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VALIDATED ASSAYS FOR HUMAN CYTOCHROME P450 ACTIVITIES

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ABSTRACT:

The measurement of the effect of new chemical entities on human cytochrome P450 marker activities using in vitro experimentation represents an important experimental approach in drug development. In vitro drug interaction data can be used in guiding the design of clinical drug interaction studies, or, when no effect is observed in vitro, the data can be used in place of an in vivo study to claim that no interaction will occur in vivo. To make such a claim, it must be assured that the in vitro experiments are performed with absolute confidence in the methods used and data obtained. To meet this need, 12 semiautomated assays for human P450 marker substrate activities have been developed and validated using approaches described in the GLP (good laboratory practices) as per the code of U.S. Federal Regulations. The assays that were validated are: phenacetin *O*-deethylase (CYP1A2), coumarin 7-hydroxylase (CYP2A6), bupropion hydroxylase (CYP2B6), amodiaquine *N*-deethylase (CYP2C8), diclofenac 4′-hydroxylase and tolbutamide methylhydroxylase (CYP2C9), (*S*)-mephenytoin 4′-hydroxylase (CYP2C19), dextromethorphan *O*-demethylase (CYP2D6), chlorzoxazone 6-hydroxylase (CYP2E1), felodipine dehydrogenase, testosterone 6β-hydroxylase, and midazolam 1′-hydroxylase (CYP3A4 and CYP3A5). High-pressure liquid chromatography-tandem mass spectrometry, using stable isotope-labeled internal standards, has been applied as the analytical method. This analytical approach, through its high sensitivity and selectivity, has permitted the use of very low incubation concentrations of microsomal protein (0.01–0.2 mg/ml). Analytical assay accuracy and precision values were excellent. Enzyme kinetic and inhibition parameters obtained using these methods demonstrated high precision and were within the range of values previously reported in the scientific literature. These methods should prove useful in the routine assessments of the potential for new drug candidates to elicit pharmacokinetic drug interactions via inhibition of cytochrome P450 activities.

Drug-drug interactions are of great interest to scientists involved in drug research, regulatory authorities who are responsible for public safety, physicians, and their patients. Since "polypharmacy," or the practice of simultaneous prescription of more than one drug to treat one or more conditions in a single patient, has become a more common practice, drug interactions have been cited as one of the major reasons for hospitalization and even death (Lazarou et al., 1998). Thus, a great deal of effort is expended by researchers engaged in new drug research in avoiding the development of compounds that will cause drug-drug interactions.

The most common mechanism underlying drug-drug interactions is the inhibition of cytochrome P450 activities. Several drugs in common use cause large increases in exposure to other drugs. Examples include ketoconazole, itraconazole, erythromycin, clarithromycin, diltiazem, and nefazodone (CYP3A); quinidine, paroxetine, and terbinafine (CYP2D6); ticlopidine (CYP2C19); enoxacin (CYP1A2); and sulfaphenazole (CYP2C9); with some drugs possessing the potential to inhibit more than one P450 enzyme: fluconazole (CYP2C9 and CYP2C19) and fluvoxamine (CYP1A2 and CYP2C19). In early drug research efforts, focus has been on the development of high-throughput assays for major drug-metabolizing enzymes to avoid progression of new chemical entities that will possess a high potential to cause drug-drug interactions and to develop structure-activity relationships useful in the design of alternate agents that will lack this potential. In this research phase, speed is important, and high-throughput approaches that use fluorogenic substrates to measure P450[1] activities have been described (Crespi and Stresser, 2001; Cohen et al., 2003), as well as "cocktail" experiments that simultaneously measure more than one P450 activity (Bu et al., 2000; Yin et al., 2000; Dierks et al., 2001).

In the later drug development stages, definitive in vitro drug interaction data are needed for clinical drug interaction study planning and for supplementing drug product labeling. Because these data can directly affect patient safety, they need to be of the highest quality possible and therefore collected in a thorough and rigorous manner (Bajpai and Esmay, 2002; Kremers, 2002). Consensus built among regulatory authorities, academic researchers, and researchers from the pharmaceutical industry has included the claim that in vitro drug interaction data can supplant the need for clinical drug interaction studies (Davit et al., 1999; Tucker et al., 2001; Yuan et al., 2002). Although it has not been stated outright that collection of such data should be subject to GLP, it has been stated that in vitro drug interaction data gathered by pharmaceutical research organizations could be subject to audit by regulatory authorities (Tucker et al., 2001). It is therefore advisable that the collection of in vitro drug interaction data that support label claims and inform prescribers and patients be done at least "in the spirit" of GLP (Bjornsson et al., 2003)

[1] Abbreviations used are: P450, cytochrome P450; GLP, good laboratory practices; PPP, 2-phenyl-2-(1-piperdinyl)propane; HPLC, high-pressure liquid chromatography; MS, mass spectrometry; LC-MS/MS, liquid chromatography/tandem mass spectrometry; HLM, human liver microsomes.

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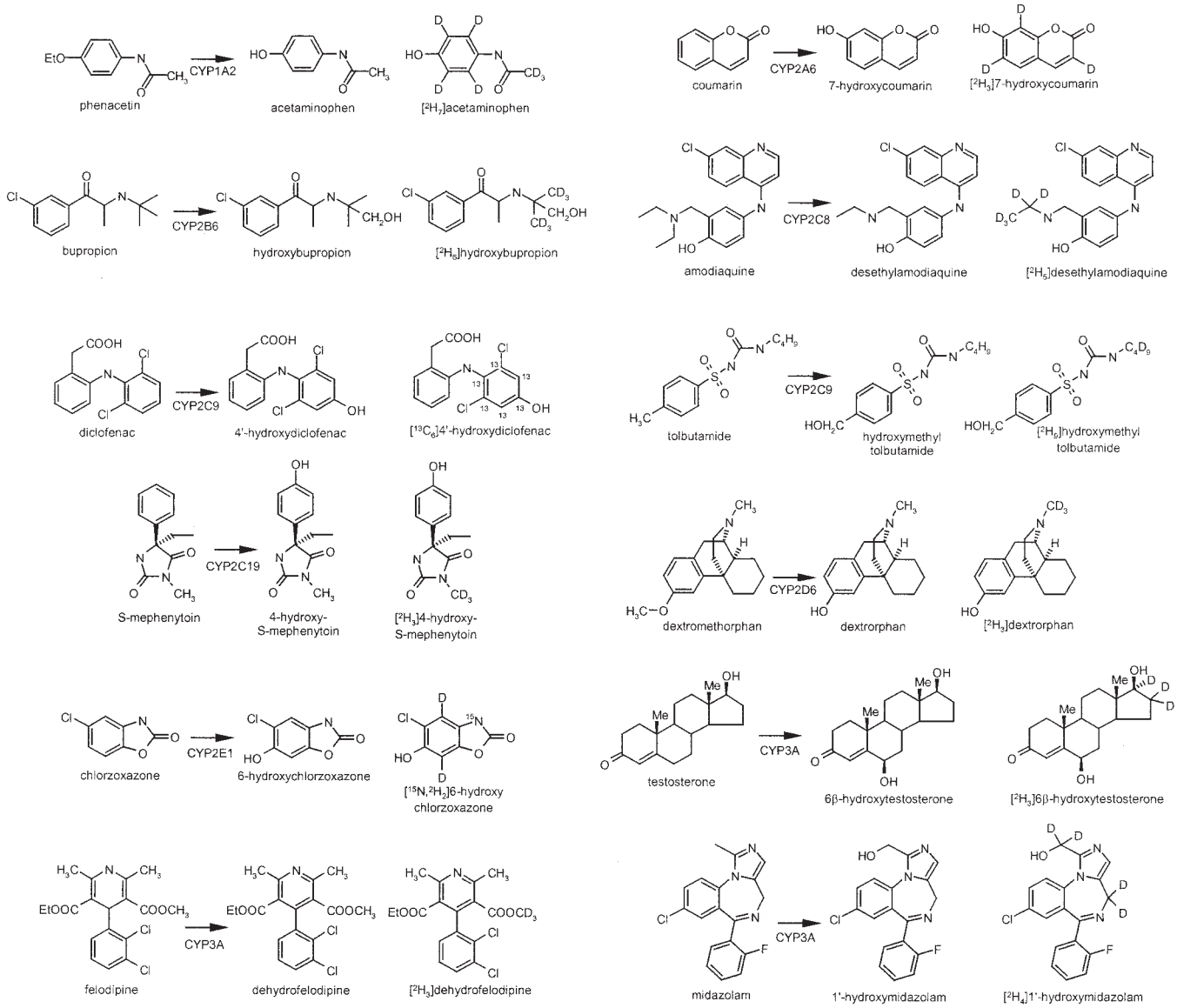

FIG. 1. *Reactions catalyzed by human cytochrome P450 enzymes.*

if not entirely compliant with GLP (Bajpai and Esmay, 2002). This means that materials and standards used are of defined and documented purity, standard operating procedures are defined for the in vitro incubation procedures, analytical methods are validated with defined assay characteristics (e.g., intra- and interassay accuracy and precision, demonstration of sample stability throughout the assay procedure, definition of analyte and internal standard recovery, demonstration of lack of assay interferences), instrumentation used in the analysis is subject to defined maintenance schedules and operates to predefined specifications, and that all data, electronic and otherwise, are documented in a readily traceable data trail (21 Code of Federal Regulations, parts 11 and 58). Although useful, good quality information can still be gathered on the inhibition of P450 enzymes in the absence of adherence to GLP, application of such practices can provide the highest possible assurance of the integrity of the data and a readily verifiable data audit trail.

In this report, we describe 12 validated assays for 10 human cytochrome P450 enzymes that are most commonly involved in the metabolism of drugs and that are most frequently subject to inhibition, which can result in drug-drug interactions (Fig. 1). Tandem mass spectrometry is used as the detection method, affording highly selective, sensitive assays with low limits of quantitation that are significantly improved over previously described assays that use ultraviolet or fluorescence detection. This has permitted lowering the concentration of microsomes in the incubation procedures, which can reduce nonspecific binding, a source of inaccuracy in inhibition and enzyme kinetic constants (Obach, 1997; Kalvass et al., 2001; Margolis and Obach, 2003). Also reported are enzyme kinetic constants for each of these reactions that were gathered using these validated methods.

Materials and Methods

Materials. Substrates, metabolite standards, internal standards, inhibitors, and other materials were obtained from the following sources: (*S*)-mephenytoin and tranylcypromine (BIOMOL Research Laboratories, Plymouth Meeting, PA); *N*-desethylamodiaquine, [$^2H_5$]*N*-desethylamodiaquine, [$^2H_3$]dextrorphan, [$^2H_3$]7-hydroxycoumarin, [$^2H_2{}^{15}N$]6-hydroxychlorzoxazone, hydroxymethyltolbutamide, [$^2H_3$]6β-hydroxytestosterone, and midazolam (Cerilliant Corp., Austin, TX); ammonium formate (purum), formic acid (purum), phenacetin, and quinidine (Fluka, Buchs, Switzerland); acetaminophen, chlorzoxazone, coumarin, dextromethor-

TABLE 1

Incubation conditions and analytical parameters for CYP1A2, 2A6, 2B6, and 2C8 assays

	Phenacetin *O*-Deethylase (CYP1A2)	Coumarin 7-Hydroxylase (CYP2A6)	Bupropion Hydroxylase (CYP2B6)	Amodiaquine *N*-Deethylase (CYP2C8)[a]
Incubation conditions				
Pooled human liver microsomes				
Protein concentration (mg/ml)	0.03	0.03	0.05	0.025
Incubation time (min)	30	6	20	10
Recombinant P450				
P450 concentration (pmol/ml)	1.2	2.1	1.8	1.7
Incubation time (min)	20	6	20	10
Analytical conditions				
Analyte	Acetaminophen	7-Hydroxycoumarin	Hydroxybupropion	Desethylamodiaquine
Internal standard: identity	[$^2H_7$]Acetaminophen	[$^2H_3$]7-Hydroxycoumarin	[$^2H_6$]Hydroxybupropion	[$^2H_5$]Desethylamodiaquine
Internal standard: final conc. (μM)	2.0	0.40	0.80	0.75
Injection volume (μl)	20	20	10	10
Mobile phase				
System	1	1	1	2
Gradient program, %B(min)	2(0)→2 (0.5)→98(2.5)	2(0)→2(0.5)→71(2.6)	2(0)→2(0.5)→71(2.7)	2(0)→8(0.5)→18(2.7)
Mass spectrometer conditions				
Mode	Positive	Negative	Positive	Positive
Capillary voltage	1.25 kV	−2.00 kV	2.00 kV	2.75 kV
Cone voltage	50 V	−50 V	30 V	40 V
Desolvation temperature	325°C	325°C	325°C	325°C
Source temperature	110°C	110°C	110°C	110°C
Collision energy	15 eV	22 eV	26 eV	20 eV
Analyte *m/z* transition	152→110	161→133	256→139	328→283
Internal standard *m/z* transition	159→115	164→136	262→139	333→283
R_t (min)	1.6	2.2	2.0	1.8
Standard curve range (nM)	25–10000	10.0–2000	5.00–2000	10.0–1500

[a] The amodiaquine method required injection syringe washes of 50/50 dimethyl sulfoxide/water to prevent assay carryover.

phan, dextrorphan, diclofenac, furafylline, 6-hydroxychlorzoxazone, 7-hydroxycoumarin, 6β-hydroxytestosterone, quercetin, sulfaphenazole, testosterone, ticlopidine, and tolbutamide (Sigma-Aldrich, St. Louis, MO); bupropion, hydroxybupropion, [$^2H_6$]hydroxybupropion, (*S*)-4′-hydroxymephenytoin (Syncom, Groningen, Netherlands); ketoconazole and NADPH (ICN Biomedicals, Aurora, OH); [$^2H_7$]acetaminophen, amodiaquine, dehydrofelodipine, (+)-*N*-3-benzylnirvanol, 4′-hydroxydiclofenac, [$^2H_9$]hydroxymethyltolbutamide, 1′-hydroxymidazolam, [$^2H_4$]1′-hydroxymidazolam, [$^{13}C_6$]4′-hydroxydiclofenac, [$^2H_3$]dehydrofelodipine, [$^2H_3$](*S*)-4′-hydroxymephenytoin, and PPP were prepared at Pfizer, Groton, CT. Felodipine was isolated from Plendil tablets (Astra Pharmaceuticals, Wayne, PA) obtained from a pharmacy. Purities were either defined by the manufacturer or, when made by custom synthesis, were subject to rigorous and defined annual analytical evaluation to provide analytical test notes. Other reagents and solvents used were from standard suppliers and were of reagent or HPLC grade. Human liver microsomes were prepared using standard procedures and represent a pool of samples from 54 individual donors. Recombinant P450 enzymes were heterologously expressed in Sf9 cells under contract with PanVera Corp. (Madison, WI). In this system NADPH/P450 oxidoreductase was coexpressed, and ratios of reductase/P450 ranged from 0.11 to 3.7. In rCYP2A6, 2C8, and 2E1, cytochrome b_5 was also coexpressed at ratios of b_5/P450 of 3.4, 1.3, and 0.9, respectively. Protein concentration was determined using the bicinchoninic assay as defined by the manufacturer (Pierce Chemical, Rockford, IL). Cytochrome P450 determinations were made using the method of Omura and Sato (1964).

Instrumentation. All analytical methods were conducted by HPLC-MS/MS. The LC-MS/MS system comprised a Micromass Quattro Ultima triple quadrupole mass spectrometer equipped with an electrospray ionization source (Micromass, Beverly, MA), two LC-10ADvp pumps with a SCL-10ADvp controller and DGU-14 solvent degasser (Shimadzu, Columbia, MD), a LEAP CTC HTS PAL autosampler with a multisolvent peristaltic self-washing system (CTC Analytics, Carrboro, NC) and a LabPro switching valve (Rheodyne LLC, Rohnert Park, CA). For 11 of the assays, HPLC separation was achieved using a LUNA C18(2) 3.00 × 30 mm 5-μm column (Phenomenex, Torrance, CA). The amodiaquine *N*-deethylase assay used an alternate HPLC column: (YMC C4, 3.00 × 50 mm, 5 μm; Waters, Milford, MA). Gradient elution at a flow rate of 0.5 ml/min was performed using one of the following mobile phase systems: system 1, A = 5 mM ammonium formate containing 0.05% formic acid and B = 95:5 acetonitrile/methanol containing 0.05% formic acid; system 2, A = 5 mM ammonium formate containing 0.3% formic acid and B = 95:5 acetonitrile/methanol containing 0.3% formic acid. Flow was diverted from the mass spectrometer to waste for the first minute of the gradient to remove nonvolatile salts.

Incubation Conditions: General. Specific aspects of the incubation condition for each assay (e.g., protein concentration, incubation time, reaction termination solvent) are defined in Tables 1 to 3. In general, microsomes at protein concentrations as defined in Tables 1 to 3 were mixed with buffer (100 mM KH_2PO_4, pH 7.4), $MgCl_2$ (3.3 mM), and substrate, and warmed to 37° ± 0.4°C in a 96-well temperature-controlled heater block. Aliquots of this mixture (0.18 ml) were delivered to each well of a 96-well polypropylene polymerase chain reaction plate maintained at 37°C, followed by addition of the inhibitor or control solvent (mixture of water and CH_3CN) as applicable. Final solvent concentrations were 1% (v/v) or less. Incubations were commenced with the addition of NADPH stock (assay concentration, 1.3 mM) to a final incubation volume of 0.2 ml and maintained at 37°C for the period defined in Tables 1 to 3. Incubations were typically terminated by acidification upon addition of 0.02 ml of termination solvent (H_2O/CH_3CN/HCOOH; 92:5:3) containing internal standard. The terminated incubation mixtures, as well as standard curve and quality control samples, composed of the same matrix materials but without NADPH or microsomes, were passed through a Millipore 96-well filtration apparatus (Millipore Corporation, Billerica, MA), containing 0.45-μm mixed cellulose membranes with mild vacuum into a receiver 96-well plate (except for the felodipine dehydrogenase assay; see below). The receiver plate was covered with a heat-sealed polypropylene film for LC-MS/MS analysis. In initial experiments in which protein concentrations and incubation times were established, incubations were conducted in a volume of 10 ml, and 0.2-ml aliquots were removed and added to 0.02 ml of termination solvents. For enzyme kinetic determinations, replicates of $n = 7$ to 8 were run at eight substrate concentrations, with the exception of midazolam in which replicates of $n = 4$ were run at 16 substrate concentrations.

Phosphate buffer (100 mM) was prepared fresh weekly and stored at ambient temperature from 100 mM mono- and dibasic potassium phosphate stock solutions that were prepared fresh every 6 months and stored at 4°C. Buffer pH was verified and adjusted daily as needed. $MgCl_2$ stock solution as a 50-fold concentrated stock was prepared fresh every 6 months and stored at 4°C. Microsomes were stored at −80°C and thawed immediately before use. Frozen stocks of liver microsomes were used twice with remaining material

TABLE 2

Incubation conditions and analytical parameters for CYP2C9, 2C19, and 2D6 assays

	Diclofenac 4′-Hydroxylase (CYP2C9)	Tolbutamide Methylhydroxylase (CYP2C9)	(*S*)-Mephenytoin 4′-Hydroxylase (CYP2C19)	Dextromethorphan *O*-Demethylase (CYP2D6)
Incubation conditions				
Pooled human liver microsomes				
Protein concentration (mg/ml)	0.03	0.1	0.2	0.03
Incubation time (min)	10	20	40	10
Recombinant P450				
P450 concentration (pmol/ml)	1.6	7.7	3.7	1.2
Incubation time (min)	10	10	20	14
Analytical conditions				
Analyte	4′-Hydroxydiclofenac	4-Hydroxytolbutamide	4′-Hydroxymephenytoin	Dextrorphan
Internal standard: identity	[$^{13}C_6$]4′-Hydroxydiclofenac	[$^2H_9$]4-Hydroxytolbutamide	[$^2H_3$]4′-Hydroxymephenytoin	[$^2H_3$]Dextrorphan
Internal standard: final conc. (μM)	0.50	1.0	2.0	0.20
Injection volume (μl)	20	20	20	20
Mobile phase				
System	1	1	1	1
Gradient program, %B(min)	2(0)→40(0.5)→78(2.5)	2(0)→20(0.5)→60(2.5)	2(0)→20(0.5)→72(2.5)	2(0)→10(0.5)→38(2.5)
Mass spectrometer conditions				
Mode	Negative	Positive	Negative	Positive
Capillary voltage	−3.00 kV	3.00 kV	−3.25 kV	2.75 kV
Cone voltage	−45 V	50 V	−50 V	60 V
Desolvation temperature	300°C	325°C	300°C	300°C
Source temperature	110°C	110°C	110°C	110°C
Collision energy	12 eV	18 eV	13 eV	40 eV
Analyte *m/z* transition	310→266	287→171	233→190	258→157
Internal standard *m/z* transition	316→272	296→171	236→193	261→157
Retention time (min)	2.0	2.0	1.7	2.0
Standard curve range (nM)	10.0–2000	10.0–5000	50.0–10,000	1.00–1000

discarded after a second thawing, whereas Sf9 microsomes containing recombinant P450s were thawed and refrozen until the activity deviated 20% from the initially measured activity. NADPH stock solution was made fresh daily. Stock solutions of analytes (i.e., metabolites) were prepared in solvent and stored at −10°C or 4°C, as shown in Table 4. Stability, as defined by less than a 10% deviation in concentration from that measured on the day of preparation, was established for a minimum of the storage period listed in Table 4.

Data Analysis. Standard curve fitting was accomplished with QuanLynx (ver4.0) software (Micromass). Data were typically fit to quadratic curves using $1/x^2$ weighting. Assay run acceptance was defined by the accuracy and precision of independently prepared quality control samples at three concentrations. Substrate interference quality control samples containing the highest concentration of the substrate used in the incubations were included to ensure lack of interference by the substrate in the quantitation of the metabolite. Additional quality control samples were included that contained the lowest analyte concentration in the presence of the highest concentration of a test inhibitor with microsomes, to ensure that the compound being tested as an inhibitor did not cause an interference in the assay. Substrate saturation curves and inhibition data were analyzed using the Enzyme Kinetics module of SigmaPlot ver8.0 (SPSS, Inc., Chicago, IL). Best-fit models were selected on the basis of the Aikake Information Criterion.

Results

General Aspects. In this report, we describe robust, GLP-validated bioanalytical methods for 12 human cytochrome P450 assays. Overall, interassay accuracy and precision for all assays were high: accuracies ranged from 96.6 to 108%, and precision ranged from 2.21 to 10.2% for all quality control samples (Table 5). No single assay appeared to demonstrate any greater inaccuracy or imprecision than any other. The use of tandem mass spectrometry with stable isotope-labeled internal standards are the most likely factors that afford this level of accuracy and precision. In a couple of cases, noted below, the substrate material contained trace quantities of the metabolite, so that samples containing the highest concentrations of substrate used in the assay (i.e., high substrate concentrations used in saturation curves) would show small peaks for the metabolite in blank samples.

In addition to potential analytical method variability, a potentially greater source of variability resides with the incubation method, and efforts were made to control for this. Linear conditions for each assay were established by conducting the incubation at three protein concentrations and measuring the formation of product over time. The incubation times were selected such that the reactions were linear with time, and the protein concentrations selected were the lowest such that the amounts of analyte formed were well within the dynamic range of the analytical methods. In the routine determination of IC_{50} values for test compounds, the assays are run repeatedly at the same substrate concentration, yielding a quantity of data from which interday accuracy and precision values can be calculated for the incubation method (Table 6).

Specific observations and attributes for each of the assays are listed below.

CYP1A2. An assay for phenacetin *O*-deethylase for CYP1A2 was developed and validated that is generally regarded as being selective for measurement of CYP1A2 activity (Tassaneeyakul et al., 1993). The K_m was determined at 47.0 ± 1.9 μM for pooled HLM and 16.7 ± 0.9 μM for rCYP1A2 (Table 7; Fig. 2). Corresponding values previously reported for HLM range from as low as 2.7 μM (Kobayashi et al., 1998) to 116 μM (Ching et al., 2001); however, most values range between 9 and 68 μM (Brosen et al., 1993; Tassaneeyakul et al., 1993; Bourrie et al., 1996; Schmider et al., 1996; Von Moltke et al., 1996a; Rodrigues et al., 1997; Agundez et al., 1998; Eagling et al., 1998; Kobayashi et al., 1999; Belle et al., 2000; Li et al., 2003) (Fig. 4). Furafylline yielded inhibition with IC_{50} values of 1.54 to 1.76 μM for this activity (Table 8). It should be noted that these results with furafylline were obtained using an experimental design in which the inhibitor was coincubated with the substrate. Different results can be obtained if furafylline is incubated with microsomes and NADPH before addition of substrate, since furafylline is a mechanism-based inactivator of CYP1A2.

CYP2A6. CYP2A6 is generally considered less important in drug metabolism reactions than other cytochrome P450 enzymes, although there are reports of some agents being metabolized by this enzyme.

TABLE 3

Incubation conditions and analytical parameters for CYP3A and CYP2E1 assays

	Chlorzoxazone 6-Hydroxylase (CYP2E1)	Felodipine Dehydrogenase (CYP3A)	Midazolam 1′-Hydroxylase (CYP3A)	Testosterone 6β-Hydroxylase (CYP3A)
Incubation conditions				
Pooled human liver microsomes				
Protein concentration (mg/ml)	0.05	0.01	0.03	0.03
Incubation time (min)	20	10	4	10
Recombinant P450				
P450 concentration (pmol/ml)	6.3	0.095/0.61	0.95/1.2[a]	2.4/6.1[a]
Incubation time (min)	20	10/10	4/4[a]	10/10[a]
Analytical conditions				
Analyte	6-Hydroxychlorzoxazone	Dehydrofelodipine	1′-Hydroxymidazolam	6β-Hydroxytestosterone
Internal standard: identity	[$^2H_2{}^{15}N$]6-Hydroxychlorzoxazone	[$^2H_3$]Dehydrofelodipine	[$^2H_4$]1′-Hydroxymidazolam	[$^2H_3$]6β-Hydroxytestosterone
Internal standard: final conc. (μM)	5.0	0.25	0.10	2.5
Injection volume (μl)	20	10	20	30
Mobile phase				
System	1	1	1	1
Gradient program, %B(min)	2(0)→15(0.5)→40(2.5)	2(0)→40(0.5)→98(2.8)	2(0)→28(0.5)→58(3)	2(0)→24(0.5)→36(2.6)
Mass spectrometer conditions				
Mode	Negative	Positive	Positive	Positive
Capillary voltage	−2.00 kV	3.25 kV	3.25 kV	3.65 kV
Cone voltage	−60 V	60 V	50 V	50 V
Desolvation temperature	300°C	325°C	275°C	350°C
Source temperature	110°C	110°C	110°C	110°C
Collision energy	40 eV	25 eV	21 eV	16 eV
Analyte *m/z* transition	184→120	382→354	342→324	305→269
Internal standard *m/z* transition	187→123	387→359	346→328	308→272
Retention time (min)	2.0	1.9	2.0	2.0
Standard curve range (nM)	100–4000	5.00–2000	1.00–1000	30.0–10,000

[a] Values represent those for recombinant CYP3A4/CYP3A5.

TABLE 4

Stabilities of stock solutions of the 12 analytes used in these assays

Assay	Analyte	Storage Solvent (CH_3CN/H_2O)	Storage Temperature	Established Stability[b]
			°C	*days*
CYP1A2	Acetaminophen	95/5	−10	109
CYP2A6	7-Hydroxycoumarin	50/50	4	39
CYP2B6	Hydroxybupropion	95/5	−10	43
CYP2C8	Desethylamodiaquine	50/50	4	64
CYP2C9	4′-Hydroxydiclofenac	95/5	−10	42
CYP2C9	Hydoxymethyltolbutamide	50/50	4	297
CYP2C19	4′-Hydroxymephenytoin	95/5	−10	41
CYP2D6	Dextrorphan	95/5	−10	69
CYP2E1	6-Hydroxychlorzoxazone	95/5	4	146
CYP3A	Dehydrofelodipine	90/5/5[a]	−10	43
CYP3A	1′-Hydroxymidazolam	75/5/20[a]	−10	50
CYP3A	6β-Hydroxytestosterone	95/5	−10	59

[a] ($CH_3CN/CH_3OH/H_2O$).
[b] Stability is defined as the period of time at which it has been demonstrated that the concentration of the stock solution has not deviated more than 10% of the value measured on the day when the stock was prepared.

Coumarin 7-hydroxylase, assessed via HPLC with fluorescence detection for the metabolite, has been reported to be a specific marker activity for the measurement of CYP2A6. The validated HPLC/MS method yielded Michaelis constants of 0.841 ± 0.037 for human liver microsomes and 0.826 ± 0.037 μM for rCYP2A6 (Table 7; Fig. 2), with corresponding tranylcypromine IC_{50} values of 0.449 and 0.895 μM, respectively (Table 8). Values for K_m in human liver microsomes that have been previously reported range from 0.2 to 2.4 μM (Pearce et al., 1992; Bourrie et al., 1996; Shimada et al., 1996; Draper et al., 1997; Li et al., 1997; Hickman et al., 1998; Inoue et al., 2000; Yin et al., 2000; Li et al., 2003) (Fig. 4).

CYP2B6. A bupropion hydroxylase assay was validated for CYP2B6. Bupropion has only recently been identified as a CYP2B6 marker substrate (Faucette et al., 2000; Hesse et al., 2000). K_m values of 81.7 ± 1.3 and 66.8 ± 1.4 μM were measured in human liver microsomes and rCYP2B6, respectively (Table 7; Fig. 2). Previously reported K_m values for human liver microsomes are 76, 89, and 130 μM (Faucette et al., 2000; Hesse et al., 2000; Li et al., 2003) (Fig. 4). PPP was used as a CYP2B6 inhibitor (Chun et al., 2000) and yielded IC_{50} values of 7.74 and 2.02 μM in pooled liver microsomes and rCYP2B6, respectively (Table 8). PPP is a mechanism-based inactivator of CYP2B6; however, the inhibitory potency reported here represents results from a coincubation of inhibitor and substrate. An investigation of the potency of PPP with preactivation of the inhibitor is being presently undertaken and will be reported in due course. An attempt was made to use (*S*)-mephenytoin *N*-demethylase as a marker activity for CYP2B6 (Heyn et al., 1996; Ko et al., 1998), but our early results suggested that other P450 enzymes contributed substantially to this activity (data not shown).

CYP2C8. An amodiaquine *N*-deethylase assay was developed and validated for CYP2C8 activity. A recent report showed convincing evidence of the selectivity of CYP2C8 for this activity (Li et al., 2002). Paclitaxel 6α-hydroxylase, which has been frequently used to measure CYP2C8 activity, was not chosen because of the high cost of

TABLE 5

Summary of analytical method performance characteristics for 12 human cytochrome P450 assays

Analyte	Enzyme	QC Concentrations			Mean Accuracy			Mean Precision		
		Low QC	Mid QC	High QC	Low QC	Mid QC	High QC	Low QC	Mid QC	High QC
			nM			*%*			*%*	
Acetaminophen	CYP1A2	50.0	2000	8000	108	105	103	8.17	5.06	5.47
7-Hydroxycoumarin	CYP2A6	30.0	400	1600	103	101	98.1	4.24	3.14	2.56
Hydroxybupropion	CYP2B6	15.0	200	1600	104	107	105	7.57	3.67	6.85
Desethylamodiaquine	CYP2C8	30.0	200	1200	101	101	97.1	3.76	2.21	3.70
4′-Hydroxydiclofenac	CYP2C9	30.0	800	1600	105	105	106	10.2	7.84	9.23
Hydroxytolbutamide	CYP2C9	30.0	400	4000	103	98.9	100	8.05	7.29	2.31
4′-Hydroxymephenytoin	CYP2C19	150	200	8000	100	98.1	97.8	5.57	4.37	4.04
Dextrorphan	CYP2D6	3.00	200	800	104	101	97.2	5.68	4.47	4.10
6-Hydroxychlorzoxazone	CYP2E1	200	800	1600	99.5	97.7	98.9	5.85	4.27	5.04
Dehydrofelodipine	CYP3A	15.0	200	1600	103	102	102	3.49	3.60	2.79
1′-Hydroxymidazolam	CYP3A	3.00	200	800	104	96.6	97.2	7.03	4.74	4.46
6β-Hydroxytestosterone	CYP3A	90.0	2000	8000	99.4	97.9	98.1	6.00	5.19	8.53

QC, quality control.
%Accuracy = 100 · [Analyte]$_{measured}$/[Analyte]$_{nominal}$.
%Precision = 100 · S.D./[Analyte]$_{measured,\ average}$.

TABLE 6

Interday precision for activity for assays in pooled human liver microsomes

Enzyme	Activity	Substrate Concentration	*N*	Interassay Precision of Reaction Velocity
		μM		%
CYP1A2	Phenacetin *O*-deethylase	50	47	4.7
CYP2A6	Coumarin 7-hydroxylase	1.0	28	9.8
CYP2B6	Bupropion hydroxylase	80	64	5.5
CYP2C8	Amodiaquine *N*-deethylase	1.9	97	5.3
CYP2C9	Diclofenac 4′-hydroxylase	4.0	44	5.6
CYP2C9	Tolbutamide hydroxylase	150	34	6.2
CYP2C19	(*S*)-Mephenytoin 4′-hydroxylase	60	63	8.4
CYP2D6	Dextromethorphan *O*-demethylase	5.0	60	5.6
CYP2E1	Chlorzoxazone 6-hydroxylase	75	38	7.6
CYP3A	Felodipine dehydrogenase	3.0	87	7.3
CYP3A	Midazolam 1′-hydroxylase	2.5	66	8.1
CYP3A	Testosterone 6β-hydroxylase	50	40	6.9

authentic standards. In initial assessments, *N*-desethylamodiaquine appeared to be subject to substantial injection-to-injection carry-over problems. This hurdle was overcome by ensuring that the glass syringe used for injection was thoroughly washed with organic solvent (50% dimethyl sulfoxide) between injections. Michaelis constants for amodiaquine *N*-deethylase were 1.89 ± 0.06 and 0.728 ± 0.040 μM for pooled human liver microsomes and rCYP2C8, respectively (Table 7; Fig. 2). The previously reported values for liver microsomes were 2.4 and 3.4 μM (Li et al., 2002, 2003). CYP2C8 lacks a highly selective chemical probe inhibitor. Quercetin has been used for CYP2C8 inhibition; however, it is not selective (Li et al., 1994; R. L. Walsky and R. S. Obach, unpublished observations). It can still be used as a positive control inhibitor for CYP2C8 activity, and IC_{50} values ranged between 3.06 and 3.33 μM for CYP2C8 activities (Table 8).

CYP2C9 Assays. Two assays were developed and validated for measurement of CYP2C9 activity: diclofenac 4′-hydroxylase and tolbutamide hydroxylase. Both have been well studied as probes of CYP2C9 activity, although some activity of other CYP2C enzymes for these substrates has been noted (Wester et al., 2000). The selection of more than one assay was done since there have been recently reported findings that CYP2C9 demonstrates unusual enzyme kinetic behavior (Hutzler et al., 2001, 2003). CYP2C9 catalyzed diclofenac 4′-hydroxylation at a very high rate, and the K_m values for this reaction were 4.04 ± 0.12 and 0.589 ± 0.020 μM for pooled human liver microsomes and rCYP2C9, respectively (Table 7; Fig. 2). Corresponding liver microsomal K_m values cited have ranged from 1.8 to 22 μM (Leemann et al., 1993; Transon et al., 1996; Yamazaki et al., 1998; Bort et al., 1999; Carlile et al., 1999; Aithal et al., 2000; Tang et al., 2000; Kumar et al., 2002; Li et al., 2003) (Fig. 4). Tolbutamide hydroxylase activity is much lower than diclofenac 4′-hydroxylase, with intrinsic clearance values that are 200 to 1100 times lower by virtue of both a higher K_m and a lower V_{max}. K_m values for tolbutamide in pooled liver microsomes and rCYP2C9 were 147 ± 4 and 82.0 ± 2.5 μM, respectively (Table 7; Fig. 2), as compared with previously cited values ranging from 60 to 580 μM (Darby and Price-Evans, 1971; Purba et al., 1987; Back et al., 1988; Miners et al., 1988; Doecke et al., 1991; Chen et al., 1993; Sharer et al., 1995; Bourrie et al., 1996; Inoue et al., 1997; Hickman et al., 1998; Lasker et al., 1998; Carlile et al., 1999; Hemeryck et al., 1999; Palamanda et al., 2000; Yin et al., 2000; Tang et al., 2002; Wang et al., 2002) (Fig. 4). Inhibition by sulfaphenazole was demonstrated, with IC_{50} values ranging between 0.169 and 0.277 μM for both activities (Table 8).

CYP2C19. (*S*)-Mephenytoin 4′-hydroxylase has been used as a marker activity for CYP2C19 both in vitro and in vivo (Meier et al., 1985). The mephenytoin hydroxylase assay was the only one to require a liver microsomal protein concentration in excess of 0.1 mg/ml, due to the slow rate of conversion, and a long incubation period (40 min) was required. Michaelis constants for this activity in pooled human liver microsomes and rCYP2C19 were 57.2 ± 2.2 μM and 17.3 ± 0.5 μM, respectively (Table 7; Fig. 3). Values cited for K_m in HLM have ranged from 23 to 169 μM (Jurima et al., 1985; Meier

TABLE 7

Summary of enzyme kinetic parameters (Mean ± S.E.) for 12 human cytochrome P450 activities in pooled human liver microsomes and recombinant heterologously expressed P450 enzymes

Enzyme	Assay	Pooled Human Liver Microsomes			Recombinant Heterologously Expressed Enzyme		
		Kinetic mechanism	K_m	V_{max}	Kinetic mechanism	K_m	V_{max}
			μM	*pmol/mg/min*		*μM*	*pmol/pmol P450/min*
CYP1A2	Phenacetin *O*-deethylase	Simple	47.0 ± 1.9	688 ± 10	Simple	16.7 ± 0.9	31.3 ± 0.7
CYP2A6	Coumarin 7-hydroxylase	Simple	0.841 ± 0.037	940 ± 10	Simple	0.826 ± 0.037	12.4 ± 0.2
CYP2B6	Bupropion hydroxylase	Simple	81.7 ± 1.3	413 ± 2	Simple	66.8 ± 1.4	12.9 ± 0.1
CYP2C8	Amodiaquine *N*-deethylase	Simple	1.89 ± 0.06	1480 ± 20	Simple	0.728 ± 0.040	11.2 ± 0.2
CYP2C9	Diclofenac 4′-hydroxylase	Simple	4.04 ± 0.12	1670 ± 20	Simple	0.589 ± 0.020	14.9 ± 0.1
CYP2C9	Tolbutamide hydroxylase	Simple	147 ± 4	276 ± 3	Simple	82.0 ± 2.5	11.9 ± 0.1
CYP2C19	(*S*)-Mephenytoin 4′-hydroxylase	Simple	57.2 ± 2.2	58.3 ± 0.8	Simple	17.3 ± 0.5	8.76 ± 0.07
CYP2D6	Dextromethorphan *O*-demethylase	Simple	4.64 ± 0.21	202 ± 3	Simple	0.196 ± 0.003	5.84 ± 0.02
CYP2E1	Chlorzoxazone 6-hydroxylase	Simple	73.9 ± 2.2	2360 ± 20	Simple	135 ± 5	27.3 ± 0.4
CYP3A	Felodipine dehydrogenase	Substrate inhibition	2.81 ± 0.61	1630 ± 180	Substrate inhibition	0.938 ± 0.137[a]	36.8 ± 3.0[a]
					Substrate inhibition	1.41 ± 0.17[b]	24.2 ± 2.0[b]
CYP3A	Midazolam 1′-hydroxylase	Substrate inhibition	2.27 ± 0.18	1220 ± 40	Substrate inhibition	0.622 ± 0.025[a]	16.3 ± 0.3[a]
					Substrate inhibition	1.53 ± 0.09[b]	54.6 ± 1.8[b]
CYP3A	Testosterone 6β-hydroxylase	Simple	46.4 ± 1.9	5260 ± 80	Substrate inhibition	31.4 ± 2.5[a]	210 ± 9[a]
					Substrate inhibition	106 ± 13[b]	164 ± 12[b]

[a] Mean ± S.E. for recombinant CYP3A4.
[b] Mean ± S.E. for recombinant CYP3A5.

et al., 1985; Shimada et al., 1985; Hall et al., 1987; Relling et al., 1989; Doecke et al., 1991; Chiba et al., 1993; Sharer et al., 1995; Schmider et al., 1996; Hickman et al., 1998; Coller et al., 1999; Yin et al., 2000; Li et al., 2003) (Fig. 4). (+)-*N*-3-Benzylnirvanol (Suzuki et al., 2002; Walsky and Obach, 2003) demonstrated inhibition of CYP2C19, with IC_{50} values of 0.414 and 0.161 μM for pooled liver microsomes and rCYP2C19, respectively (Table 8).

CYP2D6. For CYP2D6, many assays have been reported in the scientific literature. Dextromethorphan is a frequently used probe of CYP2D6 activity both in vitro and in vivo and was selected for validation in our studies. Considerably different K_m values were observed for dextromethorphan *O*-demethylase between pooled human liver microsomes (4.64 ± 0.21 μM) and rCYP2D6 (0.196 ± 0.003 μM) (Table 7; Fig. 2), but the reason for this is not known. Values reported for K_m for dextromethorphan *O*-demethylase in liver microsomes range from 2.8 to 22 μM (Broly et al., 1989; Dayer et al., 1989; Jacqz-Aigrain et al., 1993; Kerry et al., 1994; Bourrie et al., 1996; Schmider et al., 1996, 1997; Transon et al., 1996; Hickman et al., 1998; Von Moltke et al., 1998; Li et al., 2003) (Fig. 4). Quinidine is a frequently used inhibitor of CYP2D6, and an IC_{50} value of 0.0579 was measured in pooled liver microsomes, but the corresponding IC_{50} value was 1.78 μM for rCYP2D6 (Table 8).

CYP2E1. An assay for chlorzoxazone 6-hydroxylase was developed and validated for use as a CYP2E1 probe activity. This activity has been used as a CYP2E1 marker in both in vitro and in vivo studies (Peter et al., 1990: Kim et al., 1994). Michaelis constants for this activity were 73.9 ± 2.2 and 135 ± 5 μM for pooled human liver microsomes and rCYP2E1 coexpressed with cytochrome b_5, respectively (Table 7; Fig. 3), and IC_{50} values of 8.94 and 17.1 μM were measured for tranylcypromine (Table 8). The range of K_m values in human liver microsomes reported in the literature is 39 to 157 μM (Peter et al., 1990; Ono et al., 1995; Schmider et al., 1996; Court et al., 1997; Hickman et al., 1998; Yin et al., 2000; Lejus et al., 2002) (Fig. 4).

CYP3A Assays. Three assays were validated for measurement of CYP3A activity: felodipine dehydrogenase, midazolam 1′-hydroxylase, and testosterone 6β-hydroxylase. The behavior of CYP3A is complex for several possible reasons. Thorough analysis has suggested that there are different "groupings" of substrates (Kenworthy et al., 1999; Galetin et al., 2003), thus the routine determination of the potential for new compounds to inhibit CYP3A should include assessment of the effect on more than one CYP3A substrate. CYP3A activities frequently do not behave in a simple Michaelis-Menten fashion (Shou et al., 2001; Galetin et al., 2002), and inhibition kinetics can be complex. Additionally, in liver microsomes, the measurement of CYP3A activity is potentially a combination of activities contributed by two enzymes, CYP3A4 and 3A5, to varying degrees.

CYP3A is very active in the felodipine dehydrogenase assay, to the extent that only very low concentrations of microsomal protein (10 μg/ml) are needed for measurable turnover. In this assay, there was the presence of dehydrofelodipine in the felodipine substrate, so a minor correction factor was needed for velocity measurements. Also, no filtration step was used since felodipine and dehydrofelodipine appeared to stick to the polypropylene plates used for incubations for the other 11 assays. Rather, the terminated incubation mixtures were directly injected onto the HPLC-MS from silanized glass vials. Michaelis constants for felodipine dehydrogenase were 2.81 ± 0.61, 0.938 ± 0.137, and 1.41 ± 0.17 μM for pooled liver microsomes, rCYP3A4, and rCYP3A5, respectively (Table 7; Fig. 3). Values reported previously were 6.9 μM for the racemate (Baarnhielm et al., 1986) and 6.1 and 12 μM for the separate enantiomers (Eriksson et al., 1991) (Fig. 4).

Midazolam 1′-hydroxylase was also validated as an analytical method for CYP3A activity. The incubation time for midazolam was short (4 min), since it appeared that the activity declined rapidly in the initial time course experiments. This is consistent with a report of similar observations with this substrate (Khan et al., 2002). Assessment of 4-hydroxymidazolam was also attempted; however, a lack of stability of this analyte through the assay procedure prohibited validation of the method. K_m values for midazolam 1′-hydroxylase were 2.27 ± 0.18, 0.622 ± 0.025, and 1.53 ± 0.09 μM in HLM, rCYP3A4, and rCYP3A5, respectively (Table 7; Fig. 2), consistent with previously reported values of 2.4 to 12 μM for liver microsomes (Gascon and Dayer, 1991; Sharer et al., 1995; Ghosal et al., 1996; Thummel et

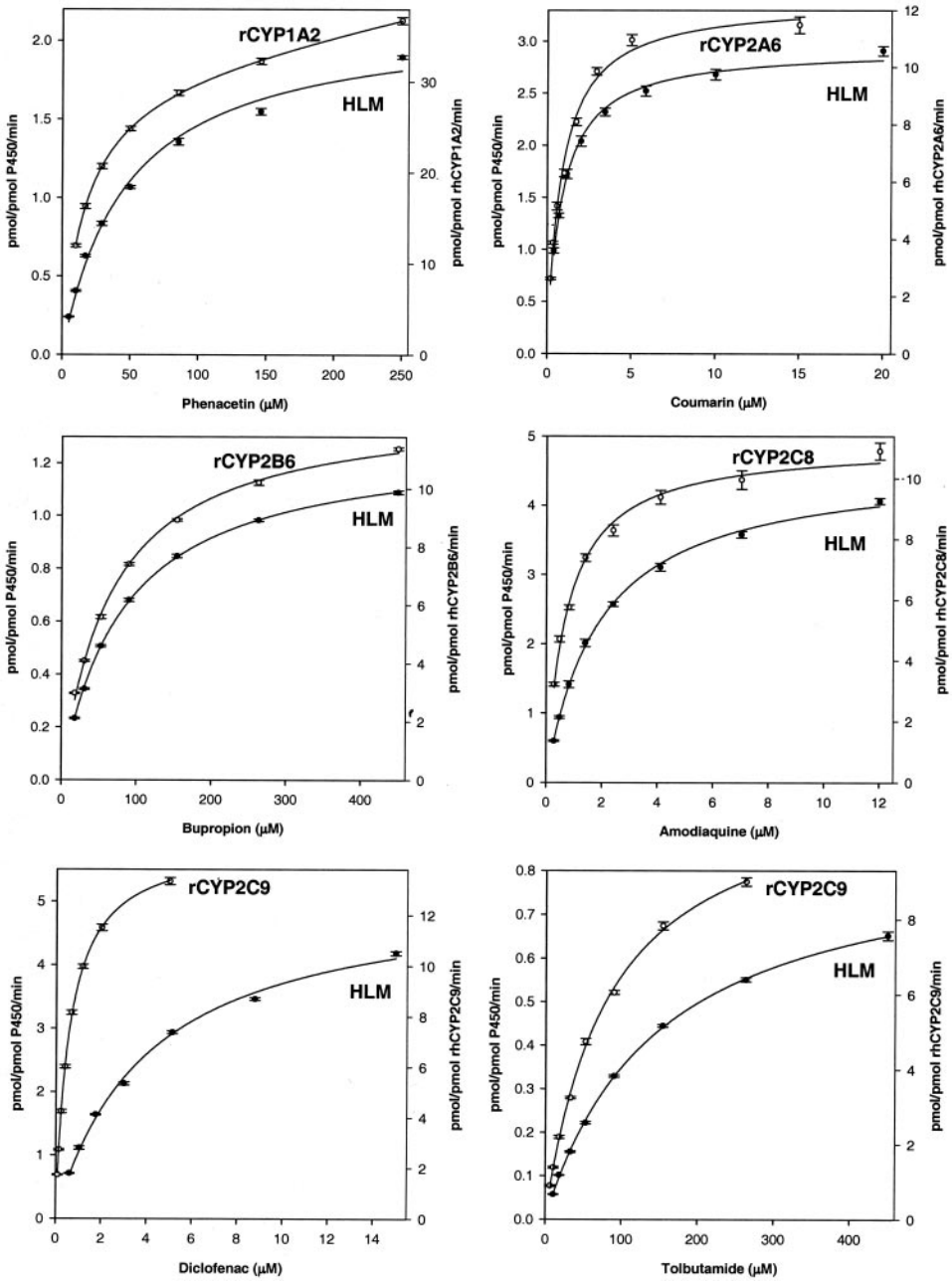

FIG. 2. *Substrate saturation curves for CYP1A2, 2A6, 2B6, 2C8, and 2C9 activities in pooled human liver microsomes and recombinant P450s.*

Solid symbols, pooled human liver microsomes; open symbols, recombinant P450s. The *y*-axes on the left side of the plots are for the liver microsome values; the ones on the right side of the plot are for the rCYPs.

al., 1996; Transon et al., 1996; Von Moltke et al., 1996b; Maenpaa et al., 1998; Wandel et al., 1998; Wang et al., 1999; Martinez et al., 2000; Warrington et al., 2000; Yin et al., 2000; Hamaoka et al., 2001; Andrews et al., 2002; Li et al., 2003) (Fig. 4), 1.0 to 8.9 μM for rCYP3A4 (Ghosal et al., 1996; Gibbs et al., 1999; Eiselt et al., 2001; Khan et al., 2002; Obach and Reed-Hagen, 2002; Williams et al., 2002; Yamaori et al., 2003), and 4.1 to 14 μM for rCYP3A5 (Gibbs et al., 1999; Williams et al., 2002; Yamaori et al., 2003).

Testosterone 6β-hydroxylase is a very well established CYP3A marker activity. Other analytical methods reported have relied on HPLC-UV with long chromatographic run times to resolve the 6β regioisomer from other hydroxylated testosterone metabolites. The presently described HPLC-MS method has a shorter run time but still delivers the requisite sensitivity and selectivity. The K_m values for testosterone 6β-hydroxylase activity in pooled liver microsomes, rCYP3A4, and rCYP3A5 were 46.4 $\pm$ 1.9, 31.4 $\pm$ 2.5, and 106 $\pm$ 13 μM, respectively (Table 7; Fig. 3). Previously reported K_m values for this activity in human liver microsomes have ranged from 31 to 206 μM (Murray et al., 1994; Lee et al., 1995; Wang et al., 1997; Draper et al., 1998; Fayer et al., 2001; Taguchi et al., 2001; Sy et al., 2002; Li et al., 2003) (Fig. 4).

Ketoconazole was used as the positive control inhibitor for all three CYP3A activities. IC_{50} values ranged from 0.0163 to 0.0261 μM in pooled human liver microsomes and were higher in rCYP3A4 and rCYP3A5 incubations (IC_{50} = 0.0619–0.492 μM) (Table 8). An explanation for this observation is not readily available.

TABLE 8

Summary of mean ±S.E. inhibitor potencies for 12 human cytochrome P450 activities in pooled human liver microsomes and recombinant heterologously expressed P450 enzymes

Enzyme	Assay	Inhibitor	IC_{50}	
			Pooled human liver microsomes	Recombinant heterologously expressed enzyme
			μM	
CYP1A2	Phenacetin *O*-deethylase	Furafylline	1.76 ± 0.28	1.54 ± 0.16
CYP2A6	Coumarin 7-hydroxylase	Tranylcypromine	0.449 ± 0.073	0.895 ± 0.262
CYP2B6	Bupropion hydroxylase	PPP	7.74±0.47	2.02 ± 0.19
CYP2C8	Amodiaquine *N*-deethylase	Quercetin	3.06 ± 0.31	3.33 ± 0.20
CYP2C9	Diclofenac 4′-hydroxylase	Sulfaphenazole	0.272 ± 0.031	0.169 ± 0.004
CYP2C9	Tolbutamide hydroxylase	Sulfaphenazole	0.277 ± 0.023	0.200 ± 0.011
CYP2C19	(*S*)-Mephenytoin 4′-hydroxylase	(+)-*N*-3-Benzylnirvanol	0.414 ± 0.035	0.161 ± 0.019
CYP2D6	Dextromethorphan *O*-demethylase	Quinidine	0.0579 ± 0.0043	1.78 ± 0.04
CYP2E1	Chlorzoxazone 7-hydroxylase	Tranylcypromine	8.94 ± 1.54	17.1 ± 2.8
CYP3A	Felodipine dehydrogenase	Ketoconazole	0.0163 ± 0.0018	0.166 ± 0.023[a] 0.374± 0.031[b]
CYP3A	Midazolam 1′-hydroxylase	Ketoconazole	0.0187 ± 0.0025	0.0619 ± 0.0089[a] 0.105± 0.012[b]
CYP3A	Testosterone 6β-hydroxylase	Ketoconazole	0.0261 ± 0.0030	0.0746 ± 0.0204[a] 0.492± 0.178[b]

[a] Mean ± S.E. for recombinant CYP3A4.
[b] Mean ± S.E. for recombinant CYP3A5.

Discussion

Over the past 2 decades, a wealth of information on the human cytochrome P450 enzymes and their roles in drug metabolism and drug-drug interactions in vitro and in vivo has been gathered. Inhibition of P450 enzymes is frequently the underlying mechanism for drug-drug interactions. Our understanding of this area has progressed to the extent that there exists confidence in the use of in vitro inhibition data for qualitative projection of drug-drug interactions in vivo; however, the science has not yet developed to the extent that quantitative projections of interactions can reliably be made from in vitro data (Yao and Levy, 2002; Yao et al., 2003). Nevertheless, in vitro inhibition data can be used in the planning of drug interaction studies in humans (Tucker et al., 2001). Positive inhibition findings for a given P450 activity can lead to the conduct of in vivo studies to determine the effect of the inhibitory drug on the pharmacokinetics of drugs that are cleared by the specific P450 enzyme affected. Negative in vitro inhibition data can be used as a justification to not conduct a clinical drug interaction study. To date, it has not been a requirement of drug regulatory authorities that the gathering of in vitro P450 inhibition data be done under the aegis of good laboratory practices (Tucker et al., 2001), and this position has been echoed in a recent position paper by scientists in the pharmaceutical research and development arena (Bjornsson et al., 2003). However, it has been suggested by others that P450 inhibition data should be gathered under the good laboratory practices (Bajpai and Esmay, 2002). This is not an unreasonable position, since important decisions affecting human safety can be made from in vitro P450 inhibition data. This is particularly true for those cases in which no inhibition is observed in vitro, and such information is used in lieu of clinical drug-drug interaction studies to guide physicians in prescribing practices.

To achieve this level of integrity, the good laboratory practices can be applied in the gathering of these data, as is the case with other types of studies (e.g., various types of toxicology studies in animal species, in vitro tests of genetic toxicity) that assist in the assessment of human risk to new drugs. Good laboratory practices are defined in the U.S. Code of Federal Regulations and are designed to ensure the integrity of scientific data. Good laboratory practices have been used for bioanalytical methods for many years, and there is broad agreement as to the various criteria to which analytical methods must satisfy (Food and Drug Administration, 2001). Such criteria include demonstration of stability of the analyte through the assay process, demonstration of specificity of the assay (i.e., no interferences in blank samples), definition of and adherence to the upper and lower limits of quantitation, demonstration of a high level of intra- and interassay accuracy (85–115%) and precision (0–15%) as assessed through the use of quality control samples that are prepared separately from standards used to construct the calibration curve (Table 5), and so forth. In addition, GLP require documentation of the purity of materials used in the analysis, detailed documentation of the analytical processes, and a data trail that could withstand an independent audit, such that the auditor could reconstruct the details of the process and that the experiments could be repeated to obtain the same results and conclusions. The analytical methods described in this report for the various products of specific P450 enzymes meet these criteria.

In addition to applying GLP aspects to the analytical methods, in this report we have also applied these types of criteria to the experimental procedures in which the samples are generated, i.e., the enzymatic incubation process. We have applied the following criteria to the incubation procedures and experimental design. 1) The determination of conditions that yield initial reaction velocity linearity with time and concentration of enzyme source (i.e., protein) is important to make. In all of these assays, careful examination was made to establish appropriate incubation times and protein concentrations for each assay. Furthermore, in no case was more than 15% of the substrate consumed during the incubation period. 2) For accurate determinations of enzyme kinetic constants, an adequate number (typically $\geq$6) and range of substrate concentrations were included that spanned the K_m value by at least 3-fold on each side (Bjornsson et al., 2003). 3) Data processing and nonlinear regression of the data were done using validated software packages, and predetermined statistical criteria were established to permit assignment of kinetic models (i.e., enzyme kinetic models and mechanism of enzyme inhibition). We selected the Aikake Information Criterion to assign enzyme kinetic models. 4) Each assay was repeated at least five times to establish standard control activities and interday variability. For each occurrence that the assay was run, the control activity was within 10% of the cumulative mean value in order for the data to be acceptable. We maintain an ongoing record of control activities and have reported interday control

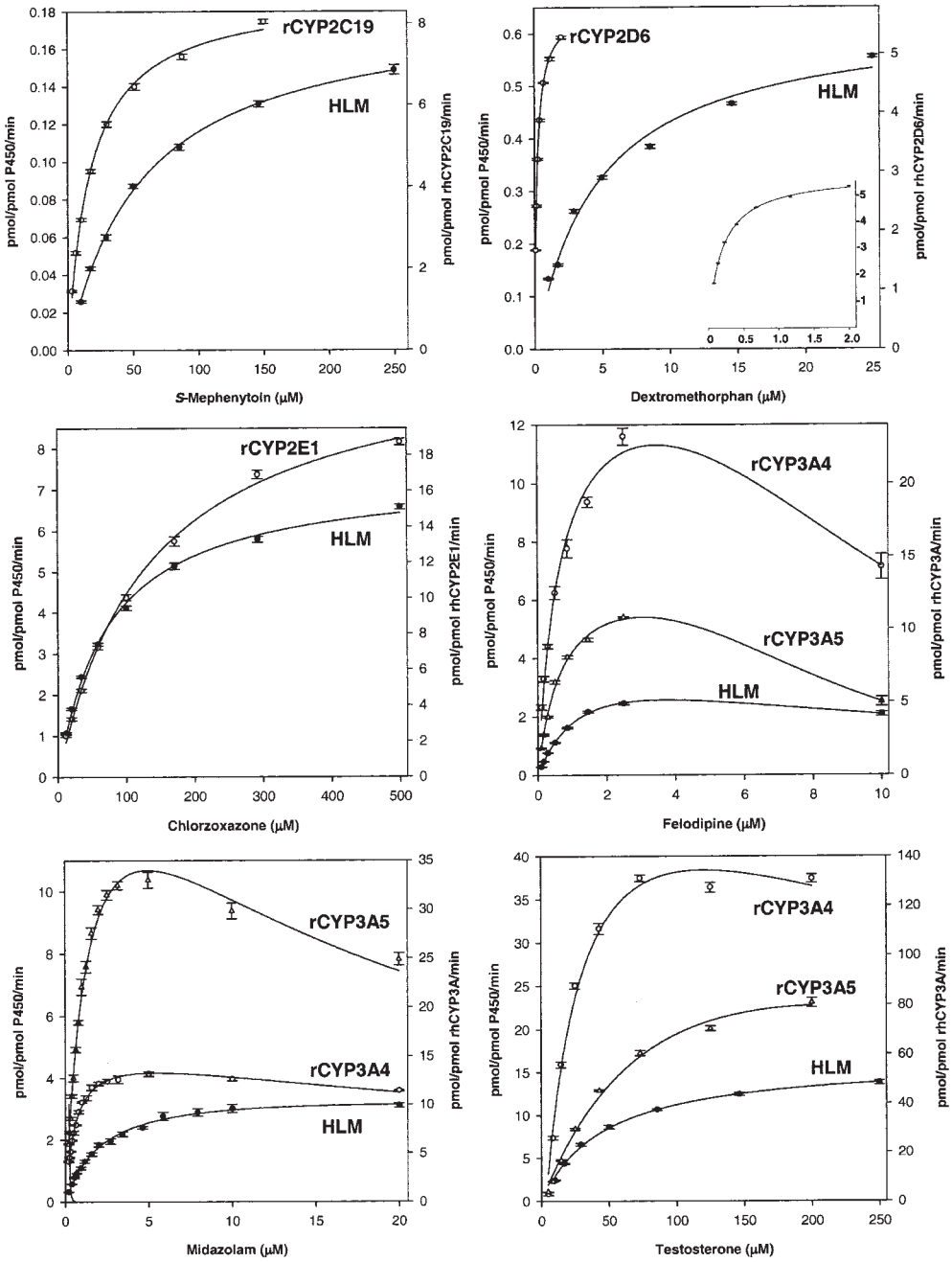

FIG. 3. *Substrate saturation curves for CYP2C19, 2D6, 2E1, 3A4, and 3A5 activities in pooled human liver microsomes and recombinant P450s.*

Solid symbols, pooled human liver microsomes; open symbols, recombinant P450s. For CYP3A: rCYP3A4, open circles; rCYP3A5, open triangles. For the CYP2D6, the inset figure represents an expansion of the substrate saturation curve for rCYP2D6. The *y*-axes on the left side of the plots are for the liver microsome values; the ones on the right side of the plot are for the rCYPs.

activity values (Table 6). 5) As each assay was developed and validated, a standard protocol was written and included in standard operating procedures.

In addition to developing and validating these assays under GLP, high precision and increased throughput has been achieved through the use of laboratory automation for sample generation and tandem quadrupole mass spectrometry as the detection method. The analytical methods that were developed were highly sensitive. This permitted the use of very low concentrations of microsomal protein, which can be important for gathering accurate enzyme kinetic data. Concentrations of human liver microsomal protein ranged from 0.01 to 0.2 mg/ml, for felodipine dehydrogenase and (*S*)-mephenytoin 4′-hydroxylase, respectively, with most assays using between 0.03 and 0.05 mg/ml. Although it is still possible to develop validated methods for P450 activities using detection techniques other than mass spectrometry (e.g., UV absorbance, fluorescence), the sensitivity and selectivity afforded by mass spectrometry can prove to be an advantage. The ability to use lower microsomal protein concentrations is an example of such an advantage afforded by the increased sensitivity of mass spectrometric detection. Also, the high selectivity of tandem mass spectrometry reduces the potential for the compound being tested as an inhibitor to interfere in the analysis. Previous work has shown that inhibition constants can be altered by increasing microsomal protein concentration, due to nonspecific binding of the inhibitor to the

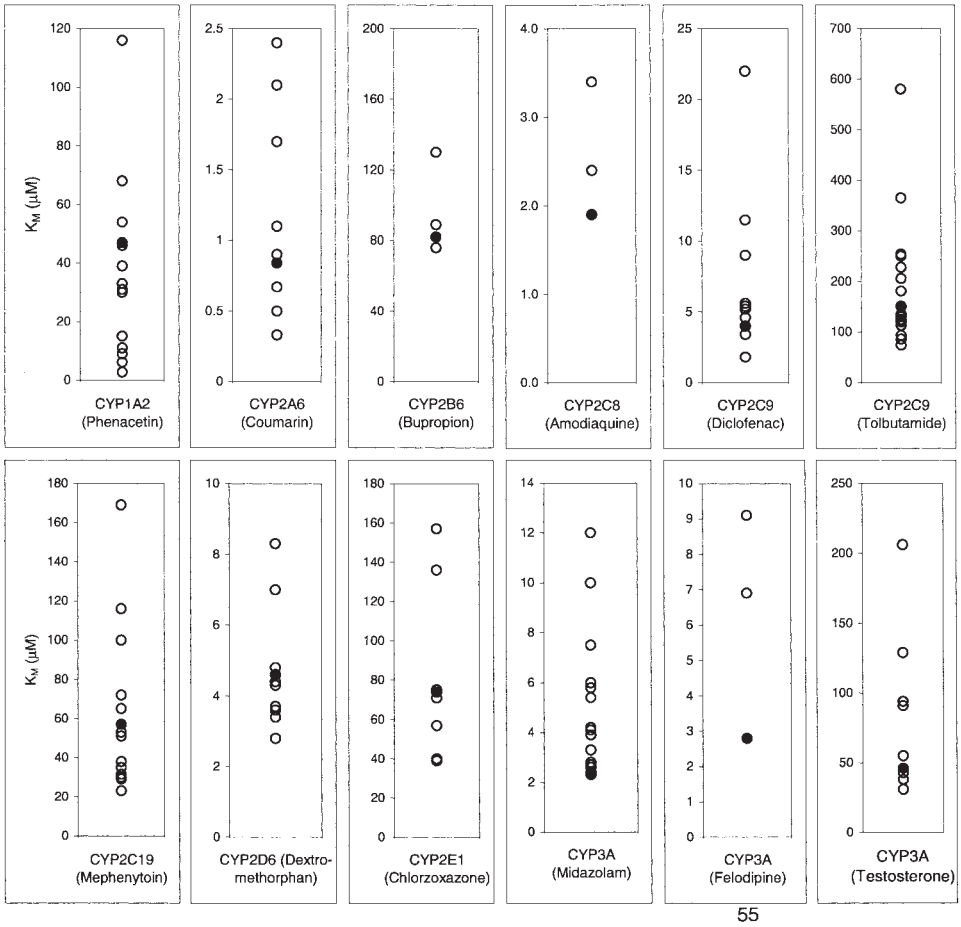

FIG. 4. *Michaelis constants measured using these assays in pooled human liver microsomes (filled symbols) compared with previously reported values (open symbols).*

phospholipid component (Margolis and Obach, 2003) and specific binding to the enzyme depleting the inhibitor (Gibbs et al., 1999; Tran et al., 2002). The use of very low microsomal protein concentrations should obviate the need to measure the free fraction of inhibitor in the in vitro matrices used.

With validation of the assays and use of chemical standards of high quality, the remaining sources of variability primarily reside with the sources of biological materials. The human liver microsomes used in these methods comprised a pool from 54 individual donors, in an attempt to overwhelm variability in activity that is observed in liver microsomal samples from individual donors. Thus, it is our expectation that when another batch of human liver microsome preparations from a similar number of donors is used, enzyme kinetic parameters will not differ substantially from the previous batch. This will be tested in the future. For recombinant P450s, enzyme kinetic parameters can depend on the expression system used and the expression of the complementary proteins, NADPH/P450 oxidoreductase and cytochrome b_5. This will be a source of variability between data presented in this report and data generated by others. Also, measurement of P450 using the classic difference spectral technique of Omura and Sato (1964) is not amenable to the types of analytical criteria put forth in GLP and is likely another source of variability and potential inaccuracy.

There are numerous reports describing enzyme kinetic and inhibition parameters for the human P450 enzymes. There will undoubtedly be differences between reports of V_{max} values in human liver microsomes that are reported per milligram of microsomal protein and are a function of the level of expression of enzyme. K_m and K_i values should be constant from preparation to preparation and comparable from one report to the next, since these parameters are an inherent property of the enzyme and should only potentially differ when the amino acid sequences of the enzymes show genetic variation, or if the activity being measured is not highly selective for one enzyme and has varying contributions of other enzymes. The rigor imparted to the incubation and analytical methods described in this report strive to measure the most accurate kinetic parameters as possible, notwithstanding aforementioned unavoidable sources of variability. The Michaelis constants measured for each reaction in human liver microsomes, when compared with previously reported values, were used to provide assurance that the assays being used were operating appropriately. The K_m values we have generated using these validated methods and the human liver microsome pool are compared with literature values in Fig. 4. In all cases, our values reside within the variability of reported values. It should be noted that K_m values measured for the same reaction using the identical GLP-validated analytical methods in this report still demonstrated some differences between pooled human liver microsomes and recombinant heterologously expressed enzymes (Table 7). Such differences are not uncommon and could be due to differences in protein concentrations used in vitro, differences in ratios of P450 reductase and/or cytochrome b_5 versus P450, differences in phospholipid composition of microsomes from expression systems versus liver, etc. However, a definitive explanation is not forthcoming. We have elected to use pooled human liver microsomes as the source of enzyme in the routine determination of inhibition of P450 enzymes for new compounds. Recombinant

P450 enzymes and human liver microsomes from individual donors are used in gathering supplementary information on inhibition.

Although many important drug-drug interactions arise via inhibition of P450 enzymes, it should be noted that effects on other drug-metabolizing enzymes can also result in drug-drug interactions. Such enzymes include others that catalyze oxidation reactions, such as monoamine oxidases A and B, aldehyde oxidase, and xanthine oxidase, as well as enzymes that catalyze conjugation reactions such as glucuronyl transferases and sulfotransferases. Future efforts will be made to develop (or adapt) and validate similar methods for these enzymes. Furthermore, the rapidly expanding knowledge of drug transport proteins will yield the need to develop rigorous methods for testing new drugs for effects on these proteins and potential drug-drug interactions. Reports of clinical drug-drug interactions involving effects on transporter proteins, P-glycoprotein in particular, have already been described (Lin and Yamazaki, 2003). It is likely that in vitro data will become an invaluable tool to study and predict these types of drug interactions, as has been the case for P450 enzymes.

In conclusion, we report the development and validation, under GLP, of 12 assays for 10 human cytochrome P450 enzymes. The rigor applied to these assays imparts reliability to the data that are gathered using them, such that important decisions concerning human safety can be made with confidence. The data obtained in this manner are suitable to include in drug product package inserts for use by physicians in prescribing medications and combinations of medications. This is most important for negative data, in which the conclusion of a lack of potential drug-drug interactions may be made solely from in vitro information.

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