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Rapid and quantitative determination of metabolites from multiple cytochrome P450 probe substrates by gradient liquid chromatography–electrospray ionization-ion trap mass spectrometry

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Abstract

A rapid quantitative assay method, developed by combining fast gradient liquid chromatography and electrospray ionization-ion trap mass spectrometry, is described for the simultaneous determination of CYP450 probe substrate metabolites (4-aminophenol for CYP2E1, acetaminophen for CYP1A2, dextrorphan for CYP2D6, 4′-hydroxymephenytoin for CYP2C19, 4-hydroxytolbutamide for CYP2C9 and 6β-hydroxytestosterone for CYP3A4) in microsomal incubations. Using this method Michaelis–Menten kinetic parameters K_m and V_{max} for the probe substrates in human liver microsomes were obtained. This LC–MS–MS method, developed with the use of LC–ESI-ion trap MS instrumentation, can efficiently be used to improve the throughput and cost-effectiveness in the preclinical drug metabolism studies.
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1. Introduction

Cytochrome P450 (CYP) enzyme family comprises multiple isoforms with different substrate specificities and catalyzes the biotransformation of a vast number of drugs [1–4]. Although CYP isoforms may exhibit partially overlapping substrate specificity, a single CYP isoform is often predominantly responsible for the drug metabolism. The relative amount and activity of the CYP isoforms are subject to induction, inhibition and genetic polymorphism, which may alter the metabolism, potential toxicity and efficacy of pharmaceutical drugs. Knowledge of the specific CYP isoform responsible for the metabolism of a drug is critical for the prediction of potential drug–drug interactions and genetically based individual variation in drug metabolism [5]. Suitable human CYP isoform-selective substrates have been identified and commonly used for probing the role of specific CYP enzymes in drug metabolism [6,7]. Examples of well-studied CYP probe substrates for in vitro drug metabolism studies are listed in Table 1.

The evaluation of CYP enzyme activities is traditionally performed for a single CYP isoform at a time. This conventional approach is labor-intensive,

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Table 1
CYP probe substrates, enzyme reactions and metabolites

Isoform	Probe substrate	Reaction	Metabolite
CYP 2E1	Aniline (AN)	4-Hydroxylation	4-Aminophenol (AP)
CYP1A2	Phenacetin (PA)	*O*-Deethylation	Acetaminophen (APAP)
CYP2D6	Dextromethorphan (DM)	*O*-Demethylation	Dextrorphan (DX)
CYP2C19	*S*-Mephenytoin (MP)	4′-Hydroxylation	4′-Hydroxymephenytoin (OH-MP)
CYP2C9	Tolbutamide (TB)	4-Hydroxylation	4-Hydroxytolbutamide (OH-TB)
CYP3A4	Testosterone (TS)	6β-Hydroxylation	6β-Hydroxytestosterone (OH-TS)

time-consuming and cost-ineffective. To increase the throughput, a mixture of CYP probe substrates can be incubated with liver microsomes and the activities of several CYP isoforms can be assessed simultaneously by monitoring the resulting probe substrate metabolites [8–10]. The success of this mixed-incubation approach requires an analytical method that allows rapid quantitative determination of multiple probe substrates and metabolites. Liquid chromatography–tandem mass spectrometry (LC–MS–MS), with its high speed, sensitivity and selectivity, is a superior tool for this purpose. Since a complete separation of analytes is not necessary, experiment cycle time can greatly be reduced in LC–MS–MS.

Ayrton et al. have developed a fast (6 min) gradient LC–MS–MS method interfaced with atmospheric pressure chemical ionization (APCI) for the determination of selected CYP probe substrate metabolites from in vitro human liver microsomal incubations [9]. Two experimental runs with two different mobile phases have been used to detect the probes under study, because the probe substrate for CYP2E1, chlorzoxazone (CZ) and its metabolite 6-hydroxychlorzoxazone (OH-CZ) could only be detected with adequate sensitivity as negative ions while other analytes were amenable to positive ion detection. Dierks et al. also reported a method of LC–MS–MS with APCI for simultaneous assessment of the activities of several CYP isoforms excluding CYP2E1 [11].

Scott et al. reported a LC–MS–MS assay based on electrospray ionization (ESI) for the determination of CYP probe substrates and metabolites in plasma and urine, in which two separate runs with one in positive and the other in negative ion mode were used [10]. Bu et al. reported a new strategy of switching electrospray (ES) polarity during the same run for simultaneous detection of the CYP probe substrates and metabolites [12]. An overwhelming background noise was reported as a result of ES polarity switching, which was mediated by setting a dummy ion transition scan after each polarity switch.

All these reported assays have been developed using a triple quadrupole mass spectrometer on the LC–MS instrument [5,9–14]. However, in many pharmaceutical laboratories, ion trap LC–MS is being routinely used due to its low cost. Here we describe the development of a LC–MS–MS method based on the LC–ESI-ion trap MS, which allows rapid quantitative determination of multiple probe substrates for CYP isoforms such as CYP1A2, 2C9, 2C19, 2D6, 2E1, and 3A4 in a single run.

Our preliminary work has shown that although it is possible to use segmented data acquisition to switch ES ion polarity during the run with the ion trap MS, it requires complete separation of the probe metabolites in positive ion detection mode from those in negative ion detection mode. This undoubtedly increases the experiment cycle time. Among the common CYP probe substrates and metabolites, CZ and OH-CZ are the only ones that require negative ion detection. C-4 hydroxylation of aniline is a specific marker of CYP2E1 activities [15–20]. Unlike CZ, aniline and its hydroxyl metabolite (4-aminophenol) can be detected with good sensitivity in positive ion mode. Therefore, aniline is used as a probe substrate for CYP2E1 isoform in the present study to facilitate simultaneous detection of the probe substrate metabolites for all the CYP isoforms under study without the need to switch ES ion polarity.

This paper describes the development and validation of a LC–MS–MS method that utilizes a fast gradient liquid chromatography and ESI-ion trap MS

for rapid quantitative analysis of multiple CYP probe metabolites in a single run. Using this method, the enzyme kinetic parameters of K_m and V_{max} for each probe substrate from in vitro human liver microsomal incubations have been obtained.

2. Experimental

2.1. Chemicals and reagents

Aniline (AN), 4-aminophenol (AP), phenacetin (PA), acetaminophen (APAP), tolbutamide (TB), dextromethorphan hydrobromide monohydrate (DM), dextrorphan (DX) D-tartrate salt, (±)-4-hydroxymephenytoin (OH-MP), testosterone (TS), 6β-hydroxytestosterone (OH-TS), bucetin, and NADPH were purchased from Sigma (St. Louis, MO, USA). *S*-(+)-Mephenytoin (MP) and 4-hydroxytolbutamide (OH-TB) were received from Gentest (Bedford, MA, USA). Formic acid and potassium phosphate dibasic were supplied by Mallinckrodt Baker (Phillipsburg, NJ, USA). HPLC-grade acetonitrile was from Burdick & Jackson (Muskegon, MI, USA). Distilled, deionized water was generated from NANOpure ultrapure water system (Barnstead/Thermolyne, Dubuque, IA, USA). Pooled human liver microsomes containing 20 mg/ml protein were obtained from XenoTech (Kansas city, KS, USA).

2.2. Microsomal incubations

Stock solutions of individual probe substrate, AN, PA, DM, MP, TB and TS, were prepared at 100 m*M* in acetonitrile. A primary stock solution of mixed probe substrates, with AN/PA/DM/MP/TB/TS at 2.5/3.0/2.0/3.0/20.0/6.0 m*M*, was prepared by mixing an appropriate amount of the individual substrate stock followed by dilution in water. A secondary stock solution of the mixed probe substrates was prepared by a 10-fold dilution of the primary stock with 50 m*M* potassium phosphate buffer (pH 7.4). The primary and secondary stock solutions were used to make working solutions at different substrate concentrations for human liver microsomal incubations, in which the organic percentage of the solution was controlled below 1%. The stock solutions were stored at −20 °C.

Incubations of mixed CYP probe substrates with human liver microsomes were carried out at 37 °C in a bench-top Lab-Line shaker (Barnstead/Thermolyne, Dubuque, IA, USA). The incubation solutions contained 50 m*M* potassium phosphate buffer (pH 7.4), 0.2–2 mg/ml microsomal proteins, 10 m*M* $MgCl_2$, 1.2 m*M* NADPH and 1.25/1.5/1.0/1.5/10/3.0 μ*M* to 0.5/0.6/0.4/0.6/4.0/1.2 m*M* AN/PA/DM/MP/TB/TS in a final volume of 100 μl. The reaction was initiated by addition of NADPH after 5-min preincubation. After a given incubation time (0–60 min), the reaction was terminated by adding 50 μl acetonitrile containing 0.1 μg/ml bucetin as an internal standard (I.S.). The samples were centrifuged (Allegra™ 6R centrifuge, Beckman Coulter, Palo Alto, CA, USA) at 5 °C for 10 min at 3000 rpm (2060 *g*) to separate protein. The supernatant was transferred to a disposable vial in an autosampler (Sample Sentinel, BAS, West Lafayette, IN, USA) at 4 °C and injected (20 μl) for LC–MS–MS analysis.

The reaction linearity was examined by incubating a sample solution of 25/30/20/30/200/60 μ*M* AN/PA/DM/MP/TB/TS with 1 mg/ml microsomal protein for different length of time (5, 10, 15, 20, 25, 30, 45, and 60 min). The effect of microsomal protein concentration and NADPH concentration on metabolite formation was investigated by varying the protein concentration in the range of 0.2–2 mg/ml and NADPH concentration in the range of 0.12–6.0 m*M* in 15-min microsomal incubations, with a fixed substrate concentration at 25/30/20/30/200/60 μ*M* AN/PA/DM/MP/TB/TS. To obtain Michaelis–Menten enzyme kinetic parameters for the probe substrate metabolites, the incubations were carried out at different substrate concentrations with 1 mg/ml microsomal proteins for 15 min. The enzymatic rate was determined by the quantity of metabolite formed per unit time per unit protein and has unit nanomol/min per mg protein. The concentration–velocity data were fitted to the Lineweaver–Burk equation to obtain K_m and V_{max} values.

2.3. Calibration standards and quality controls

A standard mixture of 100/300/25/250/100/100 μ*M* AP/APAP/DX/OH-MP/OH-TB/OH-TS was prepared by appropriately diluting 1 m*M* individual standard metabolite solutions with phosphate buffer.

This standard mixture was used as a stock to prepare calibration solutions with AP/APAP/DX/OH-MP/OH-TB/OH-TS ranging from 1.0/3.0/0.25/2.5/1.0/1.0 n*M* to 5.0/15.0/1.25/12.5/5.0/5.0 μ*M*.

Three quality control (QC) samples were prepared independently (i.e., separate weighing) from the calibration solutions, with the concentrations of AP/APAP/DX/OH-MP/OH-TB/OH-TS at 0.03/0.08/0.02/0.06/0.03/0.05, 0.15/0.4/0.1/0.3/0.15/0.25, and 1.5/4.0/1.0/3.0/1.5/2.5 μ*M*, respectively. The working solutions (100 μl) for calibration and QC contained the same components as normal microsomal incubates, i.e., 25/30/20/30/200/60 μ*M* AN/PA/DM/MP/TB/TS in 50 m*M* potassium phosphate buffer (pH 7.4), 1 mg/ml microsomal protein and 10 m*M* $MgCl_2$. The same procedure as for microsomal incubation was applied to the calibration and QC samples, i.e., the working solutions were preincubated at 37 °C for 10 min, followed by addition of 50 μl acetonitrile containing 0.1 μg/ml bucetin internal standard and 1.2 m*M* NADPH. The samples were centrifuged at 3000 rpm (2060 *g*) for 10 min at 5 °C and the supernatant (20 μl) was directly injected for LC–MS–MS analysis.

2.4. LC–MS system

The LC–MS system was equipped with a BAS PM-80 gradient pump coupled to a Finnigan LCQ Deca ion trap mass spectrometer (ThermoQuest, San Jose, CA, USA) with an electrospray ionization (ESI) source. The LC separation was carried out with a 3.5 μm, 50×2.1 mm C_8 Symmetry Shield™ column (Waters, Milford, MA, USA) at room temperature (25 °C). The mobile phase (pH 2.75) consisted of 0.1% formic acid in water (A) and in acetonitrile (B). The flow-rate was 0.8 ml/min. Samples were injected by an autosampler (Sample Sentinel, BAS) with a 20-μl injection loop. In gradient elution, the proportion of acetonitrile was linearly increased from 5 to 50% in 1 min, held at 50% for 2 min, and then returned to 5% in 0.1 min. The column was allowed to equilibrate for 3 min after each run. Only the data within the first 3.5 min were acquired by MS. The flow was diverted to waste for the first 0.5 min, to MS detector for the next 2.5 min, and to waste again for the remaining run time. The MS was operated in positive ESI mode. Nitrogen was used as both the sheath and auxiliary gas at a pressure of 80 and 20 arbitrary units, respectively. The spray voltage was set at 5.0 kV and the capillary temperature was at 350 °C.

Full-scan and product ion mass spectra of the selected CYP probe substrates and their metabolites were acquired (data not shown). The most abundant product ion of each probe metabolite was chosen for selected reaction monitoring (SRM). In LC–MS–MS experiment, helium was used as the target gas for collision-induced dissociation. The SRM transitions and collision energies are shown in Table 2. Four segments of a scan event were used for MS–MS data acquisition. The first segment (1.2 min) was used to monitor AP and APAP, while the second segment (0.8 min) was used to monitor DX. The third segment (0.75 min) was used to monitor OH-MP and bucetin, and the fourth segment (0.75 min) was used to monitor OH-TB and OH-TS.

Table 2
SRM transitions and collision energies used in LC–MS–MS for the detection of CYP probe substrate metabolites

Probe substrate metabolite	Molecular mass (MW)	SRM transition (*m*/*z*)	Collision energy (%)
4-Aminophenol (AP)	109	110→93	34
Acetaminophen (APAP)	151	152→110	30
Dextrorphan (DX)	257	258→201	34
4-Hydroxymephenytoin (OH-MP)	234	235→150	30
4-Hydroxytolbutamide (OH-TB)	286	287→188	28
6β-Hydroxytestosterone (OH-TS)	304	305→287	28
Bucetin (internal standard)	223	224→138	30

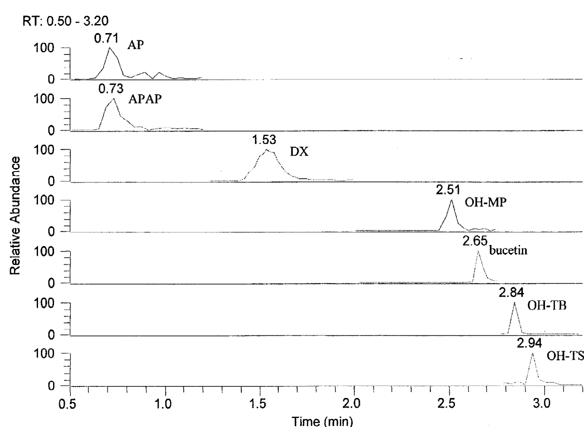


Fig. 1. SRM chromatograms of a standard metabolite mixture (0.25/0.75/0.0625/0.625/0.25/0.25 μ*M* AP/APAP/DX/OH-MP/OH-TB/OH-TS and 0.45 μ*M* bucetin).

3. Results and discussion

3.1. LC–MS–MS of standard metabolite mixture

Fig. 1 shows the SRM chromatograms of a standard metabolite mixture. The conditions of SRM transitions and collision energies for LC–MS–MS are listed in Table 2. All of the standard metabolites were detected in 3.0 min. The standard mixture has the same matrix, i.e., liver microsomes, probe substrates, $MgCl_2$, NADPH and phosphate buffer, as the normal microsomal incubates. In developing the LC–MS–MS method, there was no attempt to achieve a complete chromatographic separation because of the high selectivity of the LC–MS–MS method. However, separation of analytes may reduce potential signal suppression from co-eluting peaks during ES ionization and ion-trap MS detection processes. The LC separation in the present work has allowed the use of four segments in a LC–MS–MS scan to reduce the number of SRM transitions monitored and improve the MS–MS detection sensitivity for the metabolites.

Flow rate can affect LC separation efficiency and ES ionization efficiency. It also affects the necessary column equilibration time between runs. The MS response for metabolites such as OH-TS deteriorated when the flow-rate was above 0.8 ml/min. A flow-rate of 0.8 ml/min was used in this work to obtain a good MS response for all of the analytes and to achieve high throughput. At this flow-rate, it was found that a 3-min column equilibration was sufficient to produce a reproducible LC–MS–MS chromatogram. By using the gradient elution profile described previously, the MS data acquisition for all analytes was finished in 3.5 min and the total experiment cycle time was 6 min.

3.2. SRM chromatograms of a human liver microsomal incubate

Fig. 2 shows the SRM chromatograms of a microsomal incubate resulting from incubating a mixture of probe substrates with human liver microsomes. All the SRM peaks shown in Fig. 1 were observed in Fig. 2 at the expected retention times. The LC–MS–MS data confirm the formation of the probe substrate metabolites in human liver microsomal incubations. In the meantime, the data indicate a good reproducibility of LC separation and demonstrate that the sensitivity of the LC–MS–MS method is sufficient for the detection of the probe metabolites resulting from normal microsomal incubations.

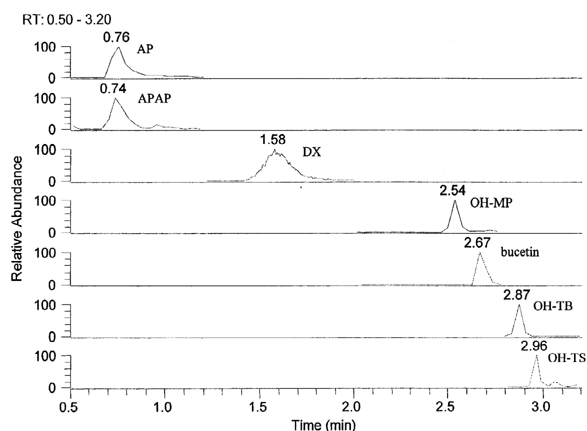
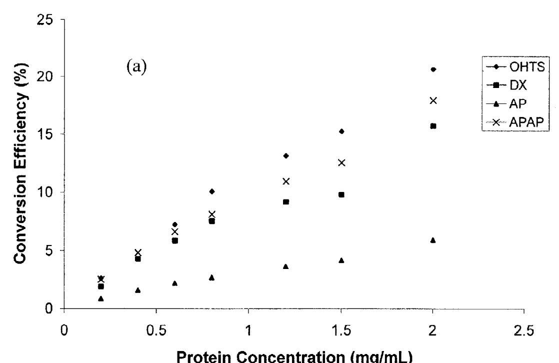


Fig. 2. SRM chromatograms of a human liver microsomal incubate: 25/30/20/30/200/60 μ*M* AN/PA/DM/MP/TB/TS, 15-min incubation, 1.0 mg/ml microsomal protein.

3.3. Reaction linearity

The linearity of enzyme reactions has been assessed by monitoring the effect of incubation time (from 5 to 60 min) and microsomal protein concentration (from 0.2 to 2 mg/ml) on metabolite production. The formation of APAP and OH-TB showed a good linearity with incubation time up to 45 min while the formation of all other probe substrate metabolites was linear with incubation time up to 20 min. The effect of protein concentration on metabolite formation is shown in Fig. 3, in which the percentage of substrates (conversion efficiency) that was converted into metabolites was plotted as a function of protein concentration. The conversion efficiency was calculated as the ratio of the moles of metabolites formed versus the substrates used in the microsomal incubations. As can be seen from Fig. 3, the formation of metabolites increased linearly with protein concentration in the range of 0.2–1.2 mg/ml. A slight deviation from linearity was observed when protein concentration was above 1.2 mg/ml. Fig. 3 shows that a maximum of ~20% TS, 18% PA, 16% DM, 6% AN, 0.5% TB and 0.9% MP probe substrates were converted into metabolites in a 15-min incubation with 2 mg/ml protein.



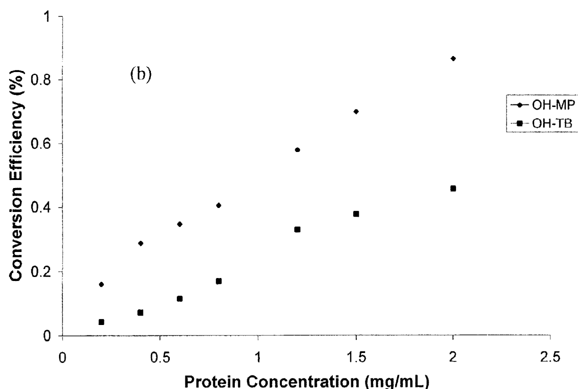


Fig. 3. Metabolite formation against protein concentration: (a) OH-TS, DX, AP, APAP; (b) OH-MP, OH-TB. Conversion efficiency was calculated as the ratio of the number of moles of metabolites formed versus substrates used in microsomal incubations. Incubation mixture contained 25/30/20/30/200/60 μ*M* AN/PA/DM/MP/TB/TS. Incubation time: 15 min.

The effect of NADPH coenzyme concentration on metabolite formation was also investigated. The CYP enzymes such as CYP1A2, 2D6 and 2E1 showed decreased activities when the NADPH coenzyme concentration was below 0.6 m*M*. Above 0.6 m*M*, NADPH had little effect on metabolite formation, indicating that the CYP enzymes achieve maximum catalytic activities under this condition. For subsequent analysis, a 15-min incubation time, 1 mg/ml microsomal protein concentration and 1.2 m*M* NADPH concentration were used in the microsomal incubations. Under this condition, the reaction is linear with respect to time and protein concentration, and the concentration of NADPH coenzyme is sufficient to maintain a maximum CYP enzyme activity.

3.4. Calibration curves

The calibration curves for each probe substrate metabolite showed good linearity with correlation coefficients greater than 0.997 for OH-MP and 0.999 for all other analytes. The lower limit of detection (LLOD) is ~5 n*M* for AP, 15 n*M* for APAP, 2.5 n*M* for OH-MP, 5 n*M* for OH-TB and 10 n*M* for OH-TS. The LLOD for DX was less than 0.25 n*M* and was not further pursued because it was below the lowest concentration of the working solutions for calibrations. The lower limit of quantification (LLOQ) is 25 n*M* for AP, 30 n*M* for APAP, 2.5 n*M* for DX, 25 n*M* for OH-MP, 10 n*M* for OH-TB and 10 n*M* for OH-TS, based on the lowest concentration within the linear range of the calibration curves that gives an acceptable accuracy of ±20% and a precision of ±20% (S/N ~3). The linear concentration range, ranging from 25/30/2.5/25/10/10 n*M* to 5.0/15.0/

1.25/12.5/5.0/5.0 μ*M* for AP/APAP/DX/OH-MP/OH-TB/OH-TS, was proved to be sufficient for the analysis of the metabolites in microsomal incubations.

3.5. Method precision and accuracy

The precision and accuracy of the LC–MS–MS method were examined by using three quality control (QC) samples prepared independently from the calibration solutions. Eight measurements were taken for each QC sample. The accuracy is calculated as the relative error (RE) and the precision as the relative standard deviation (RSD). The results are summarized in Table 3. As can be seen from Table 3, the LC–MS–MS method provides a good accuracy with RE less than 5% and good precision with RSD less than 10% for all of the analytes under study.

3.6. Determination of K_m and V_{max}

Michaelis–Menten kinetic parameters K_m and V_{max} for the reaction of each probe substrate were determined by fitting the substrate concentration, [S], and velocity, V, data into Lineweaver–Burke equation: $1/V = K_m/V_{max} \times 1/[S] + 1/V_{max}$. The velocity was calculated as the rate of metabolite production per mg protein. The values of K_m and V_{max} determined for each probe substrate in human liver microsomes are listed in Table 4. K_m reflects the substrate affinity of the enzyme and V_{max} reflects the intrahepatic concentration of the enzyme. The K_m and V_{max} values may vary significantly among different species or the same species (e.g., human) but from different sources, and the K_m data are relatively more comparable than the V_{max} data [7]. As can be seen from Table 4, the measured K_m and V_{max} values are in good agreement with the literature values obtained in human liver microsomes [7,21,22]. The V_{max} measured for MP is greater than that from the literature, which may indicate the variation of intrahepatic concentrations of the CYP2C19 enzyme in human liver microsomes. The overall agreement between the measured and the literature-reported values of K_m and V_{max} validates the LC–MS–MS method and the mixed-substrate incubation approach. The data suggest that the LC–MS–MS method combined with mixed-probe incubation can be used to efficiently obtain enzyme reaction constants with increased throughput in in vitro drug metabolism studies.

Table 3
Method accuracy and precision data ($n=8$) from QC samples

	QC sample level (n*M*)		
AP			
Nominal conc. (n*M*)	30	150	1500
Measured (n*M*)	30.50	151.8	1480.2
SD (n*M*)	1.35	5.1	53.1
Accuracy (RE)	1.7%	1.2%	1.3%
Precision (RSD)	4.5%	3.4%	3.5%
APAP			
Nominal conc. (n*M*)	80	400	4000
Measured (n*M*)	79.10	407.0	4080.5
SD (n*M*)	4.20	12.0	150.0
Accuracy (RE, %)	−1.5%	1.7%	2.0%
Precision (RSD, %)	5.3%	3.0%	3.7%
DX			
Nominal conc. (n*M*)	20	100	1000
Measured (n*M*)	20.80	97.4	985.0
SD (n*M*)	1.30	5.5	46.0
Accuracy (RE, %)	4.0%	−2.6%	−1.5%
Precision (RSD, %)	6.5%	5.5%	4.6%
OH-MP			
Nominal conc. (n*M*)	60	300	3000
Measured (n*M*)	61.05	307.8	3070.0
SD (n*M*)	3.60	13.0	125.0
Accuracy (RE, %)	1.7%	2.6%	2.3%
Precision (RSD, %)	6.0%	4.3%	4.2%
OH-TB			
Nominal conc. (n*M*)	30	150	1500
Measured (n*M*)	30.30	152.4	1562.3
SD (n*M*)	1.05	3.7	85.0
Accuracy (RE, %)	1.0%	1.6%	4.1%
Precision (RSD, %)	3.5%	2.5%	5.7%
OH-TS			
Nominal conc. (n*M*)	50	250	2500
Measured (n*M*)	49.40	245.0	2570.0
SD (n*M*)	2.4	10.5	180.0
Accuracy (RE, %)	−1.2%	−2.0%	2.8%
Precision (RSD, %)	4.8%	4.2%	7.2%

RSD values are between day values.

4. Conclusions

The present work demonstrates a LC–MS–MS method utilizing fast gradient LC and ESI-ion trap

Table 4
K_m and V_{max} determined for the enzymatic reactions of the probe substrates ($n=6$)

CYP enzyme	Probe substrate	Measured		Literature [7,21,22]	
		K_m (μ*M*)	V_{max} (nmol/min per mg protein)	K_m (μ*M*)	V_{max} (nmol/min per mg protein)
2E1	AN	24.2±1.1	1.20±0.11	24.2	1.28
1A2	PA	34.2±1.2	2.09±0.20	30.7	3.8
2D6	DM	6.2±1.7	0.11±0.01	22.9	0.13
2C19	MP	33.8±1.5	0.025±0.003	30	0.0029
2C9	TB	171.5±25.1	0.14±0.01	178	0.16
3A4	TS	69.1±5.0	0.96±0.09	67.4	1.11

MS for rapid and quantitative determination of several probe substrate metabolites for CYP2E1, 1A2, 2D6, 2C9, 2C19 and 3A4 in a single run and without sample pretreatment. Excellent linearity was observed from the calibration curves, with correlation coefficient greater than 0.997 for 4′-hydroxymephenytoin and 0.999 for all other analytes. The relative error and relative standard deviation of the measurements are less than 10%. All the analytes were detected in positive ion mode within 3.5 min in a single run. The use of aniline probe for CYP2E1 has avoided the need for switching ESI polarity during the run. The LC–MS–MS method has been validated and applied to obtain Michaelis–Menten kinetic parameters K_m and V_{max} for the probe substrates in human liver microsomes. The K_m and V_{max} values obtained by the LC–MS–MS method in conjunction with a mixed-substrate-incubation approach are in agreement with the literature-reported values.

Since LC–ESI-ion trap MS is relatively inexpensive as compared to triple quadrupole MS and is of growing use in pharmaceutical laboratories, the LC–MS–MS method developed in this work can efficiently be used to improve the throughput and cost-effectiveness for preclinical drug metabolism studies.

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