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Validated method for rapid inhibition screening of six cytochrome P450 enzymes by liquid chromatography–tandem mass spectrometry

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Abstract

In vitro drug interaction data can be used in guiding clinical interaction studies, or, the design of new candidates. To make such a claim, it must be assured that the in vitro data obtained is confident. To meet this need, a rapid liquid chromatography–tandem mass spectrometry (LC/MS/MS) method has been validated and employed for routine screening of new chemical entities for inhibition of six major human cytochrome P450 (CYP) isoforms using cDNA-expressed CYPs. Probe substrates were used near the Michaelis–Menten constant (K_m) concentration values for CYP1A2 (phenacetin), CYP2C9 (tolbutamide), CYP2C19 (*S*-mephenytoin), CYP2D6 (dextromethorphan) and CYP3A4 (midazolam and dextromethorphan). The major metabolites of CYP-specific probe substrates were quantified. The LC/MS/MS method was found to be accurate and precise within the linear range of 1.0–2000 ng/ml for each analyte in enzyme incubation mixture. The lower limit of quantification (LLOQ) was 1.0 ng/ml. The limit of detection (LOD) for the tested analytes was 0.48 ng/ml or better based on signal-to-noise ratio >3. The inhibition potential of the six CYP isoforms has been evaluated using their known selective inhibitors. The 50% inhibitory concentrations (IC_{50} values) measured by this method demonstrated high precision and are consistent with the literature values.

Keywords: Acetaminophen; 4-Hydroxytolbutamide; 4′-Hydroxymephenytoin; Dextrorphan; 1′-Hydroxymidazolam; 3-Methyoxymorphinan; 6-Hydroxychlorzoxazone

1. Introduction

Drug–drug interactions (DDIs) are of great interest to pharmaceutical scientists, clinicians, and patients. The inhibition of cytochrome P450 (CYP) activities has been recognized as the pivotal cause of the DDIs in clinic, which may alter the metabolism, potential toxicity, and efficacy of drugs. Among numerous CYP enzymes identified to date, six human hepatic CYP isoforms (CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4) have been found to play dominant roles in drug metabolism, which are involved in many clinical important drug interactions [1]. The withdrawal of terfenadine and mibefradil from the market due to inhibition-based interactions involving CYP3A4 and CYP2D6 exemplify the medical relevance of CYP inhibition [2,3]. For the inhibition assessment, a common strategy is to monitor the effect of test compounds on the metabolism of CYP probe substrates using human liver microsomes or recombinant CYPs. The data obtained from in vitro inhibition study can help guide clinical development programs by identifying drug–drug interactions that ought to be investigated, or guide the design of new candidates [4–7]. However, these data often vary considerably from one laboratory to the next, even when the experimenters claim to use the same assays and methods [5]. Therefore, a systematic and reliable approach is needed to verify the conclusions [4–7].

At present, numerous high-throughput approaches using fluorogenic substrates to measure CYP activities have been described [8,9], as well as substrate "cocktail" experiments that simultaneously measure more than one CYP activity [10–18]. Recently Kim et al. [19] described a high-throughput method that allowed the simultaneous evaluation of the metabolic activities of nine major human CYP isoforms (i.e., CYP1A2,

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CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP3A4) on their well-known specific probe substrates using human hepatic microsomes. The total run time was 6.5 min for each incubation sample. However, only one substrate (midazolam) was selected for CYP3A4 inhibition screening. CYP3A4 is the most abundant isoform in human liver. This isoform accounts for approximately 30% of the total human liver microsomal CYP protein and is involved in the metabolism of approximately 50% of all market drugs. Inhibition of this enzyme by coadministered drugs has been shown to result in adverse clinical DDIs, including fatalities [2]. CYP3A4 exhibits atypical enzyme kinetics depending on the substrate used. For some inhibitors, qualitative differences and quantitative differences of up to 300-fold in 50% inhibitory concentrations (IC_{50} values) can be obtained depending on the substrate being used in the assay [20,21]. Therefore, as a minimum requirement for in vitro inhibition studies, it was recommended that at least two CYP3A4 substrates should be used [22–24,6]. Additionally, human hepatic microsomes used in these "cocktail" experiments is the greater contributor to the effect of nonspecific binding compared with cDNA-expressed CYPs, which may result in false negative errors in prediction of the risk of drug interaction [25,26].

In this research, we developed a liquid chromatography–tandem mass spectrometry (LC/MS/MS) method for the evaluation of inhibition potential of drugs on six major human CYP isoforms using cDNA-expressed CYPs. For CYP3A4 inhibition studies, two substrates, midazolam and dextromethorphan were employed. To our best knowledge, the analytical method for simultaneous determination of 3-methoxymorphinan (3MM), dextrorphan (DX), acetaminophen (AP), 4′-hydroxymephenytoin (OH-MP) and 1′-hydroxymidazolam (OH-MDZ) in incubation has not been reported. Furthermore, the LC/MS/MS method for the simultaneous determination of multi-components in a very short run-time has been fully validated.

2. Experimental

2.1. Materials

cDNA-expressed CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP3A4 were purchased from BD Gentest (Woburn, MA, USA). All the CYPs were expressed in microsomes of insect cells (BTI-5B1-4) infected with a baculovirus containing human CYP and NADPH-CYP reductase cDNA inserts, and the cytochrome b_5 was co-expressed. The selective CYP substrates used in this method are listed in Table 1.

S-Mephenytoin, chlorzoxazone, and 3-methoxymorphinan (3MM) were purchased from BD Gentest Co. (Woburn, MA, USA). Tolbutamide, 4-hydroxytolbutamide (OH-TB), dextromethorphan, and pyridine were provided by Shenyang Pharmaceutical University (Shenyang, China). NADPH and midazolam were purchased from Roche (Basel, Switzerland). Phenacetin, ketoconazole, acetaminophen (AP), dextrorphan (DX), osalmid, omeprazole, and diphenhydramine were purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). α-Naphthoflavone was purchased from Acros Organics (Fair Lawn, NJ, USA). Sulfaphenazole, quinidine, 6-hydroxychlorzoxazone (OH-CZ), 1′-hydroxymidazolam (OH-MDZ) and 4′-hydroxymephenytoin (OH-MP) were purchased from Sigma–Aldrich (St. Louis, MO, USA). The purity of all chemicals was proved above 99%. XC302 (MW: 317, 99.6% purity) is a new anticancer drug candidate and was developed by Xinchang Pharmaceutical Factory (Zhejiang, China). Methanol (HPLC-grade) was purchased from Kangkede Chemical (Tianjin, China). Other reagents were of the highest purity available.

2.2. Incubation conditions

Incubation mixtures were prepared in a total volume of 200 μl with final component concentrations as follows: 0.1 M potassium phosphate buffer (pH 7.4) or 0.1 M TRIS–HCl buffer (pH 7.4), 1.0 mM NADPH, 25 pM recombinant CYP, test drug (XC302) or positive controls and substrates. The substrates were used at concentrations approximately equal to their respective Michaelis–Menten constant (K_m) values: 25 μM for phenacetin (CYP1A2), 100 μM for tolbutamide (CYP2C9), 100 μM for mephenytoin (CYP2C19), 5 μM for dextromethorphan (CYP2D6), 50 μM for chlorzoxazone (CYP2E1), 5 μM for midazolam (CYP3A4-M) and 50 μM for dextromethorphan (CYP3A4-D). The substrates were dissolved in methanol and added to the incubation mixture, with the final concentration of methanol <0.1%. NADPH was added after a 3-min preincubation of all other components in a water bath at 37 °C. The time of incubation used for each substrate was determined

Table 1
CYP probe substrates, enzyme reactions, and metabolites

CYP	Probe substrate	Reaction	Metabolite
1A2	Phenacetin	*O*-Deethylation	Acetaminophen (AP) [27]
2C9	Tolbutamide	4-Hydroxylation	4-Hydroxytolbutamide (OH-TB) [27]
2C19	*S*-Mephenytoin	4′-Hydroxylation	4′-Hydroxymephenytoin (OH-MP) [27]
2D6	Dextromethorphan	*O*-Demethylation	Dextrorphan (DX) [27,28]
3A4-M	Midazolam	1′-Hydroxylation	1′-Hydroxymidazolam (OH-MDZ) [27]
3A4-D	Dextromethorphan	*N*-Demethylation	3-Methoxymorphinan (3MM) [28]
2E1	Chlorzoxazone	6-Hydroxylation	6-Hydroxychlorzoxazone (OH-CZ) [27]

to be in the linear range for the rate of metabolite formation. After a given incubation time (0–60 min), the reactions were terminated by the addition of 200 μl ice-cold acetonitrile. Incubation samples were stored at −80 °C for 7 days averagely before analysis.

2.3. Chromatographic and mass spectrometric conditions instrumentation

An Agilent 1100 G1367A autosampler, a G1316A thermostatted column compartment and a G1311A quarternary pump (Palo Alto, CA, USA), were used for solvent and sample deliveries.

A Thermo Finnigan TSQ Quantum Ultra triple quadrupole mass spectrometer (San Jose, CA, USA) equipped with an electrospray ionization (ESI) or an atmospheric pressure chemical ionization (APCI) source was used for mass analysis and detection. Data were acquired using Xcalibur 1.4 software, and quantitative processing was performed using LCQuan software (Thermo Finnigan, USA).

2.3.1. Simultaneous detection of AP, OH-MP, DX, 3MM and OH-MDZ

Chromatographic separation was achieved on a Zorbax SB C_8 Column (150 mm × 4.6 mm i.d., 5 μm, Agilent, USA) with a SecurityGuard C_{18} guard column (4 mm × 3.0 mm i.d., Phenomenex, Torrance, CA, USA) using a mobile phase of methanol–water–formic acid (80:20:0.2, v/v/v), which was degassed by sonication before use. The liquid flow rate was set at 0.6 ml/min. The column temperature was maintained at 22 °C.

The HPLC system was connected to the mass spectrometer via an APCI interface. The mass spectrometer was operated in the positive ion detection mode with the corona discharge set at 4 μA. The vaporizer temperature was set at 400 °C. Nitrogen was used as the sheath (80 psi) and the auxiliary gas (3 l/min) for nebulization. The heated capillary temperature was set at 350 °C. For collision-induced dissociation (CID), argon was used as the collision gas at a pressure of approximate 1.9 Pa. Quantification was performed using selected reaction monitoring (SRM). The mass transition and collision energy selected for AP, 3MM, OH-MP, DX, OH-MDZ, and IS1 are shown in Table 2. Each analyte was monitored with a dwell time of 0.2 s per transition. The product ion mass spectra of AP, 3MM, OH-MP, DX, OH-MDZ, and IS1 are provided in Fig. 1.

Table 2
SRM parameters for the major metabolites of the seven CYP substrates and internal standards

Analyte	Transition (*m*/*z*)	Ionization	Collision energy (eV)
AP	152 → 110	$APCI^+$	20
3MM	258 → 213	$APCI^+$	30
OH-MP	235 → 133	$APCI^+$	25
DX	258 → 201	$APCI^+$	30
OH-MDZ	342 → 203	$APCI^+$	30
IS1	256 → 167	$APCI^+$	25
OH-TB	285 → 186	ESI^-	20
OH-CZ	184 → 120	ESI^-	20
IS2	228 → 210	ESI^-	25

2.3.2. Simultaneous detection of OH-TB and OH-CZ

Chromatographic separation was achieved on a Zorbax SB C_{18} Column (150 mm × 4.6 mm i.d., 5 μm, Agilent, USA) using a mobile phase of methanol–water–1% (v/v) ammonia water (80:20:0.5, v/v/v). The liquid flow rate was set at 0.5 ml/min. The other chromatographic conditions were the same as described above.

The HPLC system was connected to the mass spectrometer via an ESI interface. The mass spectrometer was operated in the negative ion detection mode with the spray voltage set at −3.8 kV. Nitrogen was used as the sheath (80 psi) and the auxiliary gas (3 l/min) for nebulization. The heated capillary temperature was set at 320 °C. For collision-induced dissociation (CID), argon was used as the collision gas at a pressure of approximate 1.9 Pa. Quantification was performed using selected reaction monitoring (SRM). The mass transition and collision energy selected for OH-TB, OH-CZ, and IS2 are also listed in Table 2. Each analyte was monitored with a dwell time of 0.2 s per transition. The product ion mass spectra of OH-TB, OH-CZ, and IS2 are provided in Fig. 1.

2.4. Calibration standards and quality control samples

The stock standard solutions of AP, OH-TB, OH-MP, DX, OH-MDZ, 3MM, and OH-CZ were freshly prepared by dissolving the accurately weighed standard compounds in methanol to give final concentration of 400 μg/ml for each analyte. The mixed stock solution was then successively diluted with a blank incubation mixture (CYP-inactive microsomes preparation) to achieve standard working solutions at concentrations of 1.0, 3.0, 10.0, 40.0, 150, 500, and 2000 ng/ml for each analyte. Quality control (QC) stock solution for each analyte was prepared individually from separate weighing in a similar fashion as standard solution. The mixed stock solution was further diluted with blank incubation mixture to obtain QC samples at the concentrations of 3.0, 40.0, and 1800 ng/ml.

Stock solutions of diphenhydramine (IS1) and osalmide (IS2) at 400 μg/ml were also prepared in methanol. The stock solutions were subsequently diluted with methanol to obtain a 200 ng/ml diphenhydramine (IS1) and a 100 ng/ml osalmide (IS2) working solution, respectively.

All the solutions were stored at 4 °C and brought to room temperature before use.

In validation and during the inhibition screening study, the standard working solutions (50 μl) were used to spike blank incubation mixture (200 μl) either for calibration curves of AP, OH-MP, DX, 3MM, and OH-MDZ or for QC, all samples were subjected to the sample preparation procedure described in Section 2.5.1; the standard working solutions (50 μl) were used to spike blank incubation mixture (50 μl) either for calibration curves of OH-TB and OH-CZ or for QC, all samples were sub-

jected to the sample preparation procedure described in Section 2.5.2.

2.5. Sample preparation

2.5.1. Simultaneous detection of AP, OH-MP, DX, 3MM, and OH-MDZ

Five incubation samples (for CYP1A2, CYP2C19, CYP2D6, CYP3A4-M, and CYP3A4-D, each containing one probe substrate) at each XC302 concentration were pooled, and an aliquot of 250 μl of each pooled sample was added to a 10-ml glass tube, followed by spiking with 50 μl of IS1 solution (200 ng/ml diphenhydramine) and 700 μl of sodium carbonate (Na_2CO_3, 0.15 M). The samples were briefly mixed, and 3 ml of a mixture of *n*-hexane–ethyl acetate (3:2, v/v) were added. The mixture was vortex-mixed for approximate 1 min and shaken on a roller-shaker for 15 min. Phase separation was achieved by centrifugation at 3500 × *g* for 5 min. The organic layer was removed and evaporated to dryness at 40 °C under a gentle stream of nitrogen, and the residue was reconstituted by an addition of 150 μl of the mobile phase. A 20-μl aliquot of the solution was injected into the LC/MS/MS system for analysis.

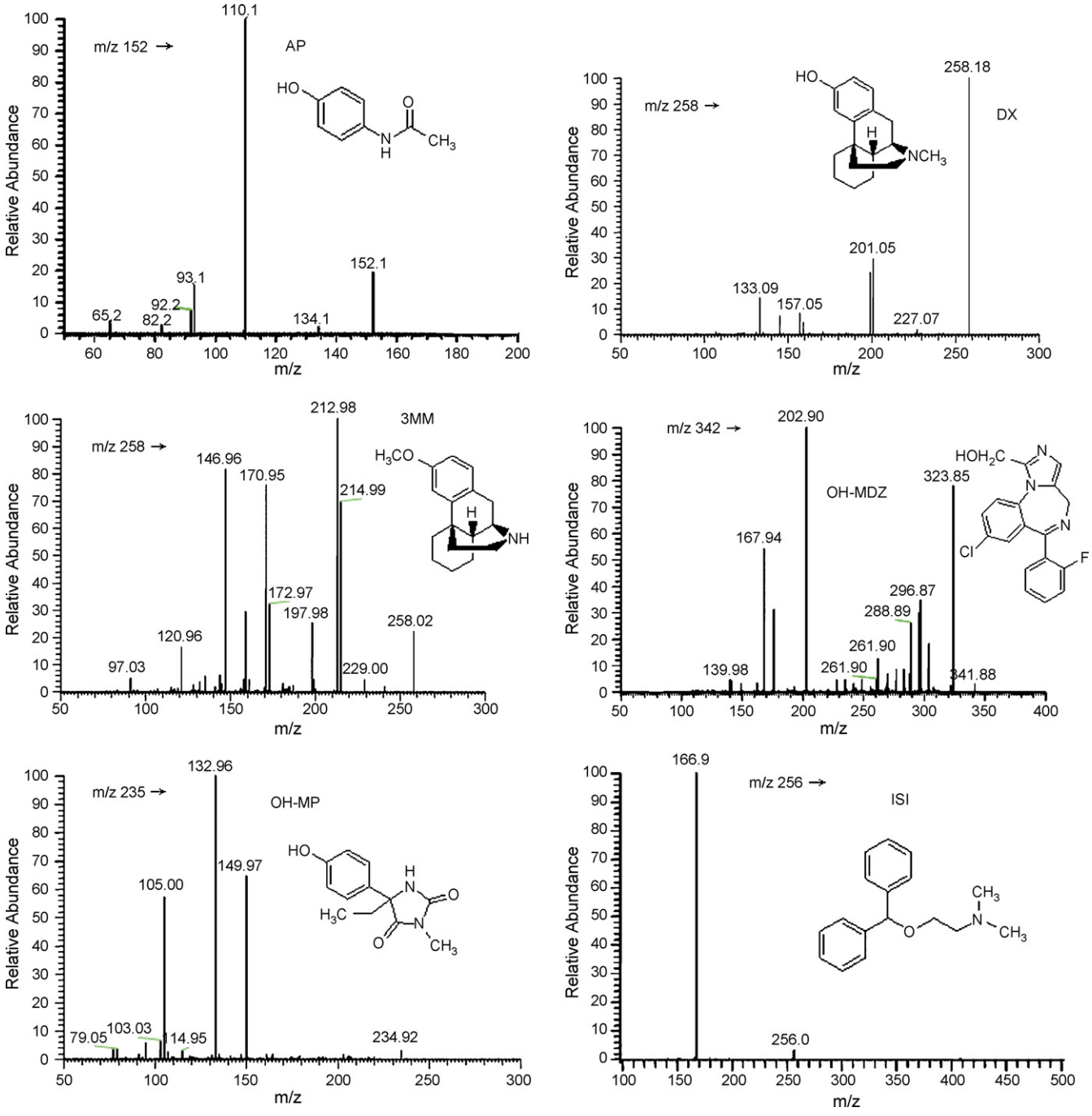

Fig. 1. Product ion scan mass spectra of the major probe metabolites and the internal standards (IS1 and IS2), respectively. For collision energy, see Table 2.

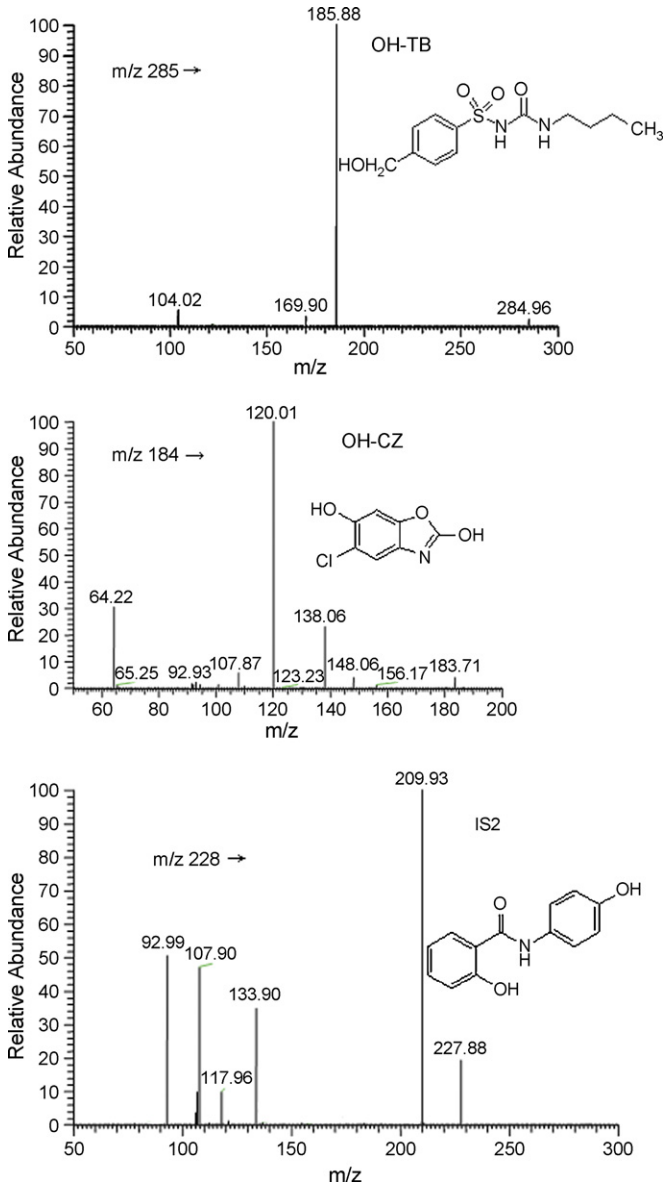

Fig. 1. (*Continued*).

2.5.2. Simultaneous detection of OH-TB and OH-CZ

Two incubation samples (for CYP2C9 and CYP2E1, each containing one probe substrate) at each XC302 concentration were pooled, and an aliquot of 100 μl of each pooled sample was added to a 10-ml glass tube, followed by spiking with 100 μl of IS2 solution (100 ng/ml osalmide) and 600 μl of phosphate buffer (30 mM, pH 7.0), respectively. The samples were briefly mixed, and 3 ml of diethyl ether was added. The following sample extraction procedures were the same as described above.

2.6. Method validation

Samples were quantified using the ratio of the peak area of analytes to that of IS as the assay parameter. Peak area ratios were plotted against analytes concentrations, and standard curves in the form of $y = A + Bx$ were calculated using weighted ($1/x^2$) least squares linear regression.

To evaluate linearity, calibration curves were prepared and assayed on 3 separate days. Precision and accuracy were determined by replicate analyses ($n = 6$) of QC samples at three concentrations in 3 separate days. The precision of the method was defined as the relative standard deviation (R.S.D.) calculated from replicate measurements of QCs. The accuracy of the assay was defined as the relative error (R.E.) of the mean of the replicate measurements of QCs from the theoretical values. The lower limit of quantification (LLOQ) was defined as the lowest concentration analyzed with acceptable accuracy (±20%) and precision (<20%). The limit of detection (LOD) was assessed as the analyte concentration at a signal-to-noise ratio >3.

The matrix effect of incubation medium was examined by comparing the MS/MS response (peak areas, B) of each analyte

(at low and high concentration levels of QCs) or IS spiked into extracts originating from CYP-inactive microsomes incubation mixture to the MS/MS response (*A*) of the same analyte or IS present in the neat mobile phase. The value ($B/A \times 100\%$) was considered as matrix effect.

The stability of metabolites in incubation mixture was assessed by analyzing triplicate QC samples at 3.0 and 1800 ng/ml stored for 2 h at ambient temperature, three freezing (−80 °C) and thawing cycles and for 20 days at −80 °C, respectively. Concentrations measured following storage were compared to those of freshly prepared samples of the same concentration. The stability of the analytes in the reconstitution solvent (mobile phase) was also assessed by re-injecting samples (three replicates of QC samples at each level for 4 h at 10 °C).

2.7. Application of the method

XC302 is a new anticancer drug candidate, which is currently in phase I clinical trial. The method developed in this paper was used to evaluate for its inhibition against six major CYP isoforms. The concentrations of XC302 ranging from 0.1 to 500 μM were selected with respect to the maximum concentrations of drug candidate in rat plasma from the preclinical pharmacokinetic study. Five incubation samples (for CYP1A2, CYP2C19, CYP2D6, CYP3A4-M, and CYP3A4-D, each containing one probe substrate) or two incubation samples (for CYP2C9 and CYP2E1) at each XC302 concentration were pooled, representing a 3.5-fold reduction in the number of samples for analysis. Each pooled sample was extracted and detected using this LC/MS/MS method.

The IC_{50} values of XC302 and positive control against six CYP isoforms were determined using nonlinear least-square regression analysis of the plot of percent control activity versus concentration of the test compound using the computer program Origin 6.1 (OriginLabCorp, Northampton, MA). The percent control activity was calculated based on a comparison between the concentration of the sample and that of the control sample (in the absence of a known inhibitor and XC302).

3. Results and discussion

3.1. Mass spectrometry

During the early stage of method development, attempt was made to simultaneously determine seven probe metabolites (AP, OH-MP, DX, OH-MDZ, 3MM, OH-TB, and OH-CZ). However, under (+) ESI conditions, OH-CZ and OH-MP produced no MS signal. When (+) APCI interface was used, intensive $[M+H]^+$ peak of OH-MP was observed, while the MS response of OH-CZ was still very low. The other analytes, including AP, DX, OH-MDZ, 3MM, and OH-TB, provided high MS response. Further research showed that OH-CZ, OH-MP, and OH-TB provided high MS response under (−) ESI condition. Therefore, it seemed that AP, DX, OH-MDZ, 3MM could be determined simultaneously using (+) APCI interface, and OH-CZ could be determined separately using (−) ESI interface, while OH-MP and OH-TB could be detected either under (+) APCI conditions or under (−) ESI conditions. The most suitable collision energy was determined by observing the maximum response obtained for the fragment ion peak *m/z*. The product ion mass spectra of the analytes and internal standards using the collision energy reported in Table 2 are presented in Fig. 1, where $[M+H]^+$ of each analyte was selected as the precursor ion. The most abundant fragment ions at *m/z* 110, 213, 133, 203, 167, 186, 120, and 210 were chosen in the SRM acquisition for AP, 3MM, OH-MP, OH-MDZ, IS1, OH-TB, OH-CZ, and IS2, respectively. Whereas the fragmentation behavior of $[M+H]^+$ of DX is strongly dependent on the collision energy. A major fragment ion at *m/z* 201 was formed by 30 eV collision energy (Fig. 1), while $[M+H]^+$ was the base ion. When higher collision energy was used, the intensity of $[M+H]^+$ was obviously reduced and more fragment ions were formed, resulting in weaker MS response. Thus, SRM was performed by monitoring the transition of $[M+H]^+ \rightarrow m/z$ 201 for DX using 30 eV collision energy.

3.2. Chromatography

Usually, it is not necessary to completely chromatographically separate coexisting analytes when highly selective SRM detection is used. Unfortunately, some $[M+H]^+$ ions of probe substrates phenacetin and dextromethorphan were source-fragmented to generate AP and DX, respectively, which interfered with the detection of intrinsic AP and DX when both substrate and metabolite co-eluted. Therefore, the chromatographic conditions were optimized to separate AP from phenacetin, as well as DX from dextromethorphan. Diamonsil C_{18} and Zorbax SB C_8 columns were tested to simultaneously determine AP and DX, and the result showed that Zorbax SB C_8 was preferred to Diamonsil C_{18} for better chromatographic separation (Fig. 2(1)), as well as shorter run time.

3.3. Sample preparation

Currently, the most widely employed incubation sample preparation is protein precipitation (PPT) [11–19]. Our initial approach for sample preparation was based on PPT, while it was found that liquid–liquid extraction provided a 10-fold higher sensitivity for the majority of the CYP probe substrate metabolites than PPT. In addition, the linearity of all analytes over the concentration range was very poor using PPT method, it seemed to be the results from ion suppression caused by high concentration buffer in incubation samples. Due to the different hydrophobic character and pK_a values among different probe metabolites, it is difficult to simultaneously extract seven metabolites from incubation mixture with organic solvents. We had tried to simultaneously extract OH-MP, AP, DX, OH-MDZ, 3MM, and OH-TB, it was found that the recovery of OH-TB was too low to be detected when using *n*-hexane–ethyl acetate (3:2, v/v) as extract solvent and adjusting the pH with 0.15 M Na_2CO_3. Under such alkaline extract condition (pH 10), AP also gave low extract recovery (about 15%) due to its weak acidity, but for high MS response of AP, good sensitivity and linearity have been obtained. Therefore, OH-MP,

AP, DX, OH-MDZ, and 3MM were extracted simultaneously as described in Section 2.5.1. Since OH-TB has high MS response under (−) ESI conditions and the similar hydrophobic character and pK_a values with OH-CZ, we developed a method for simultaneous extraction of OH-TB and OH-CZ eventually by selection of pH adjustment reagent and extract solvent. 1 M Na_2CO_3 and phosphate buffer (pH 7.0) were tested as the pH adjustment reagent during extraction procedure. The recovery of OH-TB was higher when phosphate buffer (pH 7.0) was used as pH adjustment compared with 1 M Na_2CO_3. The diethyl ether yielded higher recovery than ethyl acetate. Although OH-TB still gave poor extract recovery (about 15%) under the neutral extract condition due to its stronger acidity than OH-CZ, the sensitivity and linearity were satisfactory. Finally, OH-TB and OH-CZ were extracted simultaneously as described in Section 2.5.2.

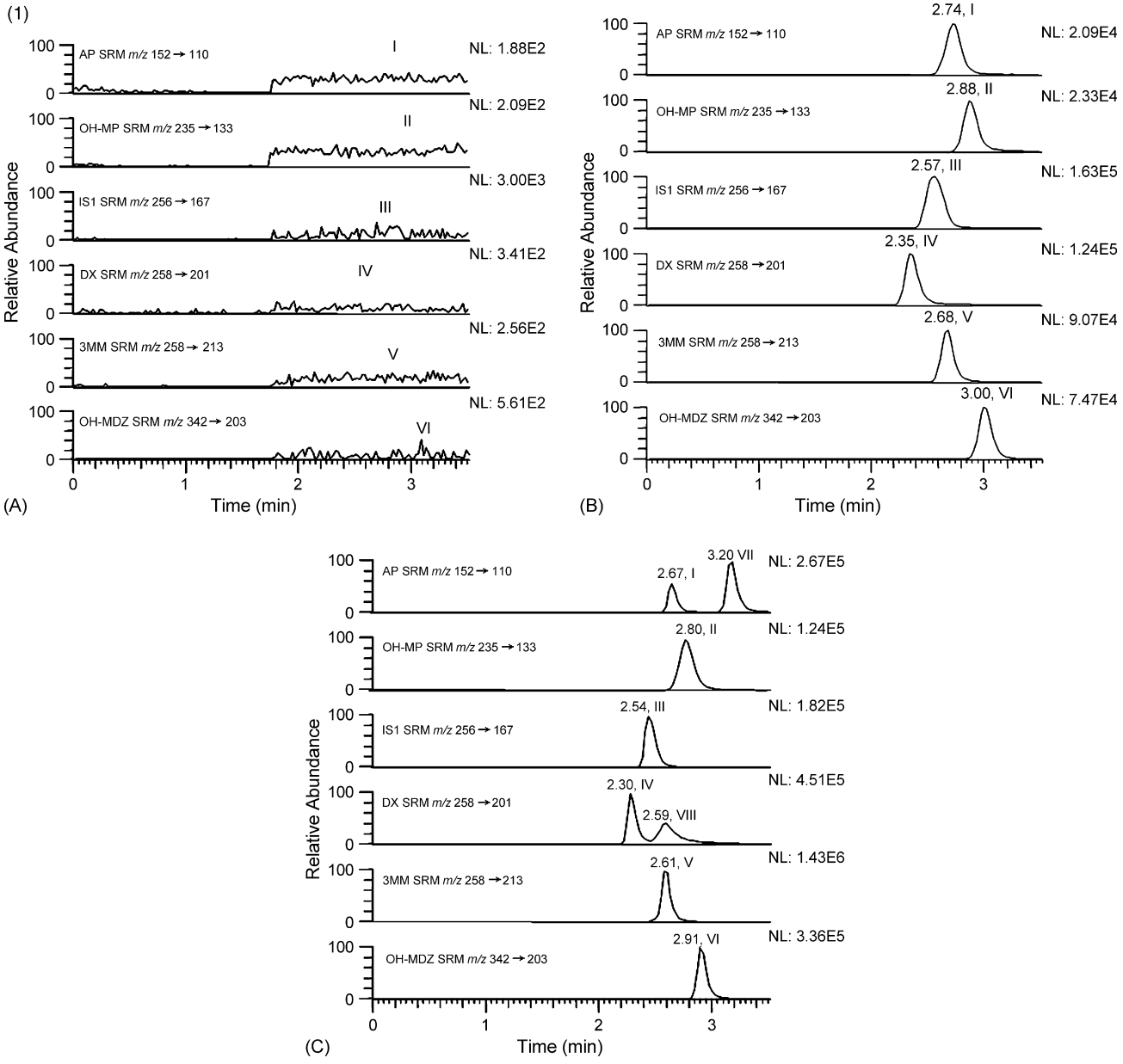

Fig. 2. (1) Representative SRM chromatograms of positive ion analytes. (A) A blank incubation sample. (B) A blank incubation sample spiked with a standard metabolite mixture (40.0 ng/ml for AP, OH-MP, DX, 3MM, and OH-MDZ, and 200 ng/ml for IS1, respectively). (C) Pooled cDNA expressed CYP incubation sample, from incubation sample initially containing 25 μM phenacetin (CYP1A2), 100 μM *S*-mephenytoin (CYP2C19), 5 μM dextromethorphan (CYP2D6), 50 μM dextromethorphan (CYP3A4) and 5 μM midazolam (CYP3A4) after incubation for 45, 30, 20, 30, and 10 min, respectively. Peak I, AP; peak II, OH-MP; peak III, IS1; peak IV, DX; peak V, 3MM; peak VI, OH-MDZ; peak VII and peak VII, AP and DX generated from the source fragment ion of phenacetin and dextromethorphan, respectively. NL: normalized level. (2) Representative SRM chromatograms of negative ion analytes. (A) A blank incubation sample. (B) A blank incubation sample spiked with a standard metabolite mixture (40.0 ng/ml for OH-CZ (CYP2E1) and OH-TB (CYP2C9), and 100 ng/ml for IS2, respectively). (C) Pooled cDNA expressed CYP incubation sample, from incubation sample initially containing 50 μM chlorzoxazone and 100 μM tolbutamide after incubation for 30 and 60 min, respectively. Peak I, OH-CZ; peak II, OH-TB; peak III, IS2.

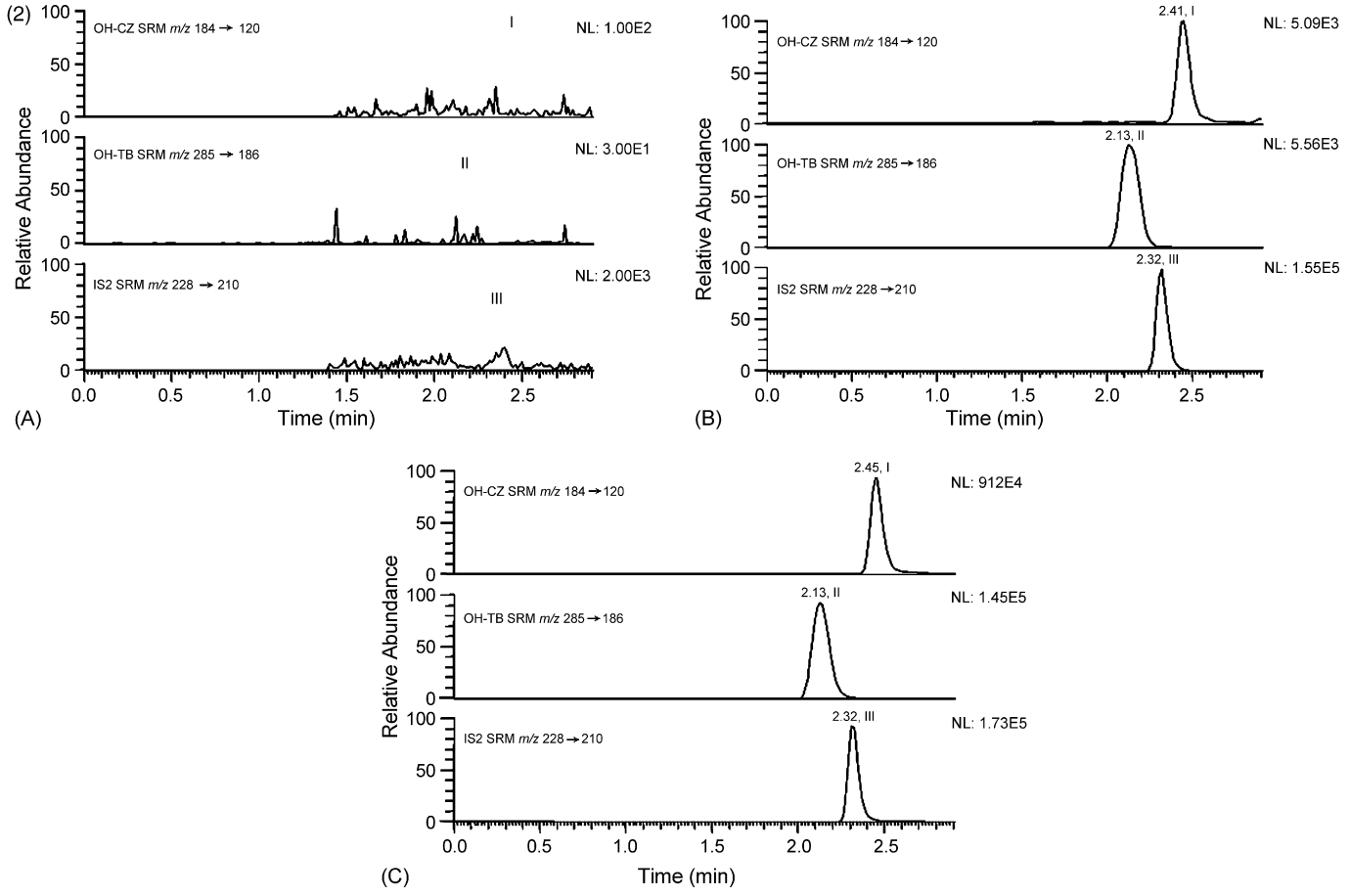

Fig. 2. (*Continued*).

3.4. Method validation

3.4.1. Assay selectivity and matrix effect

Fig. 2 shows the SRM chromatograms of representative spiked incubation matrices and cDNA expressed CYP incubation sample. As indicated in Fig. 2, all analytes were eluted in less than 3.5 min. There were no interfering peaks at the elution times for each analyte SRM channel from other analytes.

To evaluate the matrix effect in the experiment, chromatographic peak areas of each analyte from the spike-after-extraction samples at low and high concentration levels were compared to the neat standards at the same concentrations. Percent nominal concentrations ($B/A \times 100\%$) were within the acceptable limits (93.7–108.1%). The same evaluation was performed for IS1 and IS2 with the concentration of 200 and 100 ng/ml, respectively. No significant peak area differences were observed. Thus, ion suppression or enhancement from incubation matrix was negligible for this method.

3.4.2. Linearity of calibration curves and sensitivity

Linear calibration curves were obtained over the concentration range of 1.0–2000 ng/ml for each analyte in incubation matrix. Table 3 shows the slopes, intercepts and correlation coefficients obtained for typical calibration curves of all analytes.

The lower limit of quantification (LLOQ) was 1.0 ng/ml for each analyte. The accuracy at the concentrations of LLOQ for all analytes was within ±4.6%, and the precision was less than 11.8%. The limit of detection (LOD) for the tested analytes was 0.48 ng/ml or better (Table 3) with a signal-to-noise ratio >3.

3.4.3. Assay precision and accuracy

Intra- and inter-day precision and accuracy were assessed based on the results of QC samples at low, medium and high concentration levels of 3.0, 40.0, and 1800 ng/ml ($n = 18$), respectively. The mean values and R.S.D. for QC samples were calculated over three validation days. Six replicates for each QC level were determined in each day. These data were then used to calculate the intra- and inter-day precision (R.S.D.) using a one-way analysis of variance (ANOVA). The accuracy of the method was determined by calculating the percentage deviation

Table 3
Probe metabolites in incubation mixture by LC/MS/MS: LLOQ, LOD, and representative calibration curves

Analyte	LLOQ (ng/ml)	LOD (ng/ml)	Slope	Intercept	r^2
AP	1.0	0.36	0.00361	0.0164	0.9924
OH-TB	1.0	0.21	0.00133	0.000215	0.9926
OH-MP	1.0	0.45	0.00256	0.00435	0.9911
DX	1.0	0.09	0.0169	0.00934	0.9914
OH-MDZ	1.0	0.30	0.00922	0.00426	0.9945
3MM	1.0	0.11	0.0108	0.0242	0.9949
OH-CZ	1.0	0.48	0.00179	0.000732	0.9916

Table 4
Summary of precision and accuracy for the determination of probe metabolites using LC/MS/MS method

Concentration (ng/ml)	Analytes	Mean (ng/ml)	Accuracy (%)	Intra-day R.S.D. (%)	Inter-day R.S.D. (%)
3.0	AP	3.1	2.8	7.4	7.4
	OH-TB	3.0	−1.4	9.3	13.9
	OH-MP	2.9	−4.2	7.4	6.9
	DX	3.1	2.3	7.4	5.5
	OH-MDZ	3.2	7.3	6.1	3.4
	3MM	3.2	7.3	6.0	6.7
	OH-CZ	3.0	−0.5	8.6	5.0
40.0	AP	41.0	2.6	5.3	13.4
	OH-TB	38.8	−3.0	9.2	8.6
	OH-MP	40.6	1.5	7.0	12.2
	DX	39.9	−0.4	5.1	10.2
	OH-MDZ	42.3	5.8	6.5	7.0
	3MM	41.9	4.7	6.4	10.8
	OH-CZ	38.5	−3.8	9.1	8.3
1800	AP	1704	−5.3	6.1	5.1
	OH-TB	1762	−2.1	8.0	12.6
	OH-MP	1794	−0.3	7.2	12.4
	DX	1782	−1.0	6.2	10.9
	OH-MDZ	1832	1.8	6.0	5.6
	3MM	1814	0.8	6.0	5.3
	OH-CZ	1858	3.2	7.5	8.9

observed in the analysis of QC samples and expressed in the relative error (R.E.).

The intra- and inter-day precision and accuracy for seven CYP-specific probe metabolites in cDNA expressed CYP incubations are summarized in Table 4. Overall, the intra- and inter-day precision (R.S.D.) were less than 13.9% for each analyte at each QC level, and the accuracy was within ±7.3%.

3.4.4. Extraction recovery and stability

The extraction recoveries of seven CYP probe metabolites were determined by comparing the peak areas ratios of each analyte to IS in incubation samples that had been spiked with each analyte prior to extraction, with samples to which pure authentic standard solutions dissolved in the methanol at three QC levels had been added post-extraction. The results are represented in Table 5. The recoveries of AP and OH-TB were relatively low (about 15%), but the values were consistent among low, medium and high QC levels. The recoveries for other analytes were all over 41% and reproducible. The sensitivities for all analytes were satisfactory even if the extraction recoveries of some analytes were poor.

All analytes were found to be stable, and no significant degradation (<10%) was detected in incubation for at least 2 h at room temperature, for at least 20 days at −80 °C, and for three freeze/thaw cycles. After extraction, when glass vials were maintained in the autosampler at 10 °C, the compounds did not show any detectable degradation for at least 4 h (<10%).

Table 5
The extraction recoveries for seven-CYP specific probe metabolites ($n = 6$)

	3.0 (ng/ml)		40.0 (ng/ml)		1800 (ng/ml)	
	Mean (%)	S.D.	Mean (%)	S.D.	Mean (%)	S.D.
AP	15.6	0.3	15.0	0.7	12.2	0.9
OH-TB	15.8	2.0	14.0	1.4	17.5	1.0
OH-MP	59.5	1.5	56.0	2.5	57.2	2.2
DX	52.9	3.8	52.2	3.1	58.6	4.4
OH-MDZ	65.0	3.0	65.4	3.9	63.8	4.0
3MM	48.9	2.5	49.0	1.3	54.1	3.4
OH-CZ	41.2	4.7	48.5	4.9	48.3	4.7

3.4.5. Application of the method

For most substrate "cocktail" assays, incubations were performed using human hepatic microsomes, the common problem with these assays was the existence of nonspecific binding of inhibitor and substrate to microsomes, which may result in false negative errors in prediction of the risk of drug interaction. The

Table 6
The values of IC_{50} for XC302 and positive controls

Test compound	Recombinant CYP						
	1A2	2C9	2C19	2D6	2E1	3A4-M	3A4-D
XC302 (μM)	0.29 ANF	7.26 SUL	16.7 OME	34.1 QUI	>500 PRY	0.29 KET	0.90 KET
Positive control (μM)	0.069	1.02	8.9	0.046	2.81	0.041	0.23
Literature values (μM)	0.05 [16]	1.52 [16], 0.35 [18]	5.6 [25]	0.058 [20]	2.75 [18]	0.06 [18,20]	0.28 [17]

ANF, α-naphthoflavone; SUL, sulfaphenazole; OME, omeprazole; QUI: quinidine; PYR, pyridine; KET, ketoconazole.

inhibition study using cDNA expressed CYPs might be suitable to minimize the effect of nonspecific binding, and therefore, a more precise estimation of the risk of adverse drug interaction can be achieved [25,26]. The assay of pooling sample after individual incubation instead of "cocktail" incubation could further eliminate mutual drug interactions among substrates.

The IC_{50} values of XC302 and positive control against six CYP isoforms were obtained. The results are shown in Table 6. The IC_{50} values of positive control were in good agreement with published values according to the acceptable degree of accuracy [6].

4. Conclusions

This paper describes the development and validation of a new LC/MS/MS method for a rapid quantitative analysis of seven CYP probe metabolites. It offers some advantages over previously published methods for evaluation of CYP activity and inhibition. All of the substrates and metabolites are readily available commercially (e.g. phenacetin, tolbutamide, dextromethorphan, AP, OH-TB, and DX could be purchased from Sigma); high sensitivity permits decreasing the amount of cDNA expressed CYPs required for experiments. This method has proved reliable and has also significantly increased sample throughput (3.5-fold reduction in the number of samples for analysis compared with separate determination for a single probe metabolite at a time) and shortened assay time, allowing a large number of compounds to be screened rapidly for potential CYP inhibitory activity.

The application of the method should allow fast and simple assessment of any potential inhibition or induction effects drug candidates may have on the metabolism of specific CYP probe substrates. Information on the possible effects which drug candidates have on the activity of specific CYP isoforms can then be used in helping to define a strategy for any in vivo interaction studies required in the clinic. Due to these aspects of specificity, reproducibility and sensitivity, associated with a validated extraction procedure, the method can provide not only a reliable in vitro approach to rapid screening the inhibitory potential of new chemical entities but also the reliable data from the in vitro inhibition studies that can help guide clinical interactions.

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