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RAPID CHARACTERIZATION OF THE MAJOR DRUG-METABOLIZING HUMAN HEPATIC CYTOCHROME P-450 ENZYMES EXPRESSED IN *ESCHERICHIA COLI*

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ABSTRACT:

The major drug-metabolizing human hepatic cytochrome P-450s (CYPs; CYP1A2, 2C9, 2C19, 2D6, and 3A4) coexpressed functionally in *Escherichia coli* with human NADPH-P-450 reductase have been validated as surrogates to their counterparts in human liver microsomes (HLM) using automated technology. The dealkylation of ethoxyresorufin, dextromethorphan, and erythromycin were all shown to be specific reactions for CYP1A2, CYP2D6, and CYP3A4 that allowed direct comparison with kinetic data for HLM. For CYP2C9 and CYP2C19, the kinetics for the discrete oxidations of naproxen and diazepam were compared to data obtained using established, commercial CYP preparations. Turnover numbers of CYPs expressed in *E. coli* toward these substrates were generally equal to or even greater than those of the major commercial suppliers [CYP1A2 (ethoxyresorufin), *E. coli* 0.6 ± 0.2 min^{-1} versus B lymphoblasts 0.4 ± 0.1 min^{-1}; CYP2C9 (naproxen), 6.7 ± 0.9 versus 4.9 min^{-1}; CYP2C19 (diazepam), 3.7 ± 0.3 versus 0.2 ± 0.1 min^{-1}; CYP2D6 (dextromethorphan), 4.7 ± 0.1 versus 4.4 ± 0.1 min^{-1}; CYP3A4 (erythromycin), 3 ± 1.2 versus 1.6 min^{-1}]. The apparent K_m values for the specific reactions were also similar (K_m ranges for expressed CYPs and HLM were: ethoxyresorufin 0.5–1.0 μM, dextromethorphan 1.3–5.9 μM, and erythromycin 18–57 μM), indicating little if any effect of *N*-terminal modification on the *E. coli*-expressed CYPs. The data generated for all the probe substrates by HLM and recombinant CYPs also agreed well with literature values. In summary, *E. coli*-expressed CYPs appear faithful surrogates for the native (HLM) enzyme, and these data suggest that such recombinant enzymes may be suitable for predictive human metabolism studies.

Recent clinical experiences with several compounds have highlighted the importance of assessing the potential for inhibition (Honig et al., 1993; Ahmad and Wolfe, 1995) or induction of drug metabolism (Parkinson, 1996 and references therein) and has promoted heavy investment in the early prediction of the extent and route of candidate drug metabolism and pharmacokinetic parameters in humans. In addition, with the increasingly large number of compounds generated as a result of the emergence of combinatorial chemistry and high throughput screening techniques, it is becoming important to develop rapid and relatively high throughput in vitro drug metabolism models with which to assess and eradicate such unfavorable properties from a series of potential drug candidates.

Currently, a combined in vitro approach using both heterologous expression systems (eventually for all xenobiotic-metabolizing enzymes) and human hepatic tissue fractions is recommended as the most thorough route to developing safe drugs with appropriate metabolic properties (Parkinson, 1996). Identifying the specific cytochrome P-450 (CYP)[1] enzyme involved in the metabolism of drug candidates is a labor- and time-intensive process involving correlation analyses within a bank of human liver microsomes (HLM), chemical and antibody isoform-selective inhibition, and confirmation with expressed enzymes.

As confidence in the application of recombinant enzymes to such studies has increased, their use as first line approaches in human hepatic CYP inhibition screens for drug candidates (Remmel and Burchell, 1993; Crespi and Penman, 1997; Crespi et al., 1997; Moody et al., 1999) and for the prediction of HLM kinetics (Becquemont et al., 1998) has been proposed.

Many CYP isoforms have now been expressed in various heterologous cell lines including bacteria, yeast, cultured insect, and mammalian cells (Waterman et al., 1995; Gonzalez and Korzekwa, 1995). *E. coli* expression systems offer many advantages over the major commercially available materials including generally stable and easy to manipulate plasmid constructs; the expressed enzyme is easily replenished at a high yield and at relatively low cost. Furthermore, although a high level of expression may require some modification of the N-terminal amino acid sequence of the CYP protein (Larson et al., 1991; Waterman, 1993), recent data have indicated that functional expression can still be achieved with the major drug-metabolizing CYPs with properties similar to the human hepatic

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[1] Abbreviations used are: CYP, cytochrome P-450; TN, turnover number; HLM, human liver microsome(s); Cl_{int}, intrinsic clearance; EROD, ethoxyresorufin *O*-deethylation; β-NADPH, β-nicotinamide adenine dinucleotide phosphate, reduced form; RSP, robotic sample processor; SPE, solid phase extraction.

microsomal enzymes (Blake et al., 1996; Iwata et al., 1998). The availability of an in-house panel of characterized human hepatic CYP enzymes would obviate the reliance on expensive commercial supplies and provide local control of expression levels, supply, interbatch variability, and quality.

With any expressed enzyme system, it is extremely important to have confidence that the recombinant CYPs are surrogates for the human microsomal enzymes by comparative analysis of the kinetic properties of these enzymes. This characterization and validation could include an assessment of substrate and inhibitor selectivity together with comparative kinetic analyses, which represents a substantial undertaking. This process is facilitated markedly through the application of automation technology, which enables a comprehensive in-house characterization of enzyme activities and eliminates awkward interlaboratory comparisons (Moody et al., 1999).

This laboratory has recently developed fully automated inhibition screens for the major human hepatic CYPs (Moody et al., 1999). As part of an extensive collaboration between academia and major pharmaceutical companies, this paper describes the validation of the major drug-metabolizing human CYP isoforms CYP1A2, 2C9, 2C19, 2D6, and 3A4 expressed in *E. coli*. These enzymes have been characterized with respect to their kinetic properties and comparisons have been made with their counterparts in HLM as well as the most widely used commercially available recombinant CYP preparations and to the wider literature.

Materials and Methods

Chemicals. All chemicals and reagents used were of the highest available commercial grade. Ampicillin, diazepam, erythromycin, dextromethorphan, naproxen, ethoxyresorufin, resorufin, δ-aminolevulinic acid, glycerol, and β-nicotinamide adenine dinucleotide phosphate, reduced form (β-NADPH) were purchased from Sigma Chemical Co. (Poole, UK). Isopropylthio-β-D-galactoside was purchased from Gibco BRL (Grand Island, NY). Bactotryptone, yeast extract, and bactopeptone were purchased from Difco (Detroit, MI).

The ^{14}C-labeled probe substrates [*N*-methyl-^{14}C]diazepam, [*O*-methyl-^{14}C]dextromethorphan, and [*O*-methyl-^{14}C]naproxen (specific radioactivity 55 mCi/mmol, chemical purity ≥98.5%, radiochemical purity ≥99.4%) were synthesized as described previously (Moody et al., 1999). [*N*-methyl-^{14}C]erythromycin (specific radioactivity 55 mCi/mmol, radiochemical purity >97%) was purchased from NEN Life Science Products (Boston, MA).

Construction and Expression of Plasmids in *E. coli*. The LINK consortium, a collaboration between UK-based academia and industry, provided stocks of transformed cells with human CYP1A2, CYP2C9, CYP2C19, CYP2D6, and CYP3A4 individually coexpressed with human NADPH-P-450 reductase in *E. coli*. The strategy for expressing CYP1A2 (in strain JM109) and CYP2C9 (in strain DH5α) was similar to that described for CYP2D6 (Pritchard et al., 1998) and involved coexpressing each CYP with NADPH-P-450 reductase from separate compatible plasmids using ampicillin and chloramphenicol as the respective selection markers. For optimal expression of CYP1A2 and CYP2C9 the first eight codons of each CYP cDNA were replaced with eight codons of the bovine pregnenolone and progesterone 17α-hydroxylase sequence (hereafter referred to as 17α) to generate the constructs 17α-CYP1A2 and 17α-CYP2C9 (Pritchard et al., 1998).

For the expression of ompA-2C19 (in strain DH5α) and ompA-2D6 (in strain JM109) the bacterial ompA leader sequence was fused in-frame to the start of the respective CYP2C19 and CYP2D6 cDNA using polymerase chain reaction fusion and subcloned into pCW (Pritchard et al., 1998). Coexpression with NADPH-P-450 reductase using the two-plasmid system was as described previously (Pritchard et al., 1998). For the one-plasmid CYP2C19 system, both the CYP2C19 and reductase cDNAs were subcloned head-to-tail into pCW, each with its own *tac tac* promoter, using a procedure similar to that described by Blake et al. (1996).

For optimal expression of CYP3A4 (in strain JM109) a construct consisting of ompA leader followed by the two first amino acids of the mature ompA (ala-pro), followed by full length CYP3A4, was also coexpressed with NADPH-P-450 reductase from separate compatible plasmids using ampicillin and chloramphenicol as the respective selection markers.

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 essentially as described previously (Pritchard et al., 1998). Single colonies were inoculated from LB-agar plates into 1 liter of modified Terrific Broth media (containing 12 g bactotryptone, 24 g yeast extract, 2 g bactopeptone, and 4 ml glycerol) containing the appropriate selection marker (25 mg chloramphenicol/liter, 50 mg ampicillin/liter). Expression cultures were shaken at 37°C until the O.D. at 600 nm reached 0.7, when isopropylthio-β-D-galactoside (1 mM) and δ-aminolevulinic acid (0.5 mM) were added and the temperature decreased to 30°C. Cells were left for 16 h before harvesting. Harvesting of cells, preparation of spheroplasts, and membrane fractions were performed exactly as reported previously (Pritchard et al., 1998). CYP contents were estimated spectrally by the method of Omura and Sato, (1964). Protein concentrations were measured using the Randox Laboratories Ltd. (Crumlin Co., Antrim, UK) protein kit based on pyrogallol red complexing with protein in an acid environment containing molybdate ions (Watanabe et al., 1986).

Commercial CYP Sources. Microsomes prepared from human B-lymphoblastoid cells coexpressing recombinant human NADPH-P-450 reductase and human CYP1A2, CYP2C9, CYP2C19, CYP2D6, and CYP3A4 and microsomes from insect cells transfected with a baculovirus containing the cDNA for human CYP2C19 together with cytochrome b_5 (Supersomes) were purchased from Gentest Corp. (Woburn, MA). Similarly, microsomes prepared from insect cells transfected with a baculovirus expressing CYP2C19 and rabbit NADPH-P-450 reductase were purchased from PanVera Corp. (Madison, WI). Pooled HLM (batch numbers 217, 219, and 220) were supplied by IIAM (Leics, UK).

Manual Enzyme Assays. Ethoxyresorufin *O*-deethylation (EROD; Riley et al., 1995), naproxen *O*-demethylation (Rodrigues et al., 1996), dextromethorphan *O*-demethylation (Rodrigues et al., 1994) and erythromycin *N*-demethylation (Riley and Howbrook, 1998) assays were used as probe reactions for CYP1A2, CYP2C9, CYP2D6, and CYP3A4, respectively, and based on methods described previously. [^{14}C]-diazepam *N*-demethylation has been developed as an assay for CYP2C19 activity in this laboratory (Moody et al., 1999).

Substrate Selectivity. The isoform selectivity of these probe substrate reactions was investigated in incubations with *E. coli* membranes expressing CYP1A2, CYP2C9, CYP2D6, and CYP3A4 (all at 50 pmol P-450/ml) in 0.1 M potassium phosphate buffer pH 7.4 in a final volume of 200 μl. Each radiometric assay included 0.05 to 0.1 μCi of [^{14}C]naproxen, [^{14}C]dextromethorphan, [^{14}C]diazepam, or [^{14}C]erythromycin. An appropriate amount of cold substrate was added to give final substrate concentrations of dextromethorphan (30 and 3 μM), diazepam (100 and 20 μM), naproxen (2 mM and 300 μM), and erythromycin (300 and 40 μM). Substrate concentrations were chosen to reflect K_m and V_{max} conditions.

For the fluorometric assay, ethoxyresorufin was used at final concentrations of 3 and 1 μM. The probe substrate in ethanol was aliquoted and the solvent evaporated under a stream of nitrogen, before addition of protein. All incubations were carried out at 37°C and reactions were started, after preincubation for 2 min, with the addition of 1 mM NADPH. Incubation times were 15 min for EROD, naproxen *O*-demethylation, diazepam *N*-demethylation, and dextromethorphan *O*-demethylation, and 10 min for erythromycin *N*-demethylation (Table 1). Reactions were terminated by the addition of 50 μl of ice-cold trichloroacetic acid (10% w/v) and solid phase extraction (SPE) was carried out using disposable Supelclean Envi-Carb 1-ml cartridges (Supelco, Bellefonte, PA) as detailed elsewhere (Riley and Howbrook, 1998; Moody et al., 1999) and eluents were counted for 4 min using a Packard 2200CA Tri-Carb Liquid Scintillation Analyzer.

Automated K_m and V_{max} Determination. K_m and V_{max} determination assays were performed by a robotic sample processor (RSP; Genesis RSP 150; Tecan, Reading, UK). All of the assays performed by the RSP were programmed by the user and are not default program-supplied with the hardware.

The components of the radiometric assays have been described in detail elsewhere (Moody et al., 1999). Assays were carried out in 0.1 M phosphate pH 7.4; in addition, the kinetics of erythromycin *N*-demethylation by CYP3A4 was determined in TSE buffer.

TABLE 1

Experimental conditions for the five probe assays using different enzyme sources

Substrate	Isoform	Expression System	Incubation Time	Protein Concentration
			min	*mg/ml (pmol CYP/ml)*
Ethoxyresorufin	CYP1A2	*E. coli* membranes	15	0.1 (22)
		B-lymphoblasts	15	0.1 (14)
		HLM	15	0.1
Naproxen	CYP2C9	*E. coli* membranes	15	0.2 (77)
		B-lymphoblasts	5	1.4 (67)
		HLM	5	1
Diazepam	CYP2C19	*E. coli* membranes[a]	15	0.7 (40)
		Baculosomes	15	0.4 (40)
		B-lymphoblasts	15	0.2 (50)
		HLM	20	0.25
Dextromethorphan	CYP2D6	*E. coli* membranes	15	0.06 (32)
		B-lymphoblasts	15	0.24 (8.4)
		HLM	15	0.85
Erythromycin	CYP3A4	*E. coli* membranes	10	0.05 (25)
		B-lymphoblasts	10	0.1 (5)
		HLM	15	0.5

[a] Represents the one plasmid CYP2C19 expression construct.

With the exception of [^{14}C]dextromethorphan, the 0.1 μCi of probe radioactive substrate (in ethanol) was dispensed manually into a glass vial and the solvent evaporated under a steady stream of nitrogen, before addition of protein. Incubations contained 100 μl protein and probe ^{14}C-substrate mixture, 10 μl cold substrate, where appropriate, at different stock concentrations (for the dextromethorphan assay, serial dilution of ^{14}C-substrate alone was used), 70 μl of the appropriate buffer, and reactions were started by the addition 20 μl NADPH (10 mM) giving a final volume of 200 μl. Control incubations from which NADPH had been omitted were also included. After the appropriate incubation time, reactions were quenched by the addition of 50 μl of trichloroacetic acid (10% w/v).

Dilution of substrate for K_m and V_{max} assays by the RSP were programmed as follows: 1) the primary stock of each substrate (naproxen, diazepam, [^{14}C] dextromethorphan, and erythromycin) was prepared manually in acetonitrile and diluted serially by the RSP (acetonitrile) to give six secondary solutions; 2) each of these secondary solutions was further diluted 1:5 in the appropriate buffer to generate tertiary solutions; and 3) finally, 10 μl of each of the tertiary solutions were spiked into the incubation mix (200 μl total volume to give final concentrations). The final organic solvent concentration in all incubations was 1% (v/v), which demonstrated minimal inhibition (data not shown). All reactions were conducted under conditions shown to be linear with respect to time and protein concentration (Table 1).

SPE. Fully automated positive pressure SPE was performed and samples counted using the liquid scintillation analyzer as described elsewhere (Moody et al., 1999).

All determinations were performed in duplicate. Where S.D.s are presented, the experiment has been performed a minimum of three times. V_{max}, turnover numbers (TNs), and K_m values for the probe substrate reactions catalyzed by each enzyme source were calculated from both Hanes plots and by nonlinear regression analysis (Fig. 1). There was no significant difference in *Results* but the values quoted are those derived by the Hanes plots which are presented for visual clarity.

Results

***E. coli* Expression of CYP and NADPH P-450-Reductase.** Expression levels of holo-CYP determined by CO-reduced difference spectroscopy were similar to those recorded by Pritchard et al. (1997, 1998). Typical levels of spectrally active CYP (coexpressed with NADPH-reductase) recovered in *E. coli* membrane fractions were approximately 60 pmol P-450/mg protein for CYP2C19 and between 300 and 500 pmol P-450/mg protein for CYP1A2, CYP2C9, CYP2D6, and CYP3A4. NADPH-P-450 reductase activities were typically between 100 and 600 nmol cytochrome *c* reduced $\cdot$ min^{-1} $\cdot$ mg^{-1} protein.

Catalytic Activities of Recombinant CYPs. The catalytic activities of the CYPs were determined with membrane fractions of *E. coli* transformed with plasmids carrying the individual CYP and NADPH P-450-reductase. Time and protein concentration linearity profiles of all five probe assays with the different enzyme sources were determined (data not shown). Based on these data, Table 1 summarizes the experimental conditions that were adopted routinely for all assays and enzyme sources. Under these restrictions, the uppermost CYP content and incubation time (up to 15 min) were utilized to increase assay sensitivity. However, because of dissimilar CYP expression levels between enzyme sources, the overall protein concentration was variable (see Table 1).

Substrate Selectivity. Table 2 demonstrates the importance of examining substrate selectivity at several substrate concentrations. EROD and erythromycin *N*-demethylation were found to be specific reactions for CYP1A2 and CYP3A4, respectively, with minor contributions from other isoforms. Similarly, at a substrate concentration at or near the K_m for CYP2D6 (3 μM), dextromethorphan *O*-demethylation was metabolized almost exclusively by CYP2D6 (CYP2C19 activity was some 12% of that catalyzed by CYP2D6), whereas oxidation by low-affinity CYP2C isoforms was more dominant at a substrate concentration of 30 μM. The *O*-demethylation of naproxen was catalyzed principally by CYP2C9 but also by CYP1A2 and CYP2C19 (at 2 mM), whereas at a substrate concentration at or near the K_m for CYP2C9 (300 μM) catalysis by CYP1A2 was predominant. Diazepam (100 μM) was *N*-demethylated by CYP2C19 and CYP3A4 under the conditions used with negligible catalysis from the other CYPs. At 20 μM diazepam, the relative rate of metabolism by CYP3A4 was much less (approximately 30% that of CYP2C19).

Kinetics of HLM and Recombinant Human CYPs. V_{max}, TN, and K_m values for the probe substrate reactions were calculated and intrinsic clearance (Cl_{int}) values estimated from V_{max}/K_m.

Table 3 summarizes the catalytic activities and K_m measurements with different CYP sources for each reaction and compares these data with literature values. In general, all data are within the range of quoted literature values. Table 4 compares the Cl_{int} values determined from the commercial and *E. coli* source of CYPs. The activity of CYP1A2 in HLM and CYP1A2 expressed in *E. coli* and B-lymphoblasts was examined by determination of EROD activity. V_{max} and K_m values for HLM were determined to be 20 $\pm$ 3 pmol $\cdot$ min^{-1} $\cdot$ mg^{-1} and 0.5 μM, respectively. TN and K_m values for B-lymphoblasts expressing CYP1A2 were 0.4 $\pm$ 0.1 min^{-1} and 0.5 $\pm$ 0.3 μM, respectively. *E. coli* membranes expressing CYP1A2 generated similar values to B-lymphoblasts (TN of 0.6 $\pm$ 0.2 min^{-1} and K_m of 1.0 $\pm$ 0.3 μM).

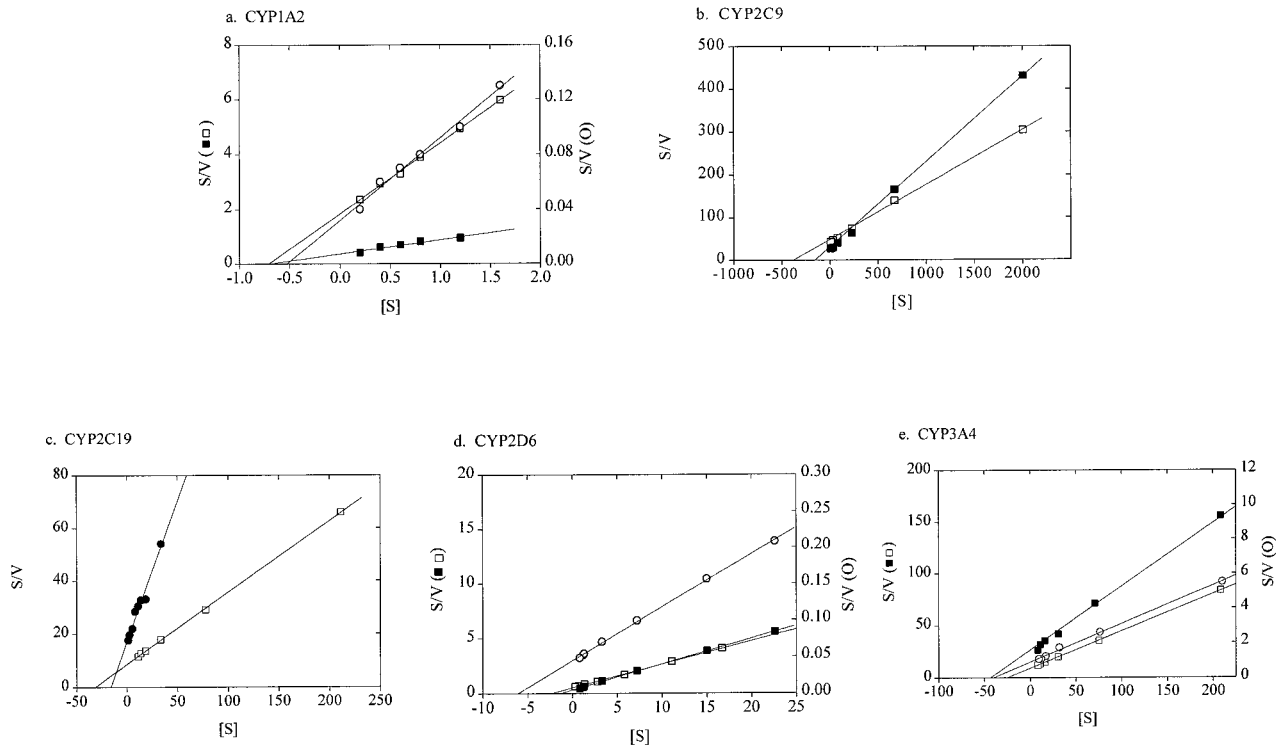

FIG. 1. *Hanes plots of the five probe assays using different enzyme sources.*

The kinetic determination was carried out as described in *Materials and Methods*. The activity of the isoforms at different substrate concentrations for probe reactions. a, CYP1A2-EROD. b, CYP2C9-naproxen *O*-demethylation. c, CYP2C19-diazepam *N*-demethylation. d, CYP2D6-dextromethorphan *O*-demethylation. e, CYP3A4-erythromycin *N*-demethylation. Full kinetic plots were determined using *E. coli* membranes expressing each individual CYP (□), B-lymphoblasts expressing CYP1A2, CYP2C9, CYP2D6, and CYP3A4 (■), baculosomes expressing CYP2C19 (●), and for the three specific probe reactions (CYP1A2, CYP2D6, and CYP3A4) HLM (○). Data shown are from one experiment typical of at least three, performed in duplicate.

TABLE 2

Selectivity of probe substrate catalysis by the five major human hepatic CYPs

Probe Reaction	Substrate Concentration	*V*				
		CYP1A2	CYP2C9	CYP2C19	CYP2D6	CYP3A4
				pmol · min^{-1} *· pmol*$^{-1}$		
EROD	3	0.40	0.01	0.05	ND	ND
	1	0.30	ND	ND	ND	ND
Naproxen *O*-demethylation	2000	3.90	6.80	4.80	0.08	ND
	300	3.46	2.36	0.87	ND	ND
Diazepam *N*-demethylation	100	ND	0.03	2.61	ND	1.43
	20	ND	0.02	1.47	ND	0.48
Dextromethorphan *O*-demethylation	30	ND	0.67	1.81	4.00	0.04
	3	ND	0.03	0.21	2.90	ND
Erythromycin *N*-demethylation	300	ND	0.09	0.23	0.31	2.59
	40	ND	ND	0.03	0.03	1.62

ND not detectable (< 0.005 min^{-1}).
Enzyme sources were *E. coli* membranes expressing CYP1A2, CYP2C9, CYP2C19 (single plasmid), CYP2D6, and CYP3A4 as described in *Materials and Methods*.

Naproxen *O*-demethylation was used as the probe reaction for CYP2C9. Because the involvement of multiple CYPs had been proposed previously (Tracy et al., 1997) and the overall shape of the HLM Michaelis-Menten plot was suggestive of multienzyme catalysis (data not shown), the data were fitted to a two-enzyme model. The estimated V_{max} and K_m values for the high-affinity component were 350 pmol · min^{-1} · mg^{-1} and 400 μM and 600 pmol · min^{-1} · mg^{-1} and 2 mM for the low-affinity component. The TN and K_m values for naproxen *O*-demethylation by B-lymphoblasts expressing CYP2C9 were 4.9 min^{-1} and 116 μM, respectively; *E. coli* membranes expressing CYP2C9 generated a higher TN (6.7 ± 0.9 min^{-1}) and K_m of 307 ± 65 μM.

Diazepam *N*-demethylation was used as the probe reaction for CYP2C19 (Ono et al., 1996; Jung et al., 1997). Again the overall shape of the HLM Michaelis-Menten plot was suggestive of multienzyme catalysis (data not shown) and the data were fitted to a two-enzyme model. The estimated V_{max} and K_m values were 350 pmol · min^{-1} · mg^{-1} and 100 μM for the high-affinity component and 1400 pmol · min^{-1} · mg^{-1} and 1.5 mM for the low-affinity component. The TN and K_m values for diazepam *N*-demethylation by baculosomes expressing CYP2C19 were 1.0 ± 0.2 min^{-1} and 17 ± 2 μM, respectively. The TN for B-lymphoblasts expressing solely CYP2C19 was markedly lower (0.2 min^{-1}) than for the cell line coexpressing CYP2C19 and cytochrome b_5 (1.6 min^{-1}) and similar to CYP2C19 and human NADPH-P-450 reductase expressed on separate plasmids in *E. coli* (TN = 0.1 min^{-1}). Membranes isolated from this

TABLE 3

Comparison of turnover number and K_m values determined in our laboratory with literature values for the five major human hepatic CYPs

Isoform	CYP Source	Astra Charnwood		Literature		References
		TN	K_m	TN	K_m	
		min^{-1}	μM	min^{-1}	μM	
	HLM	20 ± 3[a]	0.5 ± 0	1–1400[a]	0.2–0.5	Clarke et al., 1994; Pastrakuljic et al., 1997; Williams et al., 1986
CYP1A2	B-lymphoblasts	0.4 ± 0.1	0.5 ± 0.3			
	E. coli membranes	0.6 ± 0.2	1.0 ± 0.3			
	Other			0.7–2.5	0.01–1.7	Fisher et al., 1992; Eugster et al., 1993; Sandhu et al., 1993; Iwata et al., 1998
	HLM	350, 600[a]	400, 2000	538–4100[a]	92–160	Miners et al., 1996; Tracy et al., 1997; Rodrigues et al., 1996
CYP2C9	B-lymphoblasts	4.9	116	11, 41	430, 341	Tracy et al., 1997; Rodrigues et al., 1996
	E. coli membranes	6.7 ± 0.9	307 ± 65			
	HLM	350, 14000[a]	100, 1500	(267[b], 1400, 2120)[a]	20[b], 184, 557	Yasumori et al., 1993; Andersson et al., 1994; Zomorodi and Houston, 1996
	Baculosomes	1.0 ± 0.2	17 ± 2			
CYP2C19	B-lymphoblasts − b5	0.23 ± 0.08	51, 69			
	B-lymphoblasts + b5	1.6				
	E. coli membranes[c]	0.1				
	E. coli membranes[d]	3.7 ± 0.3	31 ± 3			
	Other			1.8, 2.3	21, 32	Jung et al., 1997; Ono et al., 1996
	HLM	122 ± 32[a]	5.9 ± 0.4	58–986[a,b]	2.2–9.4[b]	Gorski et al., 1994; Kerry et al., 1994; Dayer et al., 1989; Ching et al., 1995; Jacqz-Aigrain et al., 1993; Schmider et al., 1997
CYP2D6	B-lymphoblasts	4.4 ± 0.1	1.3 ± 0.1			
	E. coli membranes	4.7 ± 0.1	2.3 ± 0.2			
	Other			0.5	5.4	Ching et al., 1995
	HLM	205 ± 151	57 ± 27	63–2000[a]	44–88	Riley and Howbrook 1998; Wang et al., 1997; Gorski et al., 1994
CYP3A4	B-lymphoblasts	1.6 ± 0.1	38 ± 6	130 ± 6[a], 220 ± 10[a]	33 ± 4, 46 ± 5	Riley and Howbrook, 1998; Wang et al., 1997
	E. coli membranes	2.5 ± 0.1	18 ± 1			

[a] values in pmol/min/mg.
[b] reflects K_m and V_{max} of high-affinity component.
[c] double-plasmid system.
[d] single-plasmid system.
Probe reactions for isoforms indicated were CYP1A2-EROD, CYP2C9-naproxen *O*-demethylation, CYP2C19-diazepam *N*-demethylation, CYP2D6-dextromethorphan *O*-demethylation, and CYP3A4 *N*-demethylation.

TABLE 4

Comparison of Cl_{int} between commercial enzyme sources and E. coli membranes expressing CYPs

Probe Reaction	Isoform	Cl_{int}	
		Commercial Source	*E. coli* Membranes
		$\mu l \cdot min^{-1} \cdot nmol^{-1}$	
EROD	CYP1A2	800	600
Naproxen *O*-demethylation	CYP2C9	40	20
Diazepam *N*-demethylation	CYP2C19	60	120
Dextromethorphan *O*-demethylation	CYP2D6	340	200
Erythromycin *N*-demethylation	CYP3A4	40	140

$Cl_{int} = V_{max}/K_m$, calculated from the values in Table 3. Commercial sources of CYPs were B-lymphoblasts expressing CYP1A2, 2C9, 2D6, and 3A4, and baculosomes expressing CYP2C19.

strain typically had a NADPH-P-450 reductase activity of 100 nmol min^{-1} mg^{-1}. However, the TN in *E. coli* containing CYP2C19 and human NADPH-P-450 reductase expressed on a single plasmid was approximately 40-fold higher (3.7 min^{-1}) and the K_m was similar to the other cell lines (31 ± 3 μM). The activity of NADPH-P-450 reductase in this strain was typically 500 nmol · min^{-1} · mg^{-1}. These values correlate well with diazepam *N*-demethylase activity reported in $HepG_2$ cells expressing CYP2C19 (TN of 1.8 min^{-1} and K_m of 21 μM) (Jung et al., 1997) and in *E. coli* cells expressing CYP2C19 (TN of 2.3 min^{-1} and K_m of 32 μM; Ono et al., 1996).

Dextromethorphan *O*-demethylation was used as the probe reaction for CYP2D6 and the V_{max} and K_m values for HLM were determined to be 122 ± 32 pmol · min^{-1} · mg^{-1} and 5.9 ± 0.4 μM, respectively. TN and K_m values for B-lymphoblasts expressing CYP2D6 were 4.4 ± 0.1 min^{-1} and 1.3 ± 0.1 μM, respectively. *E. coli* membranes expressing CYP2D6 generated a TN (4.7 ± 0.1 min^{-1}) similar to B-lymphoblasts and a K_m value of 2.3 ± 0.2 μM.

The activity of CYP3A4 in HLM, *E. coli*, and B-lymphoblasts was examined by determining erythromycin *N*-demethylation. V_{max} and K_m values for HLM were calculated to be 205 ± 151 pmol · min^{-1} · mg^{-1} and 57 ± 27 μM, respectively ($n = 3$ pooled preparations). TN and K_m values for B-lymphoblasts expressing CYP3A4 in 0.1 M phosphate pH 7.4 were 1.6 ± 0.1 min^{-1} and 38 ± 6 μM, respectively. Similar values were obtained in TSE buffer (1.6 ± 0.1 min^{-1} and 36 ± 5 μM). However, CYP3A4 expressed in *E. coli* membranes appeared markedly more active in 0.1 M phosphate pH 7.4 (TN of 2.5 ± 0.1 min^{-1}, K_m of 18 ± 1 μM) compared with TSE buffer (0.32 ± 0.04 min^{-1}), although the K_m (26 ± 13 μM) remained constant.

Discussion

This study describes the characterization and validation of the five major drug-metabolizing human hepatic CYPs functionally coexpressed with human NADPH P-450-reductase in *E. coli*. This laboratory has recently developed fully automated inhibition screens for these enzymes using both HLM and recombinant human CYPs and has demonstrated that their inhibition characteristics are remarkably similar (Moody et al., 1999). This report now details an efficient investigation into the kinetic properties of CYP1A2, 2C9, 2C19, 2D6,

and 3A4 expressed in *E. coli* using this automated technology and comparison of the data with results obtained for HLM, recombinant CYPs from the major commercial sources, and the wider literature.

For the last 20 to 30 years the predominant in vitro tool for drug metabolism studies has been HLM, which have supplied qualitative (e.g., identifying which CYP isoform metabolizes the compound of interest) and quantitative (e.g., predicted Cl_{int}) information. Recent improvements in transplant technology suggest there will no longer be an inexhaustible supply of good quality human liver for the increasing amount of drug metabolism studies required by the pharmaceutical industry. As the biochemistry of the CYP family of enzymes is more fully understood, increased emphasis on cloning, expression, and the subsequent use of these enzymes is commonplace. Although initial use of expressed enzymes in vitro was as confirmation of HLM data (Aoyama et al., 1990; Tassaneeyakul et al., 1992; Rodrigues et al., 1994), sufficient confidence has recently been gained for recombinant CYP isoforms to be proposed as a first line approach (Becquemont et al., 1998; Moody et al., 1999). A major advantage of using recombinant enzymes is the potential to have a relatively low cost, inexhaustible supply of enzymes. However, assurance is required that such recombinant enzymes are faithful surrogates for HLM CYPs.

In general, the apparent K_m values, V_{max}, and Cl_{int} estimates agreed very well for the different sources of recombinant enzymes. For isoform-specific substrates such as ethoxyresorufin, erythromycin, and at low concentrations, dextromethorphan, the apparent K_m values were also equivalent to HLM and available literature data (Tables 3 and 4), demonstrating that nonspecific binding appears to be consistent for the substrates studied. The specificity of each probe reaction at high and low substrate concentrations was also determined for the five major drug-metabolizing human CYPs expressed in *E. coli*. At a substrate concentration at or near the K_m for CYP1A2, EROD catalysis by the other major human hepatic CYP isoforms studied is negligible and this reaction is specific for CYP1A2. Indeed, under V_{max} conditions for CYP1A2, of the isoforms studied, only CYP2C19 catalyzed this reaction to a minor extent (approximately 10% of CYP1A2).

In HLM, naproxen *O*-demethylation was characterized by a two-enzyme model, although this may be an oversimplification. Indeed, Korzekwa et al. (1998) have defined the atypical kinetics observed with naproxen metabolism by CYP2C9 in much more detail and invoked simultaneous binding of more than one substrate molecule to the enzyme, which was not apparent from our more limited data. Nevertheless, using recombinant CYP2C9 in *E. coli* membranes, the apparent K_m and TN obtained compared well with the high-affinity HLM component and other expression systems (Table 3). Detailed kinetic analysis suggested that CYP1A2 may exhibit a lower K_m for naproxen *O*-demethylation than CYP2C9 but also a lower V_{max}, a result in agreement with Tracy et al. (1997). Thus naproxen *O*-demethylation is suitable as a probe reaction for analysis of recombinant CYP2C9 but, even at low concentrations, not as a specific marker for CYP2C9 activity in HLM. The kinetics of the specific CYP2C9 probe, tolbutamide 4-hydroxylation, were also shown to be comparable in *E. coli* membranes (TN 2.5 min^{-1} and K_m 179 μM) and B-lymphoblasts (TN 2.8 min^{-1} and K_m 75 μM) expressing CYP2C9 and agreed well with literature values (data not shown).

Interestingly, initial data obtained with the Gentest materials indicated that diazepam *N*-demethylation might be highly dependent on the presence of cytochrome b_5. Moreover, the apparent K_m and TN for the *E. coli*-expressed CYP2C19 originating from the one plasmid construct compared well with the commercial baculovirus expression system coexpressing rabbit reductase, which suggested a pivotal role of the substantially greater NADPH P-450-reductase levels in the former system. Indeed, in vitro activities of the CYP2C family have been reported previously to be highly dependent on the supply of electrons via reductase and b_5 (Richardson et al., 1995; Crespi and Miller, 1997; Chang et al., 1997; Venkatakrishnan et al., 1998). Diazepam *N*-demethylation, under V_{max} conditions for the high-affinity CYP2C19, is catalyzed significantly by CYP3A4. At a substrate concentration nearer the pharmacological levels (20 μM) metabolism by CYP3A4 was still apparent. As the relative abundance of CYP3A may be up to 20-fold higher than CYP2C19 in an average human liver (Shimada et al., 1994; Jung et al., 1997; Inoue et al., 1997), the contribution of CYP3A4 to diazepam *N*-demethylation in HLM may be significant depending on the relative levels of these two enzymes. In HLM, two apparent K_m and V_{max} values for this reaction were determined by fitting the data to a simple two-enzyme model confirming the complex, multienzyme catalysis of diazepam *N*-demethylation in HLM (Yasumori et al., 1993; Andersson et al., 1994; Zomorodi and Houston, 1996).

Dextromethorphan was *O*-demethylated by CYP2D6, CYP2C19, and CYP2C9 under V_{max} conditions for CYP2D6. However, at a substrate concentration at or near the K_m for CYP2D6 (3 μM) the rate of dextromethorphan *O*-demethylation by CYP2C19 was approximately one-tenth of CYP2D6 activity whereas the catalysis by CYP2C9 is negligible. Thus at low dextromethorphan concentrations, this reaction would be almost exclusively CYP2D6-dependent in HLM. However, the low-affinity CYP2C19 (Moody et al., 1999) and CYP2C9 may represent the residual dextromethorphan *O*-demethylation pathway in poor metabolizers (Kroemer and Eichelbaum, 1995; Mahgoub et al., 1977).

This report confirms that erythromycin *N*-demethylation is catalyzed almost exclusively by the CYP3A family (Cook et al., 1993). Indeed, at a substrate concentration of 40 μM ($\sim K_m$ for CYP3A4), catalysis by the other human hepatic CYPs studied was negligible. Interestingly, erythromycin *N*-demethylase activity in *E. coli* membranes expressing CYP3A4 was reduced in TSE buffer (0.32 ± 0.04 min^{-1}) compared with 0.1 M phosphate pH 7.4 (2.5 ± 0.1 min^{-1}). Other reports have also determined CYP3A4 activities to be dependent on the buffer composition (Maenpaa et al., 1998) and yet this phenomenon appears to be substrate (Shet et al., 1993; Yamazaki et al., 1995) and expression system-specific (see Table 3). The selective effect on TN may indicate that the buffer composition alters the local membrane environment and electron flow via the interaction with NADPH P-450-reductase rather than effecting a conformational change of the CYP3A4 active site (Ingelman-Sundberg et al., 1996; Table 3). Indeed, the critical nature of this interaction was suggested further with data from a one-plasmid CYP3A4 system, which exhibited much higher levels of human NADPH P-450-reductase (1600 $nmol \cdot min^{-1} \cdot mg^{-1}$). This recombinant system showed nonlinear product formation with respect to time for several substrates, perhaps as a result of excess reactive oxygen species production (data not shown).

In conclusion, this paper demonstrates the characterization and validation of the five major drug-metabolizing human hepatic CYPs coexpressed with human NADPH P-450-reductase in *E. coli*. These expression systems have been validated with respect to five probe assays using predominantly marketed drug substrates and expedited by automation. The kinetic parameters of these recombinant enzymes were similar to their HLM counterparts, where directly comparable, and thus they would appear to be faithful surrogates. In addition, the activities of the optimized *E. coli*-expressed CYPs are equal to or even greater than the most widely used commercial sources. The data also suggest a pivotal role for the levels of coexpressed human NADPH P-450-reductase expression, which may be isoform- and substrate-dependent. These data make available this panel of enzymes for more

detailed biochemical and pharmacological investigations and enhanced throughput screening. This laboratory has already advocated these materials as a first line screen for early CYP inhibition analysis, and work to expand their applications in the prediction of the routes and rates of human oxidative metabolism is ongoing.

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