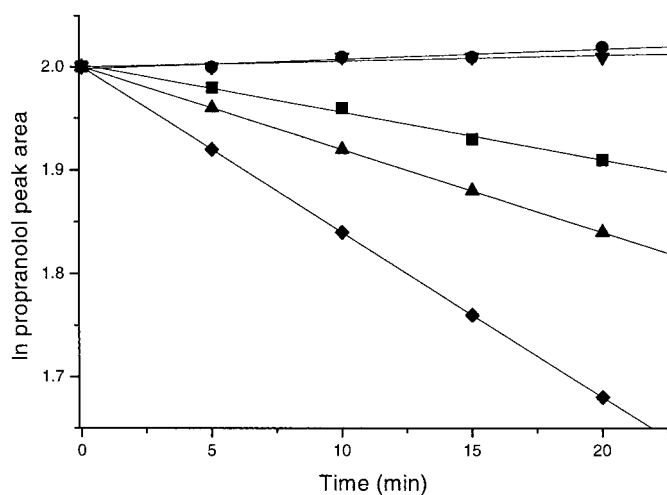


FIG. 1. CYP1A2, -2C9, -2C19, -2D6, and -3A4-dependent clearance of propranolol.

The determination of the CYP-dependent propranolol metabolism using the automated assay is as described under *Materials and Methods*. Aliquots were taken at 0, 5, 10, 15, and 20 min, and the amount of propranolol remaining in the incubation media is reflected by the peak area after HPLC-fluorescence detection. The data represent propranolol clearance by *E. coli* membranes expressing CYP1A2 (■), CYP2C9 (●), CYP2D6 (◆), CYP3A4 (▼), and baculovirus expressing CYP2C19 (▲), as described under *Materials and Methods*. The solid lines indicate linear regression of the data.

from dextromethorphan ($m/z = 272$) were dextrorphan ($m/z = 258$) and 12% were 3-methoxymorphinan ($m/z = 258$), which compares well with Von Moltke et al. (1998) (98 and 2%, respectively). The isoform responsible for dextrorphan formation was primarily CYP2D6 (92% versus 97%; as determined from Von Moltke et al., 1998) with minor contributions from CYP2C9, -2C19, and -3A4 (<3% in both laboratories). The isoforms responsible for 3-methoxymorphinan formation were CYP2C9 (43% versus 55%), CYP3A4 (42% versus 20%), CYP2C19 (8% versus 16%), and CYP2D6 (7% versus 9%). In addition, it was also determined that CYP1A2 metabolized propranolol ($m/z = 260$) to the expected *N*-deisopropylation product ($m/z = 218$) (Yoshimoto et al., 1995) and CYP2D6 metabolized propranolol to the expected hydroxylated product ($m/z = 276$), although the regiochemistry of hydroxylation was not investigated. Several other markers also generated the product profile as expected from the literature (data not shown).

Discussion

To generate confidence that recombinant CYPs may be used to predict HLM CL_{int} data, the in vitro kinetics for commonly used CYP probes were determined previously in this laboratory in both pooled HLM and CYP coexpressed with NADPH-reductase in *E. coli* cells (McGinnity et al., 1999). The kinetic parameters (including CL_{int}) of these recombinant enzymes were similar to their human liver counterparts for the enzyme substrate pairs that were directly comparable, and thus they would appear to be faithful surrogates. Indeed, Eddershaw and Dickins (1999) reported an excellent comparison between the rates of metabolism of several compounds determined from HLM and microsomes containing a mixture of the major recombinant CYPs. However, this "artificial HLM" approach gives little information as to the enzymology of metabolism.

To demonstrate the potential for predicting both the extent and route of oxidative metabolic clearance for NCEs by recombinant human CYPs, several marketed drugs were selected in which metabolism via CYP pathways was well established. Of the marketed drugs that are primarily cleared by human hepatic CYP-mediated metabolism, the vast majority were metabolized by one or more of the five isoforms, CYP1A2, -2C9, -2C19, -2D6, and -3A4 (Bertz and Graneman, 1997), and, for that reason, only these isoforms were employed in this initial study. There are limited available data on the relative levels of the five major isoforms in human hepatic microsomes (Table 2), and we have relied heavily on the seminal study by Shimada et al. (1994), which is widely cited for this purpose. The marker compounds tolbutamide, diazepam, metoprolol, ibuprofen, propranolol, dextromethorphan, omeprazole, diltiazem, testosterone, and verapamil were chosen so that metabolism by each of the five CYPs was adequately represented. The choice of DMSO as a solvent was based on its value for compounds with relatively low solubility (often encountered in early drug discovery programs) and its implementation as the solvent of choice in many liquid banks. Any inhibitory effects should not affect the comparison between recombinant CYPs and HLM.

There is a reasonable agreement between the CL_{int} of the probe substrates determined in HLM to available literature values (Table 3), although the comparison is somewhat compromised due to the large spread of the literature data. There is agreement as to whether a compound demonstrates a low, intermediate, or high CL_{int} . The limitations of such an interlaboratory comparison and the inherent variability of such an exercise are well established (Boobis et al., 1998).

TABLE 4
Determination of CYP CL_{int} of individual human CYPs to oxidative metabolism for marker substrates

Compound	CL_{int}				
	CYP1A2	CYP2C9	CYP2C19	CYP2D6	CYP3A4
	$\mu\text{l} \cdot \text{min}^{-1} \text{pmol}^{-1}$				
Tolbutamide	N.D. ^a	0.014 ^b	0.03 ± 0.01	N.D.	N.D.
Diazepam	N.D.	N.D.	0.17 ± 0.05	N.D.	N.D.
Metoprolol	N.D.	N.D.	N.D.	1.03 ± 0.07	N.D.
Ibuprofen	N.D.	0.29 ± 0.09	0.17 ± 0.01	N.D.	N.D.
Propranolol	0.34 ± 0.03	N.D.	0.66 ± 0.06	5.07 ± 2.80	N.D.
Dextromethorphan	N.D.	N.D.	0.16 ± 0.10	1.97 ± 0.24	N.D.
Omeprazole	N.D.	N.D.	6.95 ± 1.93	N.D.	0.44 ± 0.06
Diltiazem	N.D.	N.D.	0.30 ± 0.04	N.D.	0.58 ± 0.04
Testosterone	N.D.	N.D.	N.D.	N.D.	0.42 ± 0.03
Verapamil	N.D.	N.D.	N.D.	N.D.	1.19 ± 0.12

^a N.D., not detectable ($<0.02 \mu\text{l} \cdot \text{min}^{-1} \cdot \text{pmol}^{-1}$).

^b Determined from V_{max}/K_m . Mean ± S.D. for $n = 3$ separate experiments.

TABLE 5
Mean percentage contribution of individual CYPs to oxidative metabolism

Compound	CYP1A2	CYP2C9	CYP2C19	CYP2D6	CYP3A4	Literature
	%					
Tolbutamide		70	30			2C
Diazepam			100			2C19>3A
Metoprolol				100		2D6
Ibuprofen		90	10			2C9
Propranolol	26		15	59		2D6, 1A2, 2C19
Dextromethorphan			14	86		2D6>3A
Omeprazole			68		32	2C19>>3A
Diltiazem			7		93	3A
Testosterone					100	3A
Verapamil					100	3A

TABLE 6
Determination of CYP CL_{int} for marker and AR-C compounds

Compound	CL_{int}	
	HLM	CYP
	$\mu\text{l} \cdot \text{min}^{-1} \text{mg}^{-1}$	
Tolbutamide	0.7 ^a	1.3 ^a
Diazepam	3 ± 1	2 ± 1
Metoprolol	6 ± 1	7 ± 0
Ibuprofen	8 ± 2	20 ± 5
Propranolol	15 ± 0	55 ± 15
Dextromethorphan	29 ± 3	14 ± 1
Omeprazole	34 ± 14	131 ± 25
Diltiazem	45 ± 16	60 ± 3
Testosterone	60 ± 18	40 ± 3
Verapamil	256 ± 100	114 ± 11

^a Determined from V_{max}/K_m . Mean ± S.D.

Literature CL_{int} values have been obtained from a variety of sources, including V_{max}/K_m calculations, microsomes (prepared from individual as well as pooled livers), and hepatocytes [data converted to $\mu\text{l} \cdot \text{min}^{-1} \text{mg}^{-1}$ assuming 2.67×10^6 cells/mg of microsomal protein (Carlile et al., 1999)]. Variability will also result from the fact that isoform levels and activities may vary significantly between the different metabolizing sources (Boobis et al., 1998). However, the agreement is excellent where a direct comparison between two laboratories determining HLM CL_{int} for several compounds can be made (Obach, 1999).

Without exception, our data and the prevailing literature assign the same isoform to be the predominant CYP responsible for the metabolism of each marker compound. Diazepam at low micromolar concentrations was metabolized by CYP2C19, which agrees with Jung et

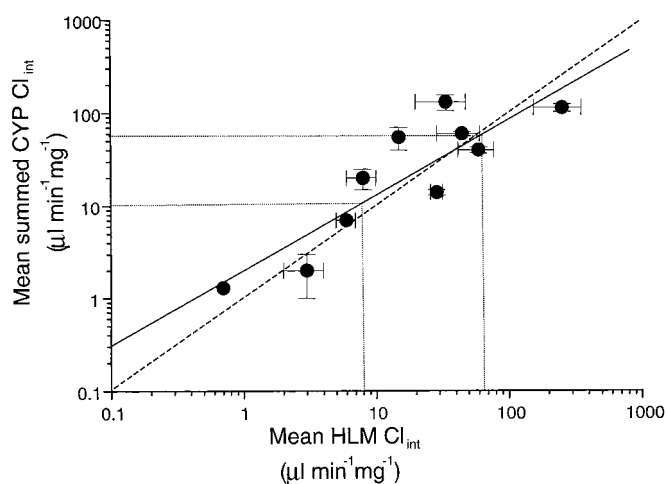


FIG. 2. Comparison of summed CYP CL_{int} with HLM CL_{int} for several probe substrates.

Both the summed CYP and HLM CL_{int} determinations were carried out as described under *Materials and Methods*. The data points represent the mean CL_{int} determinations, and the error bars reflect the standard deviation from the mean as shown in Table 6. The dotted boxes illustrate HLM CL_{int} s of $<8 \mu\text{l} \cdot \text{min}^{-1} \text{mg}^{-1}$ (low clearance) and $>65 \mu\text{l} \cdot \text{min}^{-1} \text{mg}^{-1}$ (high clearance). The solid line depicts a linear regression analysis of the data ($r^2 = 0.8$, $P < .001$) with the equation $\log \text{CYP } CL_{int} = 0.91 \times \log \text{HLM } CL_{int} + 0.3$. The dashed line indicates line of unity.

al. (1997), Yasumori et al. (1993), and Andersson et al. (1994). Indeed, detailed HLM kinetics of diazepam metabolism (not shown) suggests the involvement of multiple CYPs (e.g., CYP2C9/18, -2B6, and -3A4), but the data indicate that the high affinity component of diazepam *N*-demethylation *in vivo* may be CYP2C19. Metoprolol and

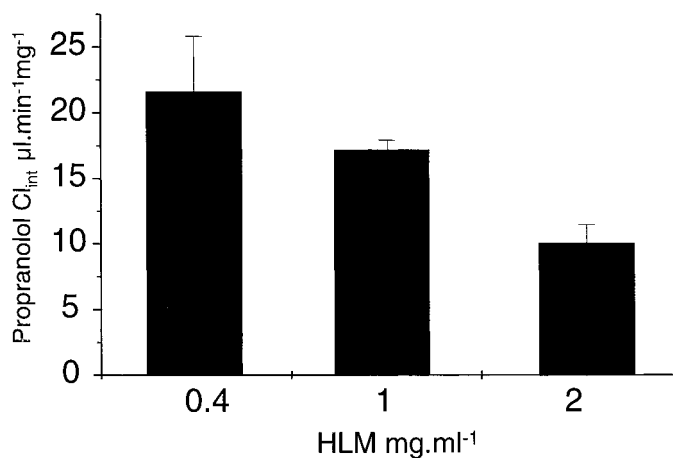


FIG. 3. Determination of propranolol CL_{int} at different concentrations of HLM.

The HLM CL_{int} determinations were carried out as described under *Materials and Methods*. The histograms reflect a mean CL_{int} , and the error bars give the standard deviation from the mean. Experiments were carried out in duplicate a minimum of three times.

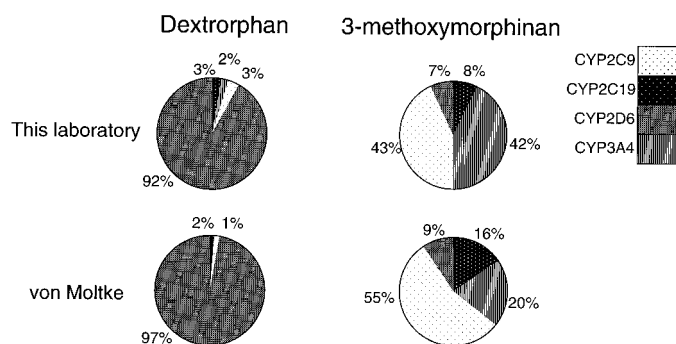


FIG. 4. Metabolite profile for the CYP-dependent clearance of dextromethorphan.

The identification of CYP-dependent dextromethorphan metabolite formation is as described under *Materials and Methods*. The data from this laboratory, where dextromethorphan was incubated at 30 μM, are compared with that of Von Moltke et al. (1998) where the data reflect CL_{int} .

dextromethorphan are primarily CYP2D6 substrates (Otton et al., 1988; Dayer et al., 1989; Jacqz-Aigrain et al., 1993; Kerry et al., 1994; Von Moltke et al., 1998). Diltiazem, testosterone, and verapamil are predominantly metabolized by CYP3A4 (Waxman et al., 1988; Pichard et al., 1990; Kroemer et al., 1993; Sutton et al., 1997; Tracy et al., 1999).

In addition, for propranolol, omeprazole, tolbutamide, and ibuprofen, there was excellent concordance between our data and the literature on the relative contribution of several isoforms in the metabolism of the respective compounds. Tolbutamide is metabolized by both CYP2C9 (70%) and CYP2C19 (30%), which agrees with Inoue et al. (1997), Wester et al. (2000), Venkatakrishnan et al. (1998), and Lasker et al. (1998). Similarly, ibuprofen is metabolized by CYP2C9 (90%) and CYP2C19 (10%) (Leemann et al., 1993; Hamman et al., 1997) and omeprazole by CYP2C19 (68%) and CYP3A4 (32%) (Andersson et al., 1993; Karam et al., 1996; Yamazaki et al., 1997; Lasker et al., 1998). Indeed, when recombinant CYP2C19 and CYP3A4 were mixed at a ratio similar to that found in HLM, the metabolism of omeprazole resembled that of HLM (Yamazaki et al., 1997). The assignment of CYP2D6 (59%), CYP1A2 (26%), and CYP2C19 (15%)-dependent metabolism for propranolol agrees with several sources (Lennard et al., 1984; Otton et al., 1990; Yoshimoto et

al., 1995), which implicate these three isoforms. In addition, the appropriate metabolites from each isoform were identified by mass spectrometry analysis.

A method based on the rate of enzyme activity (relative activity factor) of recombinant CYPs and HLM has proven useful in assigning the contribution of individual CYPs to several biotransformations (Rodrigues, 1999; Roy et al., 1999). Recently, it has been suggested that a complementary approach using the ratio of *intrinsic clearance* as a relative activity factor may be more predictive, where the kinetics for recombinant CYPs and HLM are equivalent (Nakajima et al., 1999). The correlation observed in this study between the sum of CL_{int} from the different CYP isoforms and HLM CL_{int} for the compounds tested confirms this concept. This study has additionally provided a more thorough evaluation of these recombinant proteins expressed in *E. coli*.

The summed CYP CL_{int} correctly predicted a low HLM CL_{int} (<8 μl · min⁻¹ · mg⁻¹) for tolbutamide, diazepam, and metoprolol; an intermediate HLM CL_{int} (8–65 μl · min⁻¹ · mg⁻¹) for ibuprofen, propranolol, dextromethorphan, diltiazem, and testosterone; and a high HLM CL_{int} (>65 μl · min⁻¹ · mg⁻¹) for verapamil. However, the summed CYP CL_{int} of omeprazole and propranolol did overpredict somewhat HLM CL_{int} . One possible explanation for this is an increase in "futile" binding with increased protein concentration for some compounds. For propranolol there is 50% free at 0.4 mg · ml⁻¹ and 25% at 2 mg · ml⁻¹ (Obach, 1997), which results in a 2-fold decrease of propranolol CL_{int} (Fig. 4). Typical assay conditions used 0.2 to 0.4 mg of protein/ml⁻¹ of CYPs (exact amount depended on the CYP expression level, because all incubations contain 100 pmol of CYP/ml⁻¹) and 1 mg · ml⁻¹ HLM. The HLM CL_{int} of propranolol at 0.4 mg · ml⁻¹ was determined to be 22 ± 4 μl · min⁻¹ · mg⁻¹, which compares more favorably with the summed CYP CL_{int} at the same protein level (55 ± 15 μl · min⁻¹ · mg⁻¹).

Generally, lower protein levels in the recombinant CYP assay may allow a more accurate reflection of unbound CL_{int} and provide a greater dynamic CL_{int} range when discriminating between large numbers of compounds. There is likely to be no significant differences between the extent of futile binding for HLM and recombinant CYPs at the same total protein concentration (Venkatakrishnan et al., 2000). Differential protein binding between in vitro matrices for predicting in vivo CL_{met} is currently under investigation.

In our experience, an accurate determination of a wide range of CL_{int} is achieved at an incubation concentration for recombinant CYP of 100 pmol of CYP/ml⁻¹, which may be subsequently optimized. The molar ratio of NADPH-P450 reductase to recombinant CYP has been manipulated for the *E. coli* expression constructs to produce optimal reaction kinetics for probe substrates (McGinnity et al., 1999). For example, optimal CYP2C19-mediated diazepam *N*-demethylation can be achieved, in the absence of cytochrome *b*₅, by increasing the molar ratio of NADPH-P450 reductase:CYP2C19 to approximately 20:1 (McGinnity et al., 1999). Indeed, to optimize CYP expression systems, further elucidation of the role and importance of ancillary electron transporters such as *b*₅ in the metabolism of xenobiotics is required (Yamazaki et al., 1999).

A correlation between HLM and CYP CL_{int} allows compounds to be ranked with respect to metabolic stability, should expedite knowledge of the pharmacophore of individual CYP isoforms, and may facilitate more rational compound synthesis to achieve greater metabolic stability. Furthermore, an underprediction of HLM CL_{int} from the five major human hepatic isoforms should prompt an investigation into possible metabolism by the more minor human hepatic CYPs, i.e., CYP2A6, -2B6, -2C8, or -2E1 (Houston, 1994).

This automated assay is being used early in drug discovery at

AstraZeneca R&D Charnwood, a strategy distinct from the comprehensive isoform profiling of a drug later in the development process by other groups (Machinist et al., 1998; Fischer et al., 1999; Nakajima et al., 1999; Roy et al., 1999). The early identification of the major CYP isoforms involved in the metabolism of a drug candidate is useful for several purposes, including understanding ligand-enzyme structure-activity relationships, expanding the database for substrates of the polymorphic isoforms, assessing the potential intersubject variability, and predicting the drug-drug interactions and, ultimately, the direction of clinical trials.

These data indicate that recombinant CYPs may be used to predict HLM CL_{int} . Furthermore, it may prove feasible to scale human CL_{int} data to the fractional metabolic clearance encountered clinically (Iwatsubo et al., 1997; Obach, 1999). Therefore, although very much in its infancy, data in this report demonstrate that *E. coli*-expressed CYPs may be useful as an early approach for the prediction of the enzymology of human CYP metabolism. Further efforts to examine the differential nonspecific binding between the separate *in vitro* models and the effects on CL_{int} are underway.

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