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Development and full validation of six inhibition assays for five major cytochrome P450 enzymes in human liver microsomes using an automated 96-well microplate incubation format and LC–MS/MS analysis

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Abstract

Substrate inhibition assays for five of the major CYP enzymes (phenacetin for CYP1A2, diclofenac for CYP2C9, (*S*)-mephenytoin for CYP2C19, dextromethorphan for CYP2D6 and midazolam and testosterone for CYP3A4) in human liver microsomes were developed. Fully automated incubations were conducted in a 96-well format under optimized enzyme kinetic conditions. Metabolites of probe substrates were analyzed with rapid LC–MS/MS methods. The assays were fully validated following the procedure for validating bioanalytical methods recommended by regulatory agencies. Quality control samples and a positive control CYP inhibitor were included in each assay. The IC_{50} values determined for typical CYP inhibitors were reproducible and consistent with those reported in the literature. The high quality and throughput of these assays make them ideally suited for providing information for decision making in late drug discovery and early development and for providing labeling input for new drug registrations.
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Keywords: CYP inhibition; Human liver microsomes; Assay validation; Automation; Filtration; LC/MS/MS analysis

1. Introduction

The cytochromes P450 enzymes (CYP) are a super family of hemethiolate enzymes responsible for the metabolic clearance of a wide variety of drugs [1]. Inhibition of CYP enzyme activity by a drug can significantly increase exposure of co-administrated drugs that are metabolized by the same CYP enzyme, which can result in significant adverse events [2]. This type of clinical drug–drug interaction has caused several drugs to be withdrawn from the market or have significant limitations placed on their use [3–5]. In the 1990s, several guidances were issued from regulatory agencies on *in vitro* and *in vivo* drug interaction studies, including the assessment of *in vitro* inhibition of the major CYP enzymes by drug candidates [6,7]. The guidance documents, along with an increased understanding of the use of *in vitro* data to predict the outcome of CYP-mediated drug–drug interactions have enabled screening for drug–drug interaction liabilities during optimization and candidate selection phases of drug discovery, allowed pharmaceutical companies to investigate thoroughly *in vitro* and *in vivo* drug–drug interactions of drug candidates in development.

To address inhibition of CYP enzymes at different stages of drug discovery and development, a variety of CYP inhibition assays have been developed. Microtiter plate assays using a single recombinant CYP enzyme and a fluorescent product (non-chromatographic) are widely used for high throughput screening for CYP inhibition and rank ordering of compounds. The CYP inhibition results can be used to track structure activity relationships and support optimization of lead compounds in early drug discovery [8,9]. However, since most probes used in fluorescent assays are not CYP enzyme-specific, the fluorescent CYP

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inhibition assays cannot be used for studies with human liver microsomes (HLM).

Inhibition assays for specific CYP enzymes using selective probe substrates and HLM have been developed for the definitive evaluation of inhibitory effects of drug candidates [10–14]. Results from these assays play an important role in the selection of development candidates, guiding clinical drug–drug interaction studies and regulatory filings of new drugs. The experimental results from CYP probe substrate assays, in some cases, are significantly different from those generated from fluorescent assays [15,16], especially when dealing with CYP3A4 inhibition which is often substrate-dependent [17–19]. Additionally, many *in vitro* probe substrates, such as theophylline (CYP1A2), S-warfarin (CYP2C9), omeprazole (CYP2C19), dextromethorphan (CYP2D6), and midazolam (CYP3A4) are preferred probe substrates for clinical drug–drug interaction studies, and thus, can be used as clinical probes as well.

The CYP substrate cocktail assays employ a mixture of probe substrates to assess the inhibition of several CYP forms simultaneously [20–23]. Metabolites of multiple substrates in the incubations are determined by a single LC–MS/MS run. Alternatively, HLM incubations are carried out separately, each of which uses a single probe substrate, and samples from each incubation are pooled and analyzed by a single LC–MS/MS run [24]. Recently, Turpeinin et al. reported comparisons between several assays including single substrate, cocktail (n-in-one technique) and fluorescent probe with recombinant CYPs. They demonstrated that all three assays yielded comparable results, although some unexplained differences were noted. It was suggested that the single substrate assay would still be the recommended choice if detailed and more accurate information is warranted [25].

The development of fully validated CYP inhibition assays run “in the spirit of GLP” has been recommended by representatives of regulatory agencies, academia and industry [26–28]. To address this need, Walsky et al. reported 12 specific probe CYP inhibition assays for 10 human CYP enzymes, which were validated based on GLP requirements [29]. In the analyses, a CYP-specific probe substrate and test compound were incubated with HLM or a recombinant CYP enzyme, followed by LC–MS/MS quantification of the metabolites using the respective stable isotope analog as an internal standard (IS). Recently Lim et al. reported an automated screening assay for confirmation of mechanism-based inactivation of five major CYP enzymes in HLM. The assay was developed and validated using three separate 96-well plates, followed by LC/MS/MS analysis [30].

In this report, we describe the development and full validation of six assays for the assessment of CYP inhibition (CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A) in HLM. The selective substrates used, related metabolic reactions, and internal standards are shown in Fig. 1. The incubations were run in a fully automated fashion (96-well format) and under kinetically rigorous conditions. Metabolites of probe substrates were analyzed by LC–MS/MS. The IC_{50} values of selective CYP inhibitors generated from the assays were consistent with those reported in the literature, demonstrating that the CYP inhibition results obtained from these assays can be used reliably to enable the prioritization of clinical drug–drug interaction studies and drug regulatory registrations. The procedures described herein offer two main advantages over current reported assays. First, procedures are described for the treatment of incubation plates to overcome the non-specific binding issues and detailed methods are described for automated control of a (Tecan) liquid handling system. Second, a newly designed filtration plate was adopted to accelerate sample processing and reduce assay variability.

2. Experimental

2.1. Materials

Midazolam, 1-hydroxymidazolam, (*S*)-mephenytoin, (*S*)-4′-hydroxymephenytoin, (+)-*N*-3-benzylnirvanol, and pooled human liver microsomes (HLM) were from BD Biosciences (Woburn, MA, USA). Flufenamic acid, 4′-hydroxydiclofenac, phenacetin, (*R*)-(+)-propranolol, phenytoin, quinidine, acetaminophen, dextromethorphan, α-hydroxytriazolam, testosterone, 6β-hydroxytestosterone, 4-hydroxybutyranilide, sulfaphenazole, diclofenac, dextrorphan, ketoconazole, α-naphthoflavone (α-NF) and NADPH were purchased from Sigma–Aldrich (St. Louis, MO, USA). Acetonitrile (HPLC-grade) was obtained from Burdick and Jackson (Muskeson, MI, USA). 6β-Hydroxyprogesterone was from Steraloids, Inc. (Newport, RI, USA). Water was purified by a Mill-Q-System from Millipore Corp. (Milford, MA, USA). Formic acid (analytical-grade) was from J.T. Baker (Phillipsburg, NJ, USA). All other reagents were of analytical grade.

Ninety six-well reaction plates (300 μl) were purchased from Axygen Scientific (Union, CA, USA). 96-Well preparation plates (2 ml) and 96-well receiver plates (2 ml) were purchased from BD BioScience (San Jose, CA, USA). Hydrophobic and hydrophilic 96-well filtration plates (0.45 μm, polytetrafluoroethylene) were purchased from Millipore Corp (Billerica, MA, USA).

2.2. Instrumentation

A Shimadzu HPLC system with two LC-10ADvp pumps, a SCL-10ADvp controller, and a DGU-14 solvent degasser (Columbia, MD, USA), was used for solvent delivery. A Perkin-Elmer series 200 autosampler (Norwalk, CT, USA) was used for sample delivery. The mass spectrometer was an Applied Biosystems MDS Sciex (Toronto, Canada) API-365 triple Quadrapole mass spectrometer equipped with a turbo ion spray ionization source. A Tecan Genesis RSP 200 liquid handling workstation (Tecan, Triangle Park, NC, USA) equipped with eight tips along with shaking and temperature control (heating block) was used for sample transfer, dilution, and incubation. Gemini software version 4.0 was used for programming all automated steps. An Eppendorf 5810R centrifuge (Eppendorf, Hamburg, Germany.) equipped with a 96-well plate rotor (A-Z-DWP) was used to conduct the filtration process to separate precipitated proteins.

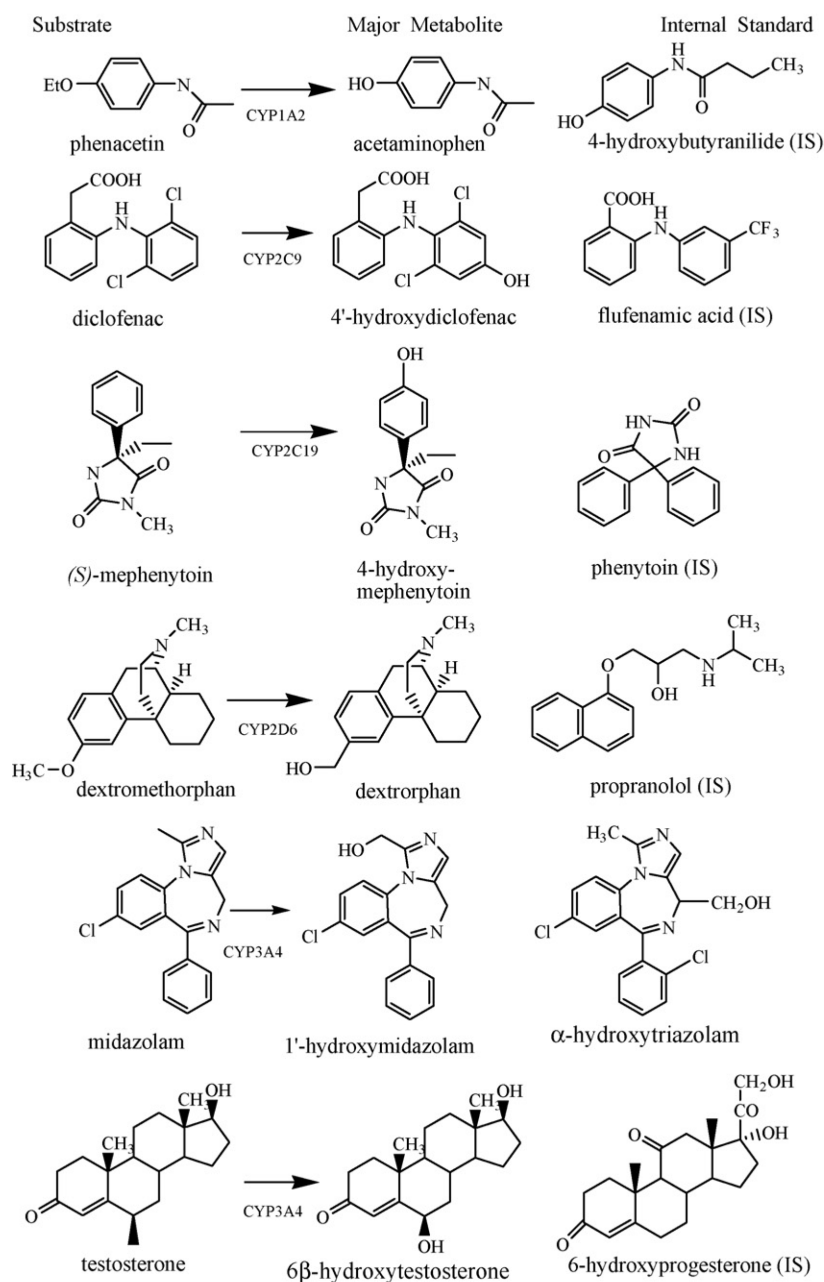

Fig. 1. Structures of six CYP-specific substrates, their corresponding metabolites and the internal standards.

2.3. Chromatographic conditions

Two HPLC columns were used for chromatographic separation. For CYP3A4 assays (testosterone and midazolam), HPLC separation was achieved using an Agilent Zorbax SB-C18 150 mm × 4.6 mm, 5 μm column (Wilmington, DE, USA). For all other assays, a Waters YMC ODS AQ 2.1 mm × 50 mm 3 μm column (Milford, MA, USA) was used. An HPLC solvent system that consisted of mobile phase A (acetonitrile:water:formic acid, 5/95/0.1, v/v/v) and B (acetonitrile:water:formic acid, 95/5/0.1, v/v/v) was used for all assays. Three HPLC gradient programs were used for these CYP assays.

HPLC gradient program I, employed for CYP1A2, CYP2C9 and CYP2C19 assays, was as follows: (1) mobile phase B was held at 5% for 0.2 min, (2) a linear gradient was run to 90% B in 1.8 min, (3) solvent composition was held for 0.5 min and (4) solvent composition was returned to 5% B in 0.1 min for reequilibration. The total run time was 4.5 min with a flow rate of 0.3 ml/min.

HPLC gradient program II, used for CYP2D6 inhibition assay, was as follows:(1) mobile phase B was held at 5% for 0.5 min, (2) a linear was run to 90% in 1.5 min, (3) solvent composition was held for 1 min and (4) solvent composition was returned to 5% B in 0.1 min for re-equilibration. Total run time was 4.5 min with a flow rate of 0.3 ml/min.

HPLC gradient program III used for both CYP3A4 inhibition assays was as follows: (1) Mobile phase B was held at 20% for 0.1 min, (2) a linear was run to 60% B in 4.5 min, then

Table 1
Analytical parameters for CYP1A2, CYP2C9, CYP2C19, CYP2D6, and CYP3A4 assays

P450	Monitored metabolite and internal standard (IS)	Concentration range (μM)	Retention time (min)	Transition (*m/z*)	Collision (eV)
CYP1A2	4-Hydroxyphenacetin	0.025–10	1.65	152.13 > 109.94	21
	4-Hydroxybutyranilide (IS)	2	2.14	180.16 > 110.01	23
CYP2C9	4′-Hydroxydiclofenac	0.025–10	2.18	312.10 > 265.8	19
	Flufenamic acid (IS)	1	2.66	282.10 > 263.93	19
CYP2C19	4′-Hydroxymephenytoin	0.0125–2.5	2.12	235.25 > 149.99	21
	Phenytoin (IS)	2	2.44	253.15 > 182.10	21
CYP2D6	Dextrorphan	0.025–10	2.06	258.18 > 157.08	49
	Propranolol (IS)	2	2.22	260.17 > 183.04	20
CYP3A4	1′-Hydroxymidazolam	0.005–1.25	2.75	326.14 > 291.13	29
	α-Hydroxytirazolam (IS)	1	3.50	359.12 > 340.72	25
CYP3A4	6β-Hydroxytestosterone	0.06–36	2.73	305.28 > 269.18	19
	6-Hydroxyprogesterone (IS)	5	4.72	331.26 > 295.43	19

increased to 90% B in 0.1 min, (3) solvent composition was held for 0.5 min and (4) solvent composition returned to 20% B in 0.1 min for re-equilibration. Total run time was 6.5 min with a flow rate of 0.5 ml/min.

The HPLC flow was diverted from the mass spectrometer to the waste for the first and last minute of the gradient.

2.4. MS/MS detection

For quantitation, the Sciex API-365 mass spectrometer was used in the positive mode (multiple reaction-monitoring, MRM, mode) to monitor for a metabolite and an internal standard with a dwell time set to 150 ms for each transition. After optimization, heated nebulizer parameters were set as follows (arbitrary units): nebulizer, 10; curtain: 8; and temperature, 300 °C. The flow rate of heated gas (gas 2) was operated at 5 l/min. The mass transition and collision energy for each metabolite and internal standard can be found in Table 1. Data were collected and processed using Sciex Analyst 1.1 data collection and integration software.

2.5. Pretreatment of reaction and preparation plates

The reaction plates and preparation plates were dipped in acetonitrile and sonicated for 5 min. Then the plates were rinsed with water, and placed in a centrifuge upside down and centrifuged to dryness prior to use.

2.6. Preparation of substrates, positive controls, test compounds, standards (STD) and quality control (QC) samples

Working solutions of HLM were prepared by diluting pooled HLM (20 mg/ml, purchased from BD Biosciences) with 100 mM phosphate buffer (pH 7.4) to form solutions of 0.11–0.28 mg/ml (solutions were referred to as HLM-1). Stock solutions of the metabolites were prepared in acetonitrile/water, and then further diluted with HLM-1 to obtain the highest concentration standard and QC samples (Table 2). A second HLM working solution (referred to as HLM-2) was prepared by diluting a probe substrate at a concentration close to its Michaelis Menten constant (K_m) value, with HLM-1. Positive control (inhibitor) or test compound stock solutions were prepared in DMSO and then 2.5 μl of the stock solutions was dissolved in HLM-2 at their highest concentration used. All working solutions were stored on ice before being transferred and diluted. Serial dilutions were performed by the Tecan liquid handler for all samples. Seven concentrations for the standard and four concentrations for QC samples were prepared for calibration and quality control. Eight concentrations were prepared for positive inhibitors and test compounds.

2.7. Automated incubation procedure for IC_{50} determination

2.7.1. Incubation conditions

Phosphate buffer (100 mM KH_2PO_4, pH 7.4) containing 1 mM EDTA was prepared from 400 mM mono- and dibasic potassium phosphate stock solutions that were prepared fresh every six months and stored at 4 °C. Frozen stock solutions of liver microsomes (BD Biosciences) were used once after thawing and were not refrozen. NADPH stock solutions (10 mM) in phosphate buffer were made fresh daily. Stock solutions of analytes (i.e., metabolites) were prepared in solvent and stored at −20 °C or 4 °C. Internal standards were dissolved in acetonitrile and further diluted with acetonitrile or 1% formic acid and acetonitrile (7:3, v/v) to prepare working solutions (Table 2).

2.7.2. Automated sample preparation and incubation

The assay was designed to run six compounds (five test compounds, and one positive control) for each CYP enzyme. A known inhibitor for each CYP enzyme was run alongside the test compounds. Eight concentrations of each inhibitor run in triplicate were used to calculate the IC_{50} value. Two plates were used to determine IC_{50} values for five test compounds. One plate contained standard, two test compounds, and one positive control while the other plate contained QC samples and three additional test compounds. Fig. 2 details the plate layout for the process of sample preparation, incubation and filtration.

The highest concentration of standard, positive control, and two test compounds were manually prepared and spiked into

Table 2
Preparation of stock and working solution of substrate, standard, QC, positive control, test compound and internal standard

	Substrate	STD	QC	Positive control	Test compound	IS
CYP1A2 assay						
Chemical name	Phenacetin	Acetaminophen	Acetaminophen	α-Naphthoflavone	X	4OH-Butyranilide
Stock solution (mM) (vehicle)	60 (ACN:H_2O = 1:1)	3.33 (ACN:H_2O = 1:1)	2.67 (ACN:H_2O = 1:1)	0.67 (DMSO)	30 (DMSO)	30 (ACN)
Highest working solution (μM) (vehicle)	50.00 (HLM-1)	5.56 (HLM-1)	4.44 (HLM-1)	1.11 (HLM-2)	50 (HLM-2)	2 (ACN/1%FA = 30/70)
Final concentration (μM)	45.00	5.00	4.00	1.00	45.00	
CYP 2C9 assay						
Chemical name	Diclofenac	4-OH-Diclofenac	4-OH-Diclofenac	Sulfaphenazole	X	Flufenamic acid
Stock solution (mM) (vehicle)	10 (ACN:H_2O = 1:1)	6.67 (ACN)	4.67 (ACN)	13.33 (DMSO)	30 (DMSO)	1 (ACN)
Highest working solution (μM) (vehicle)	11.11 (HLM-1)	11.11 (HLM-1)	7.78 (HLM-1)	22.22 (HLM-2)	50 (HLM-2)	1 (ACN)
Final concentration (μM)	10.00	10.00	7.00	20.00	45.00	
CYP2C19 assay						
Chemical name	(*S*)-Mephenytoin	4-OH-Mephenytion	4-OH-Mephenytion	*N*-3-Benzylnirvanol	X	Phenytoin
Stock solution (mM) (vehicle)	40 (ACN:H_2O = 1:1)	1.67 (ACN:H_2O = 1:1)	1.33 (ACN:H_2O = 1:1)	13.33 (DMSO)	30 (DMSO)	*2* (ACN)
Highest working solution (μM) (vehicle)	61.05 (HLM-1)	2.78 (HLM-1)	2.22 (HLM-1)	22.22 (HLM-2)	50 (HLM-2)	1 (ACN)
Final concentration (μM)	55.00	2.5	2.0	20.00	45.00	
CYP2D6 assay						
Chemical name	Dextromethorphan	Dextrorphan	Dextrorphan	Quinidine	X	Flufenamic acid
Stock solution (mM) (vehicle)	10 (ACN)	6.67 (ACN:H_2O = 1:1)	4.67 (ACN:H_2O = 1:1)	6.67 (DMSO)	30 (DMSO)	*2* (ACN)
Highest working solution (μM) (vehicle)	11.11 (HLM-1)	11.11 (HLM-1)	7.78 (HLM-1)	11.11 (HLM-2)	50 (HLM-2)	2 (ACN)
Final concentration (μM)	10.00	10.00	7.00	10.00	45.00	
CYP3A4 assay						
Chemical name	Midazolam	1-OH-Midazolam	1-OH-Midazolam	Ketoconazole	X	α-OH-Triazolam
Stock solution (mM) (vehicle)	24 (DMSO)	1 (ACN:DMSO = 1:1)	1 (ACN:DMSO = 1:1)	3.33 (DMSO)	30 (DMSO)	2.5 (ACN)
Highest working solution (μM) (vehicle)	5.56 (HLM-1)	1.39 (HLM-1)	1.11 (HLM-1)	5.56 (HLM-2)	50 (HLM-2)	5 (ACN)
Final concentration (μM)	5	1.25	1.00	5.00	45.00	
CYP3A4 assay						
Chemical name	Testosterone	6-OH-Testosterone	6-OH-Testosterone	Ketoconazole	X	6-OH-Progesterone
Stock solution (mM) (vehicle)	24 (ACN)	24 (ACN)	16 (ACN)	3.33 (DMSO)	30 (DMSO)	2.5 (ACN)
Highest working solution (μM) (vehicle)	83.33 (HLM-1)	40.00 (HLM-1)	26.66 (HLM-1)	5.56 (HLM-2)	50 (HLM-2)	5 (ACN)
Final concentration (μM)	75.00	36.00	24.00	5.00	45.00	

Note: ACN, acetonitrile; DMSO, dimethyl sulfoxide.

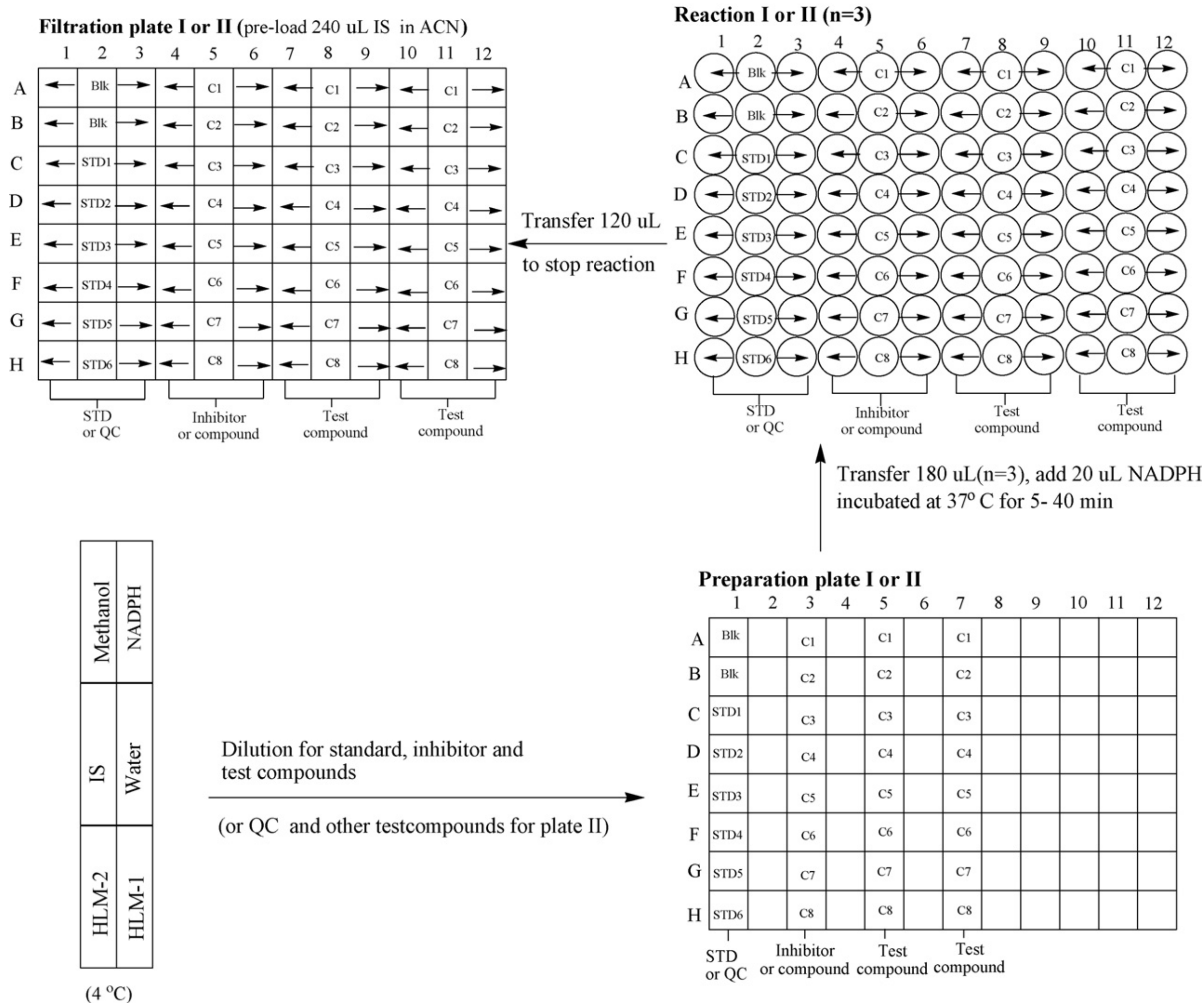

Fig. 2. Tecan layout for sample preparation, incubation and filtration.

the last well (H) of each column (1, 3, 5 and 7) of a 2-ml 96-well preparation plate I for serial dilution by Tecan. For those serial dilution samples in the preparation plate, 1–1.33 μl of DMSO was added to each diluted sample to maintain the same amount of organic solvent, 0.16% (v/v). Then blank HLM-1 was transferred to column 1 for standard dilution and HLM-2 was transfer to column 3, 5, and 7 except the last well of each column. The test compounds were diluted serially to form eight concentrations and mixed well by Tecan before transferring.

After serial dilution by Tecan, 180 μl of mixtures located in column 1, 3, 5, and 7 were transferred to an incubation plate in triplicate. After pre-incubation at 37 °C in a 96-well temperature-controlled heater block for 5 min, 20 μl of NADPH (10 mM in 100 mM phosphate buffer) was added to each well of the reaction plate to give a final volume of 200 μl and initiate the reaction. The plates were maintained at 37 °C for the time period defined in Table 3 for each assay.

Table 3
Summary of enzyme kinetic parameters (Mean ± S.E.) for six human CYP activities in pooled human liver microsomes

Enzyme	Assay	Incubation condition		V_{max}/K_m determination		Literature range for K_m (μM) [29]
		Time (min)	Protein concentration (mg/ml)	V_{max} (pmol/mg/min)	K_m (μM)	
CYP1A2	Phenacetin *O*-deethylase	10	0.15	722 ± 65	45.0 ± 3.8	9–68
CYP2C9	Diclofenac 4′-hydroxylase	7	0.15	5300 ± 190	9.8 ± 0.5	1.8–22
CYP2C19	(*S*)-Mephenytoin 4′-hydroxylase	40	0.25	56 ± 2.3	55.6 ± 2.8	23–169
CYP2D6	Dextromethorphan *O*-demethylase	7	0.15	493 ± 38	10.9 ± 2.2	2.8–22
CYP3A4	Midazolam 1′-hydroxylase	5	0.1	1756 ± 274	4.13 ± 0.3	2.4–12
CYP3A4	Testosterone 6β-hydroxylase	10	0.15	5147 ± 296	83.3 ± 3.3	31–206

2.7.3. Sample filtration

To prepare the filter plates, 240 μl of acetonitrile containing internal standard was transferred into a filter plate (or 100 μl of 30% acetonitrile in 1% formic acid for CYP1A2 assay). After incubation, 120 μl of the reaction mixtures (or 150 μl for CYP1A2 assay) from the wells containing positive control and test compound were transferred in the filter plate to stop the reaction. An aliquot (108 μl) from the wells containing standard sample was then transferred to the filter plate along with an additional 12 μl of NADPH. Also, 1 μl of the five test compounds (5 mM) was added to the blank at positions A-1 to A-3 and B-1 to B-2 in the filtration plate, respectively, as a control to monitor the interference of the test compound to the analysis of the corresponding metabolite of each substrate. The filter plate containing terminated incubation mixtures was then stacked on a 2 ml 96-well receiver plate which were preloaded with 360 μl of 0.1% formic acid in water, vortexed for 30 s, and all mixtures were passed through a 0.45 μm hydrophobic or hydrophilic (CYP1A2 assay only) PTFE membrane by centrifugation for 5 min at 2000 × *g* into the receiving plate. Finally, the receiver plate was vortexed, sealed with a polypropylene film, and 10–25 μl of sample was injected on the LC–MS/MS for quantitation.

The second preparation plate and reaction plate (plate II) were generated in the same fashion as the preparation plate I except standard, positive control, and two test compounds were replaced by QC samples and three other test compounds.

For determination of enzyme kinetic parameters, replicates of $n = 3$ were run at eight substrate concentrations with a total of four to five separate experiments for K_m and V_{max} determination. Subsequently, experiments were run with replicates of $n = 3$ at eight inhibitor concentrations with a total of four separate experiments for IC_{50} determination.

2.8. Automated incubation procedure for K_i determination (CYP2C9 inhibition as an example)

Two 96-well plates were used to determine the K_i value of one test compound, which was similar to the IC_{50} determination. Instead of measuring six compounds, six concentrations of the substrate were used for the K_i determination. An example was given here to describe the K_i determination for CYP2C9 inhibition. Briefly, six concentrations of diclofenac in HLM (55.6, 38.9, 27.8, 17.8, 8.9, and 4.4 μM) were manually prepared to form HLM-2 to HLM-7 solutions. The highest concentrations of inhibitor, sulfaphenazole, or test compound, were prepared from HLM-2 to HLM-7, respectively. Each of the solution with the highest inhibitor concentration was added to the position of H3, H5, and H7 in preparation plate I (or H3, H5, and H7 in plate II for the other three concentrations). Then HLM-2 to HLM-4 were added to column 3, 5, and 7 from well-A to well-G in plate I (HLM-5 to HLM-7 was added to plate II) by Tecan. Serials dilutions of inhibitor were conducted by the Tecan liquid handler for each individual concentration of substrate. Standard and QC preparation remained the same as described previously and the subsequent procedures were similar to the IC_{50} determination.

2.9. Assay validation procedure

2.9.1. Calibration curve and linearity

The validation for the establishment of the CYP inhibition assays was based on the guidance for industry for bioanalytical method validation from the FDA [31]. For the calibration curve and linearity, a six-point calibration curve was constructed by plotting peak area ratio (y) of metabolite to the internal standard *versus* metabolite concentrations (x). The regression parameters of slope, intercept and correlation coefficient were calculated by weighted ($1/x$) linear regression (Analyst 1.1 software). The concentrations of calibration standards, analyzed in triplicate, were then back calculated. Linearity was evaluated by comparing the correlation coefficient (r^2), residuals and errors between theoretical and back-calculated concentrations of calibration standard samples.

2.9.2. Lower limit of quantitation

The lower limit of quantitation (LLQ) was evaluated by preparing metabolites ($n = 6$) in pooled human liver microsomes at the lowest concentration of standard curve, assaying them as unknown samples against the standard curve.

2.9.3. Intra-assay and inter-assay precision and accuracy

Intra-assay and inter-assay precision and accuracy were evaluated by determining the metabolite concentrations in six replicates of QC samples prepared at four different concentrations on three separate days. The four different concentrations were 3 times the LLQ, and a low, median and high QC each selected from one third of the standard curve range. Each run consisted of calibration standards in triplicate, QC samples in six replicates, and blank samples with and without internal standard in triplicate. The analysis was run daily on three separate days to evaluate assay performance.

The accuracy of the assay was evaluated by determining percent deviation (%DEV) from nominal concentration using the formula: %DEV = 100 × (mean back calculated concentration − nominal concentration)/nominal concentration. Intra- and inter-assay precisions were obtained by one-way analysis of variance (ANOVA), and reported as percent relative standard deviation (%R.S.D.) for each QC. Acceptance criteria for the assays were: (a) accuracy of less than 15% DEV; and (b) precision of less than 15% R.S.D. at every concentration studied, except for the lower limit of quantitation (LLQ) where 20% DEV and 20% R.S.D. were acceptable.

2.9.4. Specificity

Twelve blank samples were processed with or without the internal standard and with or without the substrate to evaluate the presence of interfering peaks.

2.9.5. Stability

The stability of reconstituted samples for each metabolite was assessed by using QC samples at two different concentrations at room temperature for 24–48 h or 4 °C for 72–96 h in the autosampler. Freshly processed standard samples were used

to quantitate all QC samples. All stability QC samples were analyzed in six replicates.

2.9.6. Enzyme kinetics and data analysis

In order to establish accurate kinetic parameters, some commonly accepted practices were utilized. First, reaction time course experiments were performed in which the incubation was conducted at a single concentration of protein near the lowest probe substrate concentration. Secondly, the protein concentrations used should be in a linear range with respect to reaction rate. Lastly, consumption of substrate should be less than 20%. Eight substrate concentrations were used in our studies, and the substrate concentrations span a range from $1/3K_m$ to $3K_m$. K_m values were determined by nonlinear regression of enzyme activity versus substrate concentration. Substrate saturation curves and inhibition data were analyzed using the enzyme kinetics module of Grafit v. 5.0 (Erithacus Software Ltd, Horley Surrey, UK).

3. Results and discussion

3.1. Optimization of reaction conditions

Enzyme kinetic results for each of the six CYP inhibition assays are shown in Table 3. The formation of major metabolites was linear with incubation time up to 20 min for the CYP1A2, CYP2D6, CYP2C9, CYP3A4 (testosterone) assays, up to 10 min for the CYP3A4 (midazolam) assay, and up to 50 min for CYP2C19 assay. The formation of major metabolites was linear with protein concentration from 0.1 to 0.3 mg/ml (CYP1A2, CYP2D6, CYP2C9, and CYP3A4 assays), and from 0.1 to 0.45 mg/ml (CYP2C19 assay). To better evaluate the kinetic parameters for each CYP enzyme, especially for the highly protein bound compounds, the protein concentrations were kept as close to 0.15 mg/ml as possible, the largest deviation being the CYP2C19 assay due to the very low metabolic rate of (*S*)-mephenytoin metabolism. Also because non-specific binding of drug in microsomes may lead to changes of free fraction in the *in vitro* system, which will affect the kinetic parameters [32], the lowest protein concentration was used to minimize this effect. Measured kinetic parameters, K_m and V_{max}, were consistent with reported literature values [33].

Over time, automation of screening assays has been considered an advantage because it relieves human burden and reduces error involved with manual operations. However, reports describing fully validated CYP inhibition assays, employing automated liquid handling, are few in number [30,34]. As described herein, it was possible to use a commercially available liquid handling device for sample preparation, reaction incubation with shaking and temperature control, and filtration for LC/MS sampling. The technology makes the assay robust, reliable and precise, which provides high quality data for submission of regulatory documents.

3.2. Pretreatment of reaction and preparation plates

In preliminary experiments, a large variation was noticed when untreated reaction and preparation plates were used. The major reason for the large variation might come from non-specific binding in the plates. To reduce the variation, we developed a procedure to treat the plates prior to use, which was described in the experimental section. The result showed that the variation was reduced significantly. This improvement might be due to the deactivation of the surface of each well by an organic solvent, consequently reducing non-specific binding.

3.3. Use of filtration microplates

In order to lessen the large variation observed when using direct centrifugation methods for removal of proteins, a filtration plate method was explored. Filtration technology has been used to separate soluble metabolites from biological matrices for many years [35–37]. However, the technology has not gained wide acceptance because some technical issues have not been addressed. These include choice of membrane, membrane leakage, when organic solvents are preloaded. Recently, Millipore Inc. developed a new 96-well filter plate, which employs a chemically-resistant material, polytetrafluoroethylene (PTFE), as the membrane (0.45 μm). This filter plate can process up to 1.8 ml of partial aqueous (organic) solutions. Using these filter plates, precipitated proteins can be successfully removed and a very clean filtrate can be obtained. Samples in the 96-well filtration plate can be transferred quantitively onto a common 96-well collection plate using either a vacuum manifold or centrifuge equipped with a plate carrier. After adoption of this filtration technology in our studies, the separation step for protein-removal was reduced tremendously, from 1 h to 5 min. The collection plate can be used directly in subsequent LC–MS injections, thus eliminating a sample transfer step. Moreover, the samples were much cleaner than samples prepared by direct centrifugation, which reduces the LC/MS data variability. Finally, the filtration technology has an advantage over solid phase extraction methods because it eliminates the evaporation and reconstitution steps [38].

3.4. Chromatography and specificity

Since some probe substrates, such as testosterone, have multiple isomeric metabolites, attempts were made to achieve maximal chromatographic resolution in order to minimize interferences. For example, multiple isomers of hydroxytestosterone have been reported when testosterone was used as a substrate in HLM (hydroxylation at the 2-, 6-, 15- and 16-positions) [39,40]. Under the optimized HPLC conditions described, four isomers were completely separated by a SB C18 Zorbax column (2.1 mm × 150 mm, 5 μm) within a 6 min run time (Fig. 3A). Also, we confirmed that >82% of total metabolite at a retention time of 2.75 min was 6β-hydroxytestosterone and approximately 13% was 2-hydroxytestosterone (Fig. 3B). When midazolam was incubated with HLM, two hydroxyl metabolites of midazolam were formed with 1′-hydroxymidazolam as the major metabolite. Both 1′-hydroxy- and 4′-hydroxymidazolam were well separated by the same Zorbax column and the elution conditions described. Additionally, all metabolites from other substrates were separated from the analytes of interest using a

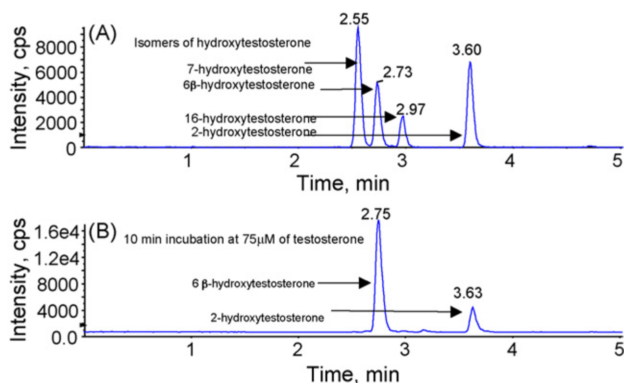

Fig. 3. MRM chromatograms from the analysis of mono-hydroxyl metabolites of testosterone. (A) Separation of four standards of mono-hydroxyl testosterone; (B) an incubation of testosterone with HLM in presence of NADPH.

shorter column (YMC AQ C18, 2.1 mm × 50 mm, 3 μm). Fig. 4a and b show each corresponding LC–MS profile for individual metabolites near the LLQ and also the internal standard used for each assay. The total run times were from 4.5 to 6 min. Blank samples from pooled HLM showed no significant interfering peaks at the retention times of metabolites or internal standards. Since the peak shape of acetaminophen in the CYP1A2 assay was very sensitive to the presence of organic solvent in the reconstituted solution, acetonitrile and 1% formic acid (30/70, v/v) was used as the precipitation solvent and a hydrophilic filter plate was used for filtration. The filtrate was then further diluted with water to lower the organic concentration below 10%. (Fig. 4a, A1). In addition, all substrates were separated from their metabolites under our current HPLC conditions for each CYP assay.

3.5. Validation of analytical methods

3.5.1. Linearity and lower limit of quantification

Calibration curves were linear over the concentration range of metabolites of each substrate (correlation coefficients ≥0.99) in HLM and slope values were consistent when evaluated by weighed (1/x) linear regression. In addition, residuals were randomly distributed when plotted against concentration. At the lowest concentration in each assay, the accuracy was within 20% and the precision was within 20%. Therefore, the LLQ of each metabolite of probe substrate in HLM was established (Table 2). Typical chromatograms of LLQ samples are shown in Fig. 4a and b.

Fig. 4. Elution profiles of metabolites at the level of LLQ using the LC/MS/MS methods: (a) CYP1A2 assay (A1 and A2), CYP2C9 assay (B1 and B2) and CYP2C19 assay (C1 and C2); (b) CYP2D6 assay (D1 and D2), CYP3A4 midazolam assay (E1 and E2) and 3A4 testosterone assay (F1 and F2).

Table 4
Assay accuracy and precision results of metabolites ($n = 18$)

	QC Sample Level (μM)			
Acetaminophen (CYP1A2 assay)				
Nominal concentration (μM)	0.075	0.50	2.00	4.00
Mean measured (μM)	0.074	0.45	1.89	4.09
Accuracy (mean DEV, %)	−0.99	−10.04	−5.66	2.21
Precision				
Between-run (R.S.D., %)	4.66	3.68	0.43	0.45
Within-run (R.S.D., %)	7.25	3.32	4.93	3.23
4-Hydroxydiclofenac (CYP2C9 assay)				
Nominal concentration (μM)	0.075	2.00	4.00	8.00
Mean measured (μM)	0.077	2.08	4.00	8.64
Accuracy (mean Dev, %)	3.04	3.96	0.03	8.03
Precision				
Between-Run (R.S.D., %)	2.21	7.39	6.10	2.92
Within-Run (R.S.D., %)	7.90	4.96	5.46	7.39
4-Hydroxymephenytoin (CYP2C19 assay)				
Nominal concentration (μM)	0.075	0.50	2.00	4.00
Mean measured (μM)	0.071	0.45	1.93	4.15
Accuracy (mean Dev, %)	−4.86	−10.00	−3.73	3.76
Precision				
Between-Run (R.S.D., %)	3.46	3.07	9.29	2.62
Within-Run (R.S.D., %)	5.40	6.28	7.30	7.84
Dextrorphan (CYP2D6 assay)				
Nominal concentration (μM)	0.075	2.00	4.00	8.00
Mean measured (μM)	0.080	2.09	3.99	7.97
Accuracy (mean Dev, %)	7.22	4.58	−0.30	−0.43
Precision				
Between-Run (R.S.D., %)	3.92	7.39	6.69	0.74
Within-Run (R.S.D., %)	6.39	4.96	1.71	8.44
1′-Hydroxymidazolam (CYP3A4 assay)				
Nominal concentration (μM)	0.015	0.125	0.50	1.00
Mean measured (μM)	0.017	0.130	0.50	1.08
Accuracy (mean Dev, %)	11.92	5.04	0.84	7.65
Precision				
Between-Run (R.S.D., %)	7.98	7.39	3.60	0.39
Within-Run (R.S.D., %)	5.32	4.96	3.82	3.77
6β-Hydroxytestosterone (CYP3A4 assay)				
Nominal concentration (μM)	0.180	3.00	12.00	24.00
Mean measured (μM)	0.200	3.14	13.15	25.96
Accuracy (mean Dev, %)	11.05	4.74	9.55	8.15
Precision				
Between-Run (R.S.D., %)	1.34	2.25	2.31	2.32
Within-Run (R.S.D., %)	4.35	5.24	3.08	3.34

All data were summarized from three runs. R.S.D. = Relative standard deviation; DEV = Deviation.

3.5.2. *Precision and accuracy*

Table 4 illustrates the within- and between-assay accuracy and precision for all metabolites from different substrates. The methods developed were found to be accurate with less than 11.9% deviation from the nominal values and precision less than 9.3% (between-run) and less than 8.0% (within-run) at each concentration of QC sample tested.

3.5.3. *Stability*

Processed samples were stable up to 24 h in the autosampler tray with no significant loss (Table 5). Since standard and QC samples were freshly prepared in HLM, it was not necessary to conduct storage and freeze/thaw stability tests on these samples. For all six assays, the stability of the metabolite stock solutions (up to 2 months) was tested and found to be acceptable, which was consistent with reported data [29].

Table 5
Stability of processed samples in the autosampler at room temperature or 4 °C

		%DEV[a]		%DEV[a]
Acetaminophen (CYP1A2 assay)				
Nominal concentration (μM)	**0.25**		**1.00**	
Predicted concentration (μM) at 0 h	0.22	−10.67	0.93	−6.68
Predicted concentration (μM) at 24 h at room temperature	0.26	4.93	1.11	11.33
Predicted concentration (μM) after 72 h @ at 4 °C	0.25	0.93	1.01	1.33
4-Hydroxydilofenac (CYP2C9 assay)				
Nominal concentration (μM)	**2.00**		**4.00**	
Predicted concentration (μM) at 0h	2.04	1.83	4.32	7.88
Predicted concentration (μM) at 24 h at room temperature	1.92	−4.17	4.24	6.00
Predicted concentration (μM) after 72 h @ at 4 °C	1.88	−6.00	4.05	1.33
4-Hydroxymephenytoin (CYP2C19 assay)				
Nominal concentration (μM)	**0.075**		**0.50**	
Predicted concentration (μM) at 0h	0.072	−4.67	0.47	−6.03
Predicted concentration (μM) at 24 h at room temperature	0.082	8.67	0.50	−0.13
Predicted concentration (μM) after 72 h @ at 4 °C	0.078	4.62	0.49	−2.41
Dextrorphan (CYP2D6 assay)				
Nominal concentration (μM)	**0.075**		**2.00**	
Predicted concentration (μM) at 0 h	0.077	3.11	2.06	2.97
Predicted concentration (μM) at 24 h at room temperature	0.082	9.56	2.29	14.58
Predicted concentration (μM) after 72 h @ at 4 °C	0.080	7.07	2.12	6.15
1′-Hydroxymidazolam (CYP3A4 assay)				
Nominal concentration (μM)	0.25		1.00	
Predicted concentration (μM) at 0 h	0.24	−4.90	0.94	−6.1
Predicted concentration (μM) at 48 h at room temperature	0.23	−6.00	0.94	−6.2
6β-Hydroxytestosterone (CYP3A4 assay)				
Nominal concentration (μM)	**0.18**		**3.00**	
Predicted concentration (μM) at 0 h	0.20	11.11	3.21	6.97
Predicted concentration (μM) at 24 h at room temperature	0.19	6.94	2.91	−3.07
Predicted concentration (μM) after 72 h @ at 4 °C	0.21	13.89	2.78	−7.43

All samples were analyzed in six replicates. Mean values are reported.
[a] %DEV: percentage of deviation from nominal concentration.

3.6. *Determination of IC_{50} values for CYP inhibition*

After kinetic parameters were determined for each substrate, the IC_{50} value of a known specific CYP inhibitor was measured near the K_m value of each respective probe substrate. Table 6 shows that IC_{50} values measured at eight concentrations

Table 6
Summary of IC_{50} values of inhibitors for five human cytochrome P450 enzymes in pooled human liver microsomes

Enzyme	Substrate	Inhibitor		IC_{50} (μM)	
		Name	Tested concentration (μM)	Mean[a] ± S.D. (in pooled HLM)	Accepted range
CYP1A2	Phenacetin	α-Naphthoflavone	0, 0.0004, 0.0016, 0.008, 0.04, 0.2, 1, 5	0.0141 ± 0.0016	0.0071–0.0282
CYP2C9	Diclofenac	Sulfaphenazole	0, 0.0016, 0.008,0.04, 0.16, 0.8, 4, 20	0.478 ± 0.085	0.239–0.956
CYP2C19	(S)-Mephenytoin	(+)-*N*-3-Benzylnirvanol	0, 0.0016, 0.008,0.04, 0.16, 0.8, 4, 20	0.395 ± 0.079	0.198–0.790
CYP2D6	Dextromethorphan	Quinidine	0, 0.001, 0.004, 0.02, 0.08, 0.4, 2, 10	0.076 ± 0.022	0.0645–0.258
CYP3A4	Midazolam	Ketoconazole	0, 0.0005, 0.002, 0.01, 0.04, 0.2, 1, 5	0.0323 ± 0.0015	0.162–0.0646
CYP3A4	Testosterone	Ketoconazole	0, 0.0005, 0.002, 0.01, 0.04, 0.2, 1, 5	0.0477 ± 0.007	0.0238–0.0954

[a] $n = 5$.

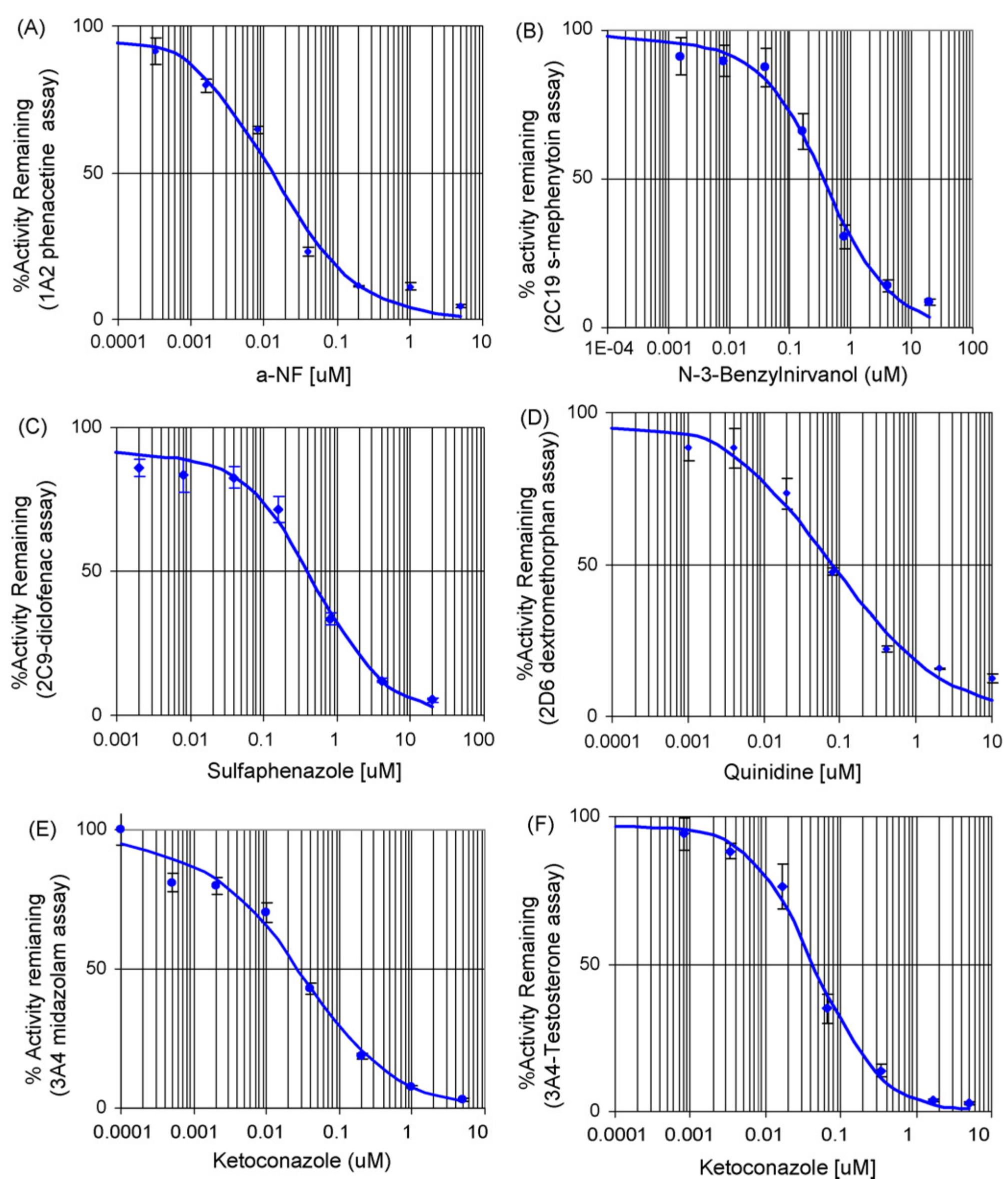

Fig. 5. Inhibition curves obtained using individual CYP probe substrates. Each point is the mean of four experiments. (A) Inhibition of phenacetin O-deethylation by α-NF; (B) inhibition of diclofenac 4′-hydroxylation by sulfaphenazole; (C) inhibition of dextromethorphan O-demethylase by quinidine; (D) inhibition of (*S*)-mephenytoin 4-hydroxylase by (*S*)-(+)-*N*-3-benzylnirvanol; (E) inhibition of midazolam 1′-hydroxylase by ketoconazole; (F) inhibition of testosterone 6β-hydroxylation by ketoconazole.

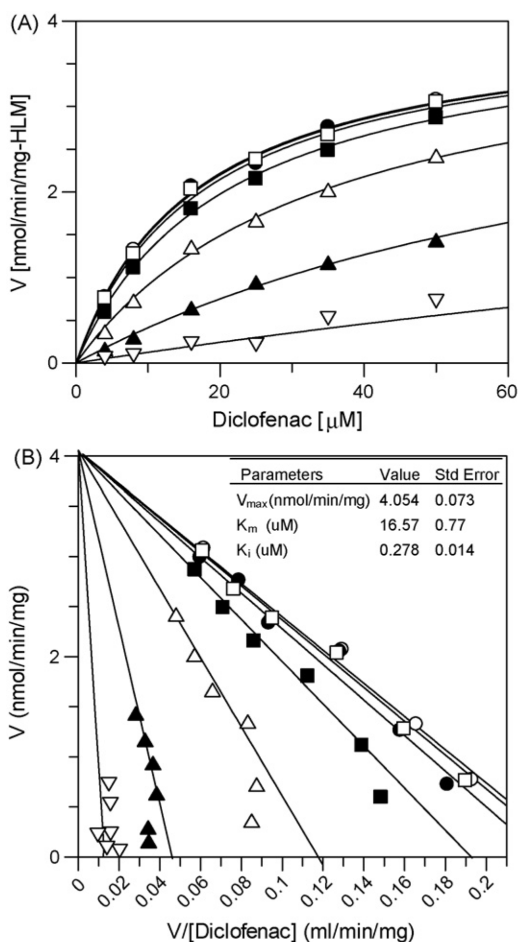

Fig. 6. Concentration-dependent inhibition of diclofinac 4′-hydroxylase in human liver microsomes by various concentrations of sulfaphenazole. (A) direct plot, (B) Eadie-Hofstee plot.

for known inhibitors are consistent with values reported in the literature [29,33]. Inhibition curves obtained using individual substrates are shown in Fig. 5A–F.

Based on the variation of tested IC_{50}, the acceptance criterion was set for the resulting IC_{50} value of each positive control inhibitor, (0.5- to 2-fold of the mean IC_{50} value established during the assay validation).

After completion of the validation, it was possible to successfully apply the assays and assess the CYP inhibition potential of a number of proprietary (in-house) compounds. In this instance, none of the compounds were classified as potent inhibitors ($IC_{50} > 45\ \mu M$) of the different human CYP forms tested.

3.7. Determination of K_i values for CYP2C9 inhibition

Through minor modifications of the Tecan liquid handling program, the procedure for IC_{50} measurement can be used to determine the K_i values for test compounds or known inhibitors. For example, it was possible for determine the K_i for sulfaphenazole towards CYP2C9. In this experiment, five different substrate concentrations with eight different inhibitor concentrations were prepared. The inhibition K_i value of sulfaphenazole toward CYP2C9-mediated diclofenac hydroxylation is shown as an example along with the Eadie-Hofstee plot (Fig. 6). The diclofenac concentration was in the range of 4.0–50 μM, while sulfaphenazole concentrations were from 0.00375 to 15 μM. The K_i value of sulfaphenazole for CYP2C9-mediated diclofenac hydroxylation was calculated to be 0.27 μM, which was consistent with the value reported in the literature [33].

4. Conclusions

This paper describes the development and full validation of six CYP inhibition assays in HLM. *In vitro* incubations were carried out using a liquid handling system (96-well format) under optimized kinetic conditions. The probe substrates used in these assays were selected based on the recommendations from regulatory agencies and PhRMA: phenacetin for CYP1A2, diclofenac for CYP2C9, (*S*)-mephenytoin for CYP2C9, dextromethorphan for CYP2D6 and midazolam and testosterone for CYP3A4. Post-incubation samples were prepared using a 96-well plate filtration technique for quick removal of precipitated proteins, followed by fast LC/MS/MS analyses. In addition, these assays were fully validated with respect to calibration curve linearity, lower limits of quantitation, intra-assay and inter-assay precision and accuracy, specificity of analyte detection and stability of analytes prior to analysis. Furthermore, quality control samples to examine analytical accuracy, and a positive control inhibitor specific to the corresponding CYP enzyme as a biological control sample, were included in each analysis.

The IC_{50} values generated using the assays were consistent with those reported in the literature. In addition, the assays can be adapted for K_i determination with slight modifications to the procedure used for IC_{50} determination and proper LC/MS/MS sensitivity. The fully validated assays, together with quality control procedures, allow resultant CYP inhibition data to be used reliably to enable the prioritization and design of clinical drug–drug interaction studies and support drug registrations.

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