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Liquid chromatography/tandem mass spectrometry method for simultaneous evaluation of activities of five cytochrome P450s using a five-drug cocktail and application to cytochrome P450 phenotyping studies in rats

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ABSTRACT

A reliable liquid chromatography/tandem mass spectrometry has been developed for simultaneous evaluation of the activities of five cytochrome P450s (CYP1A2, CYP2C9, CYP2C19, CYP2D6, and CYP3A) in rat plasma and urine. The five-specific probe substrates/metabolites include phenacetin/paracetamol (CYP1A2), tolbutamide/4-hydroxytolbutamide and carboxytolbutamide (CYP2C9), mephenytoin/4′-hydroxymephenytoin (CYP2C19), dextromethorphan/dextrorphan (CYP2D6), and midazolam/1′-hydroxymidazolam (CYP3A). Internal standards were brodimoprim (for phenacetin, paracetamol, midazolam and 1′-hydroxymidazolam), ofloxacin (for 4′-hydroxymephenytoin, dextromethorphan and dextrorphan) and meloxicam (for tolbutamide, 4-hydroxytolbutamide and carboxytolbutamide). Sample preparation was conducted with solid-phase extraction using Oasis® HLB cartridges. The chromatography was performed using a C_{18} column with mobile phase consisting of methanol/0.1% formic acid in 20 mM ammonium formate (75:25). The triple–quadrupole mass spectrometric detection was operated in both positive mode (for phenacetin, paracetamol, midazolam, 1′-hydroxymidazolam, brodimoprim, 4′-hydroxymephenytoin, dextromethorphan, dextrorphan and ofloxacin) and negative mode (for tolbutamide, 4-hydroxytolbutamide, carboxytolbutamide and meloxicam). Multiple reaction monitoring mode was used for data acquisition. Calibration ranges in plasma were 2.5–2500 ng/mL for phenacetin, 2.5–2500 ng/mL for paracetamol, 5–500 ng/mL for midazolam, and 0.5–500 ng/mL for 1′-hydroxymidazolam. In urine calibration ranges were 5–1000 ng/mL for dextromethorphan, 0.05–10 μg/mL for dextrorphan and 4′-hydroxymephenytoin, 5–2000 ng/mL for tolbutamide, 0.05–20 μg/mL for 4-hydroxytolbutamide and 0.025–10 μg/mL for carboxytolbutamide. The intra- and inter-day precision were 4.3–12.4% and 1.5–14.8%, respectively for all of the above analytes. The intra- and inter-day accuracy ranged from −9.1 to 8.3% and −10 to 9.2%, respectively for all of the above analytes. The lower limits of quantification were 2.5 ng/mL for phenacetin and paracetamol, 5 ng/mL for midazolam, 0.5 ng/mL for 1′-hydroxymidazolam, 5 ng/mL for dextromethorphan, 50 ng/mL for dextrorphan and 4′-hydroxymephenytoin, 5 ng/mL for tolbutamide, 50 ng/mL for 4-hydroxytolbutamide and 25 ng/mL for carboxytolbutamide. All the analytes were evaluated for short-term (24 h, room temperature), long-term (3 months, −20 °C), three freeze–thaw cycles and autosampler (24 h, 4 °C) stability. The stability of urine samples was also prepared with and without β-glucuronidase incubation (37 °C) and measured comparatively. No significant loss of the analytes was observed at any of the investigated conditions. The current method provides a robust and reliable analytical tool for the above five-probe drug cocktail, and has been successfully verified with known CYP inducers.

1. Introduction

Cytochrome P450 (CYP) system represents drug-metabolizing enzymes involved in phase I (oxidative) metabolism. Among the various CYP isozymes, CYP1A2, 2C9, 2C19, 2D6 and 3A are the major isoforms responsible for the metabolism of more than 90% of market drugs [1,2]. Administration of specific probe drugs followed by measurement of metabolism of those particular substrates can

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be used to determine the real-time activities of CYP450 [3–8]. Compared to the individual administration of specific probes in multiple studies, the "cocktail" approach can minimize the confounding influence of inter-subject and intra-subject variability over the time [9]. Therefore, a number of drug metabolism cocktails (particularly five- or six-drug cocktails) have been proposed and developed [3–5,10–13]. Recently, Jerdi et al. and Yin et al., respectively reported analytical methods for the simultaneous determination of CYP1A2, 2C9, 2C19, 2D6 and 3A substrates [14,15]. However, their cocktails still included some undesirable probe drugs (i.e. debrisoquine and flurbiprofen), so the practical application of both cocktails was limited. First of all, studies suggested that debrisoquine might not correlate well with the CYP2D6 probe sparteine *in vivo* [16,17]. Additionally, debrisoquine had the potential of causing significant hypotension [18]. Secondly, the use of flurbiprofen as a CYP2C9 marker is undesirable due to the weak correlation observed between the formation clearance of flurbiprofen to its CYP2C9-mediated metabolites and genotype [19]. Thirdly, omeprazole has been used as a probe drug for CYP2C19, but besides the CYP2C19-mediated 5-hydroxylation of omeprazole, CYP3A4-mediated sulfoxidation of both omeprazole and hydroxyomeprazole also occurred [20]. Thus the activity of CYP2C19 reflected by omeprazole and 5-hydroxylation is probably not objective.

The current study was proposed to (1) develop a modified phenotyping cocktail with superior probes; (2) choose the most appropriate biological matrix for accurate metabolic analysis of probes; (3) investigate a specific analytical method for the simultaneous evaluation of *in vivo* activities of five major CYP isozymes for drug biotransformation (1A2, 2C9, 2C19, 2D6 and 3A) in one test system. The probe drugs selected for the present study were phenacetin, tolbutamide, mephenytoin, dextromethorphan and midazolam. The chosen substrates have demonstrated as superior (in terms of safety, sensitivity and specificity of enzyme metabolism) to the probes included in previous studies of phenotyping cocktails. To the best of our knowledge, this is the first work combining these five probe drugs for these five CYPs. In this paper we describe the development and validation of a sensitive and selective liquid chromatography/mass spectrometry (LC–MS/MS) method for the simultaneous analysis of the probe drugs (phenacetin, tolbutamide, dextromethorphan and midazolam) and metabolites (paracetamol, 4-hydroxytolbutamide, carboxytolbutamide, 4′-hydroxymephenytoin, dextrorphan and 1′-hydroxymidazolam) in rat plasma and urine (Fig. 1). This method has also been successfully verified with known CYP inducers.

2. Experimental

2.1. Chemicals and animals

Midazolam, 1′-hydroxymidazolam, 4′-hydroxymephenytoin, dextrorphan, carboxytolbutamide, 4-hydroxytolbutamide and lyophilized β-glucuronidase (type VII–A) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Phenacetin was obtained from Tianjin Li Sheng Pharmaceutical Co. Ltd. (Tianjin, China). Paracetamol was kindly provided by Shen Yang Pharmaceutical University (Shenyang, China). Dextromethorphan, tolbutamide, brodimoprim, ofloxacin and meloxicam were supplied by Tianjin Institute of Pharmaceutical Research (Tianjin, China). All the above standard compounds possess purity of better than 99%. *S*-Mephenytoin was kindly provided by Tianjin Medical University (Tianjin, China). Phenobarbital was purchased from Shanghai Jin Shan Pharmaceutical Co. Ltd. (Shanghai, China). Rifampicin was obtained from Shenyang Hong Qi Pharmaceutical Co. Ltd. (Shenyang, China). HPLC-grade methanol was obtained from Tianjin Concord Tech Reagent Co. Ltd. (Tianjin, China). All other reagents were purchased from Shanghai Chemical Reagent Co. Ltd. (Shanghai, China) and were of analytical grade. Deionized water was prepared using a SYZ550 quartz pure water distiller (Tianjin Xinzhou Tech Co. Ltd., China) and used throughout the study. Oasis® HLB solid-phase extraction (SPE) cartridge (1 ml, 30 mg) were purchased from Waters Corp. (Milford, MA, USA).

Male Wistar rats weighting 180–220 g were purchased from Center of Experiment Animals, Tianjin Institute of Pharmaceutical Research (Certificate No. 2007-0001), Tianjin, China. Animals were fasted overnight before dose.

2.2. Instrumentation

Samples were analyzed by LC–MS/MS using a Thermo Electron (San Jose, CA, USA) LC–MS/MS system consisting of a Surveyor quaternary narrowbore LC pump, a Surveyor autosampler, fitted with a tempered tray and a column oven, coupled to a TSQ Quantum triple quadrupole tandem mass spectrometer which was equipped with an electrospray ionization (ESI) source. Instrument control and data acquisition was performed with the Xcalibur 1.1 software (Thermo Finnigan). Peak integration and calibration curves were made with LCQuan software (Thermo Finnigan). MS/MS conditions for analytes were optimized by infusing pure solutions (concentrations were approximately 100 ng/mL of each analyte) using the Quantum Tune Master® software (Thermo Electron).

2.3. LC–MS/MS conditions

The chromatographic separation was performed on a Shiseido C18 column (150 mm × 4.6 mm i.d., 5 μm; Shiseido Fine Chemicals, Japan). The column temperature was maintained at 35 °C. The tray temperature in the autosampler was kept at 4 °C. The mobile phase consisted of methanol and 20 mM ammonium formate containing 0.1% formic acid (75:25) with a flow rate of 0.4 mL/min. The total run time for each injection was 16 min (9.0 min for positive mode followed by 7.0 min for negative mode).

The electrospray ionization source was operated in either positive mode (4200 V) or negative mode (−4000 V). The capillary temperature was maintained at 280 °C. High purity nitrogen served both as sheath and auxiliary gas and set to 30 and 10 (arbitrary units), respectively. Argon (1.6 mTorr) was used as the collision-induced dissociation (CID) gas. Detection was carried out in the multiple reaction monitoring (MRM) mode. The MS/MS transitions and fragmentation conditions selected for individual analytes are shown in Table 1. The peak full width at half maximum (FWHM) was set at 0.7 Th for both Q1 and Q3. The scan time for each analyte was 0.2 s.

2.4. Calibration standards and quality control samples

The primary stock solutions of each probe drug and its metabolite were prepared at 1 mg/mL in methanol, except for 1′-hydroxymidazolam, 4′-hydroxymephenytoin and carboxytolbutamide which were 0.5 mg/mL in methanol. The stock solutions of internal standards, brodimoprim, ofloxacin and meloxicam, were prepared at 1 mg/mL in methanol, respectively. All the stock solutions were stored at −20 °C, and were stable for at least 6 months. The working solution of each analyte was prepared by diluting the stock solution with methanol/water (50:50).

Calibration standards (CSs) and quality control (QC) samples were prepared by spiking working solution of each analyte into blank plasma or urine. Calibration samples ($n = 3$) were made to achieved the final plasma concentrations of 2.5, 5, 25, 100,

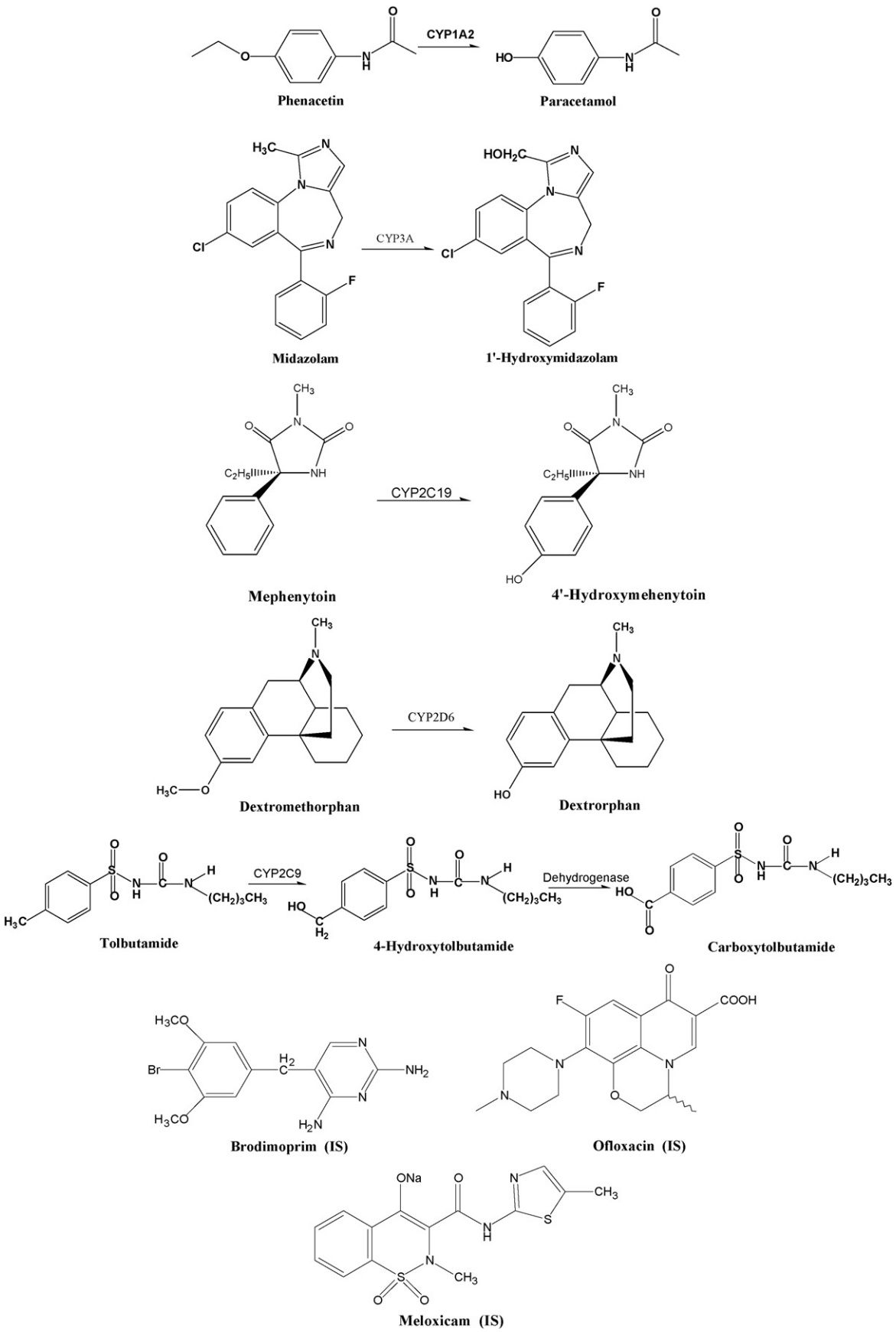

Fig. 1. Probe drugs used in the study, their *in vivo* metabolic conversions and the structures of internal standards.

Table 1
MS/MS transitions and collision energies for the detection of the analytes and internal standards

Analyte	Molecular mass (MW)	Precursor (m/z)	Product (m/z)	Ionization mode	Collision energy (eV)
Phenacetin	179	180	138	ESI^+	20
Paracetamol	151	152	110	ESI^+	20
Midazolam	325	326	291	ESI^+	30
1′-Hydroxymidazolam	341	342	324	ESI^+	25
Brodimoprim (IS)	338	339	281	ESI^+	35
4′-Hydroxymephenytoin	234	235	150	ESI^+	17
Dextromethorphan	271	272	215	ESI^+	25
Dextrorphan	257	258	199	ESI^+	25
Ofloxacin (IS)	361	362	318	ESI^+	25
Tolbutamide	270	269	170	ESI^-	20
4-Hydroxytolbutamide	286	285	186	ESI^-	21
Carboxytolbutamide	300	299	92	ESI^-	40
Meloxicam (IS)	351	350	286	ESI^-	15

500, 1000 and 2500 ng/mL for phenacetin and paracetamol; 5, 10, 20, 50, 100, 200 and 500 ng/mL for midazolam, and 0.5, 1, 5, 20, 100, 200 and 500 ng/mL for 1′-hydroxymidazolam. For urine the final concentrations of calibration standards ($n = 3$) were 50, 100, 500, 1000, 2000, 5000 and 10000 ng/mL for 4′-hydroxymephenytoin and dextrorphan; 5, 10, 50, 100, 200, 500 and 1000 ng/mL for dextromethorphan; 5, 10, 50, 100, 500, 1000 and 2000 ng/mL for tolbutamide; 50, 100, 500, 1000, 5000, 10,000 and 20,000 ng/mL for 4-hydroxytolbutamide, and 25, 50, 250, 500, 2500, 5000 and 10,000 ng/mL for carboxytolbutamide. Quality control samples ($n = 5$) were prepared at low, medium and high concentrations of 5, 100 and 1000 ng/mL for phenacetin and paracetamol; 10, 50 and 200 ng/mL for midazolam; 1, 20 and 200 ng/mL for 1′-hydroxymidazolam; 100, 1000 and 5000 ng/mL for 4′-hydroxymephenytoin and dextrorphan; 10, 100 and 500 ng/mL for dextromethorphan; 10, 100 and 1000 ng/mL for tolbutamide; 100, 1000 and 10,000 ng/mL for 4-hydroxytolbutamide, and 50, 500 and 5000 ng/mL for carboxytolbutamide. Aliquots of spiked plasma/urine samples were stored at −20 °C prior to use.

2.5. Sample preparations

2.5.1. Plasma samples

An aliquot of 10 μL of internal standard solution (2 μg/mL brodimoprim) was added to 0.200 mL plasma. After shaking the samples for 0.5 min on a vortex shaker, the mixture was then loaded onto an Oasis® HLB cartridge, which was already conditioned with 1 mL methanol followed by 1 mL water. After washing with 1 mL water and 1 mL 5% methanol in water, the analytes were eluted with 2× 1 mL methanol. The eluate was collected and evaporated to dryness at 37 °C under a gentle stream of nitrogen. The residue was reconstituted with 150 μL mobile phase, vortex-mixed for 1.0 min and transferred to a clean autosampler vial. A 20-μL aliquot was subsequently injected into the LC–MS/MS system.

2.5.2. Urine samples

All CS and QC samples were analyzed without previous treatment with β-glucuronidase. An aliquot of 50 μL urine was diluted with 450 μL water. To the above diluted urine sample, 500 μL of 20 mM ammonium formate (pH 4.75) were added, followed by addition of 10 μL of 5 μg/mL ofloxacin solution (IS for 4′-hydroxymephenytoin, dextromethorphan and dextrorphan) and 10 μL of 1 μg/mL meloxicam (IS for tolbutamide, 4-hydroxytolbutamide and carboxytolbutamide). The sample was mixed for 0.5 min on a vortex shaker and then loaded onto a preconditioned Oasis® HLB cartridge and extracted using the same procedure as described for plasma. After washing with 1 mL water and 1 mL 5% methanol in water, the analytes were eluted with 2× 1 mL methanol. The eluate was collected and evaporated to dryness at 37 °C under a gentle stream of nitrogen. The residue was reconstituted with 150 μL mobile phase by vortexing 1 min and then the sample was transferred into a clean autosampler vial. A 20-μL aliquot was subsequently injected into the LC–MS/MS system.

2.6. Method validation

The quantification method was validated according to FDA guidelines [21]. The specificity was investigated by analyzing blank rat plasma or urine from six different sources and comparing the potential interferences at the LC peak region for each analyte and IS, each in duplicate. Calibration curves were constructed using the analyte/IS peak area ratio versus the analyte concentration, and were fitted by a weighted ($1/x^2$) linear regression. To assess linearity, deviations of the mean calculated concentrations over three runs were set at ±15% of nominal concentration, except for the lower limit of quantification (LLOQ) where a deviation of ±20% was permitted. The extraction recovery were determined at low, medium and high concentrations by comparing the areas of plasma or urine samples spiked before extraction with those of blank plasma or urine extracts spiked after extraction. Method precision was expressed as relative standard deviation (R.S.D.), and accuracy was assessed as the percentage bias from the nominal concentration (% bias). QC samples (at three different concentrations) in five replicates were analyzed on the same day to determine the intra-day precision and accuracy, and were analyzed on each of three separate days to determine inter-day precision and accuracy. The acceptable intra- and inter-day precision and bias were set at ≤15%. The matrix effect (ME) was assessed in the following two ways introduced by He et al. and Matuszewski et al. [22,23]: to assess the absolute matrix effect, i.e. the potential ion suppression/enhancement due to matrix components, six different sources of each of blank rat plasma or urine were extracted by SPE and spiked with the selected analytes at QC concentrations. The absolute matrix effect was defined as (1-signal of post-extraction spiked sample/signal of pure solution) × 100%. To assess the co-elution effects, i.e. the potential ion suppression/enhancement effect of co-eluting analytes, pooled blank plasma/urine was spiked with each analyte or IS separately, and the corresponding peak area was compared to that from the spiked sample with mixture analytes. The experiments were preformed in triplicate at the medium QC concentrations. The stability of the analytes in rat plasma or urine under different conditions was assessed at three QC levels ($n = 3$). The freeze/thaw stability was determined after three freeze (−20 °C) and thaw (20 °C) cycles on separate days. The long-term stability was evaluated after storage of the test samples at −20 °C for 3 months, and short-term stability for 24 h at room temperature. To assess the injector stability of the processed samples, the test samples were extracted and placed in the autosampler at 4 °C

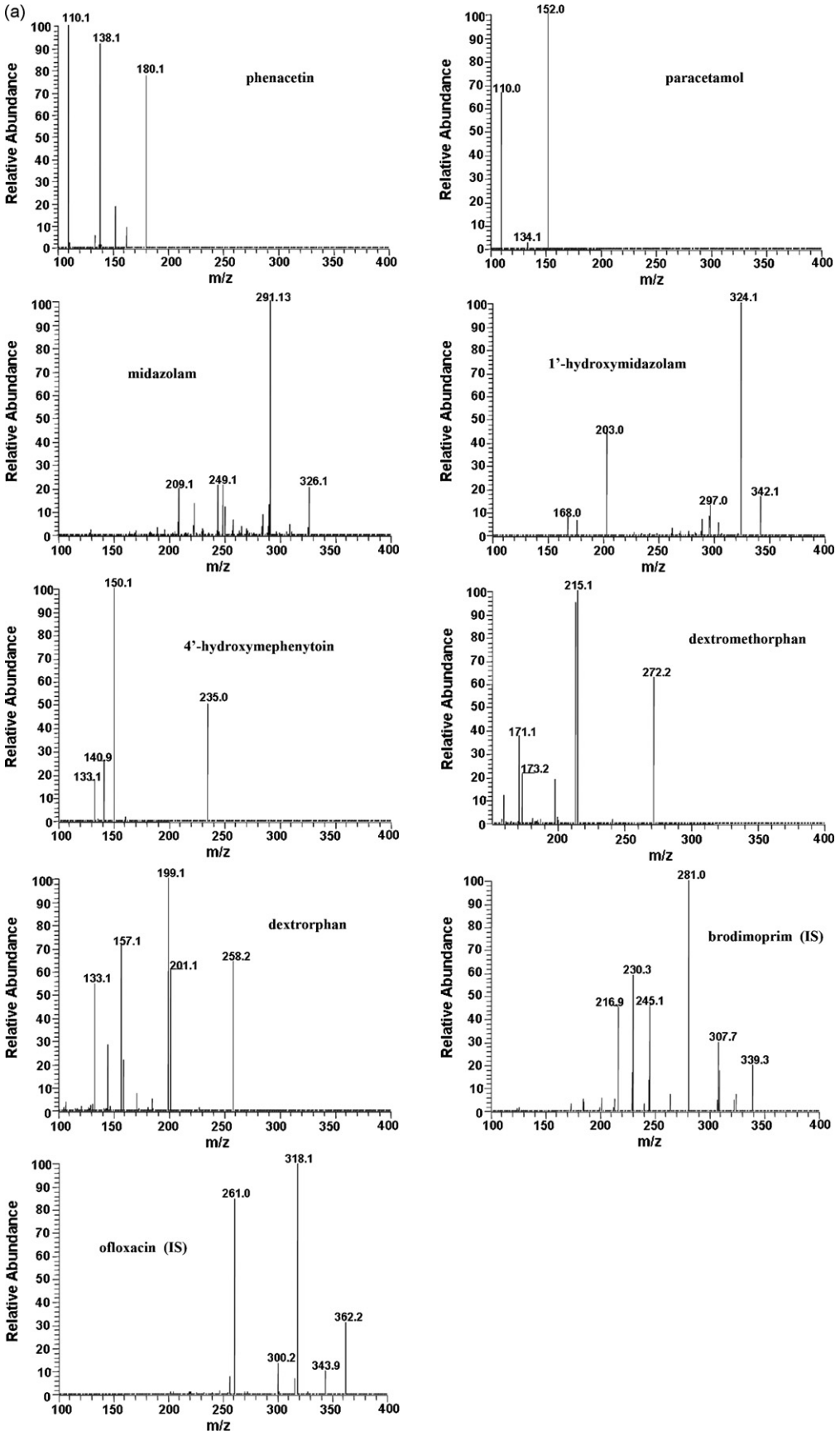

Fig. 2. Product ion mass spectra of the probe drugs, metabolites and internal standards: (a) positive ion analytes and internal standards and (b) negative ion analytes and internal standard.

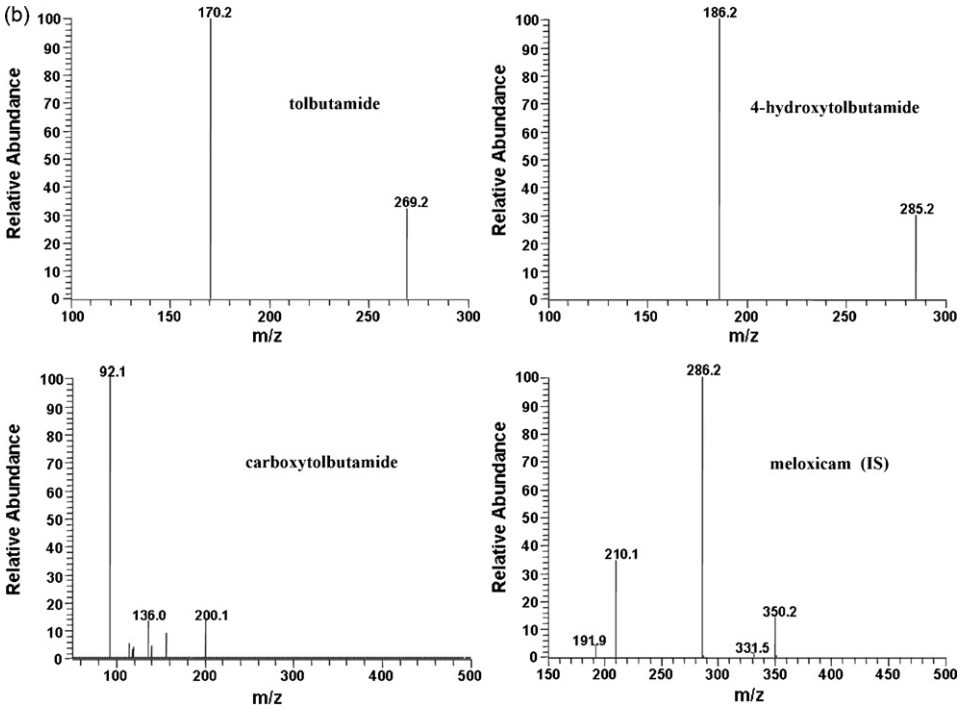

Fig. 2. (*Continued*).

Table 2
Calibration curves, extraction recoveries and sensitivity of the assay

Analyte	Matrix	Calibration range (ng/mL)	r^2	Recovery ($n = 9$) (%)[a]	LLOQ (ng/mL)
Phenacetin	Plasma	2.5–2500	0.9982 ± 0.0045	96.9 ± 3.5	2.5
Paracetamol	Plasma	2.5–2500	0.9985 ± 0.0030	81.6 ± 0.4	2.5
Midazolam	Plasma	5–500	0.9904 ± 0.0004	67.0 ± 13.8	5
1′-Hydroxymidazolam	Plasma	0.5–500	0.9915 ± 0.0015	93.0 ± 3.1	0.5
Dextromethorphan	Urine	5–1000	0.9994 ± 0.0035	95.1 ± 3.2	5
Dextrorphan	Urine	50–10,000	0.9950 ± 0.0019	85.2 ± 3.1	50
4′-Hydroxymephenytoin	Urine	50–10,000	0.9992 ± 0.0020	86.2 ± 8.7	50
Tolbutamide	Urine	5–2000	0.9994 ± 0.0015	98.4 ± 3.0	5
4-Hydroxytolbutamide	Urine	50–20,000	0.9937 ± 0.0026	98.3 ± 1.4	50
Carboxytolbutamide	Urine	25–10,000	0.9968 ± 0.0017	75.0 ± 5.0	25
Brodimoprim (IS)	Plasma	–	–	88.7	–
Ofloxacin (IS)	Urine	–	–	83.6	–
Meloxican (IS)	Urine	–	–	97.0	–

[a] For each analyte, the recovery experiment was performed with three QC concentrations (low, medium, and high), with triplicate determinations for each concentration.

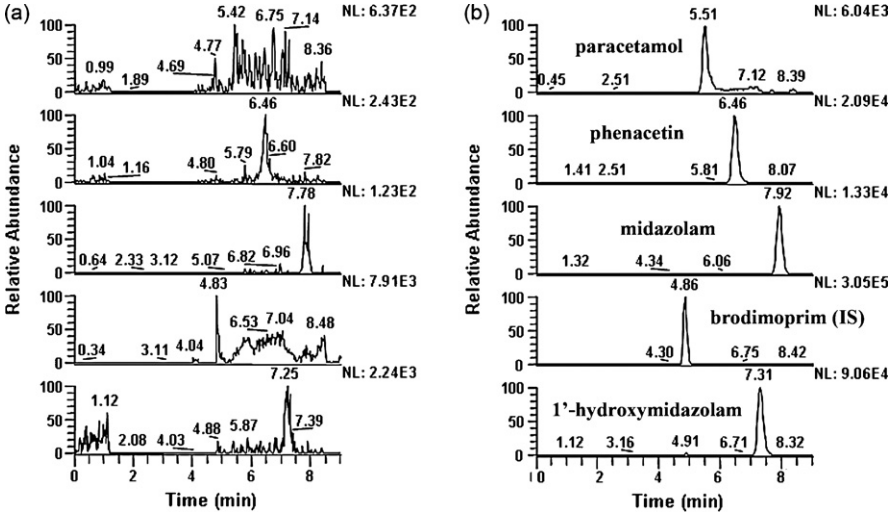

Fig. 3. MRM chromatograms: (a) of a 'blank' rat plasma sample and (b) of a QC plasma sample containing phenacetin (5 ng/mL), paracetamol (5 ng/mL), midazolam (10 ng/mL), 1′-hydroxymidazolam (1 ng/mL) and brodimoprim (100 ng/mL).

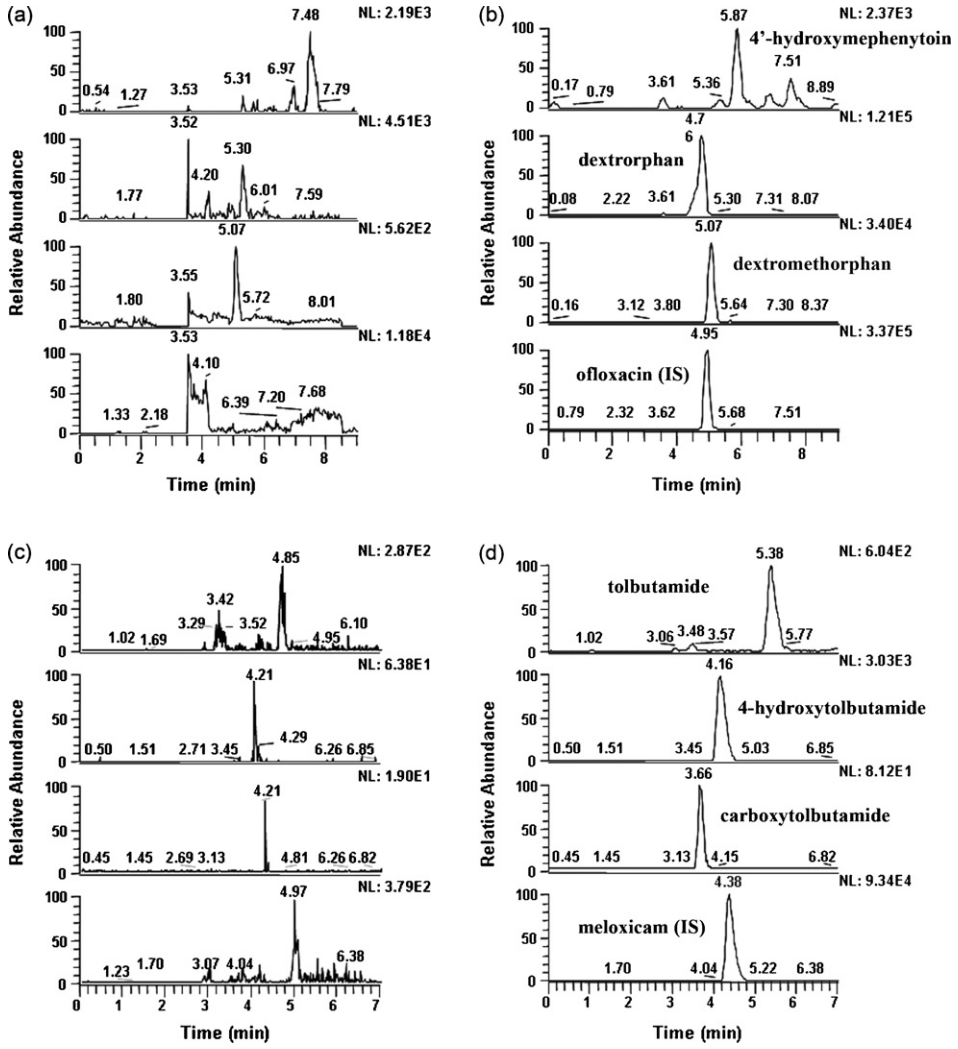

Fig. 4. MRM chromatograms: (a) of a 'blank' rat urine sample for positive ion analytes; (b) of a QC urine sample containing 4′-hydroxymephentoin (100 ng/mL), dextromethorphan (10 ng/mL), dextrophan (100 ng/mL) and off ofloxacin (1 μg/mL); (c) of a 'blank rat urine sample for negative ion analytes and (d) of a QC urine sample containing tolbutamide (10 ng/mL), 4-hydroxytolbutamide (100 ng/mL), carboxytolbutamide (50 ng/mL) and meloxican (200 ng/mL).

for 24 h, and then injected into the LC–MS/MS system for analysis. The measured concentrations were then compared to those of the same QC samples that had been analyzed immediately after processing and the percentage concentration deviation was calculated.

Urine samples at three QC levels were prepared with and without β-glucuronidase incubation (37 °C) and measured comparatively. This was done to test whether a time- and material-saving renunciation of β-glucuronidase would influence LC–MS/MS analysis.

Table 3
Matrix effect of the developed assay

Analyte	Matrix	Absolute ME[a] (%) ($n = 6$)		Co-elution effect[b] ($n = 3$)	
		Low QC	Medium QC	High QC	Medium QC
Phenacetin	Plasma	12.85	9.50	2.64	0.98
Paracetamol	Plasma	8.70	6.62	7.40	1.03
Midazolam	Plasma	23.97	22.11	20.23	1.04
1′-Hydroxymidazolam	Plasma	3.33	5.69	3.04	1.02
4′-Hydroxymephenytoin	Urine	−5.06	2.59	2.38	0.96
Dextromethorphan	Urine	29.55	28.12	23.48	0.98
Dextrorphan	Urine	5.84	−0.15	2.95	0.89
Tolbutamide	Urine	14.77	17.95	14.98	1.00
4-Hydroxytolbutamide	Urine	4.81	−6.54	−9.24	1.04
Carboxytolbutamide	Urine	−12.16	−3.83	−5.89	1.08
Brodimoprim (IS)	Plasma		5.45		1.02
Ofloxacin (IS)	Urine		12.09		0.93
Meloxicam (IS)	Urine		5.66		1.06

[a] Absolute ME was evaluated using six different lots of blank rat plasma/urine.
[b] Co-elution effect was evaluated using pooled blank rat plasma/urine.

Table 4
Intra- and inter-day precision and accuracy (n = 15, 5 replicates per day for 3 days)

Analyte	Matrix	Nominal concentration (ng/mL)	Intra-day			Inter-day		
			Measured concentration (ng/mL)	Accuracy (% bias)	Precision (% R.S.D.)	Measured concentration (ng/mL)	Accuracy (% bias)	Precision (% R.S.D.)
Phenacetin	Plasma	5	5.2	4.0	5.4	5.1	2.0	9.2
		100	108.3	8.3	7.8	105.9	5.9	4.5
		1000	946.5	−5.4	6.0	957.4	−4.3	4.4
Paracetamol	Plasma	5	5.0	0.0	6.7	4.9	−2.0	5.9
		100	106.9	6.9	6.8	105.8	5.8	3.2
		1000	965.9	−3.4	5.7	981.6	−1.8	12.7
Midazolam	Plasma	10	9.5	−5.0	6.7	10.0	0.0	14.2
		50	47.3	−5.4	12.4	49.4	−1.2	9.5
		200	211.8	5.9	5.4	213.2	6.6	6.8
1′-Hydroxymidazolam	Plasma	1	1.0	0.0	4.6	0.9	−10.0	14.8
		20	20.7	3.5	7.5	20.4	2.0	7.6
		200	197.4	−1.3	6.3	202.4	1.2	6.3
Dextromethorphan	Urine	10	10.7	7.0	6.4	10.0	0.0	13.1
		100	92.4	−7.6	5.6	97.4	−2.6	9.9
		500	502.6	0.5	9.0	513.9	2.8	14.0
Dextrorphan	Urine	100	100.2	0.2	9.0	96.3	−3.7	7.8
		1000	986.4	−1.4	9.3	986.5	−1.4	3.9
		5000	4594.7	−8.1	7.2	4579.4	−8.4	13.9
4′-Hydroxymephenytoin	Urine	100	91.2	−8.8	8.2	96.1	−3.9	14.8
		1000	909.2	−9.1	6.4	969.5	−3.1	14.0
		5000	5039.8	0.8	5.3	5276.2	5.5	9.4
Tolbutamide	Urine	10	9.8	−2.0	7.1	9.8	−2.0	1.5
		100	96.8	−3.2	5.0	98.9	−1.1	4.2
		1000	1001.2	0.1	4.4	993.3	−0.7	6.7
4-Hydroxytolbutamide	Urine	100	107.7	7.7	7.5	105.2	5.2	13.4
		1000	978.7	−2.1	5.5	967.4	−3.3	2.6
		10000	9877.1	−1.2	4.9	10175.7	1.8	5.7
Carboxytolbutamide	Urine	50	53.3	6.6	5.1	54.6	9.2	9.7
		500	488.4	−2.3	4.3	463.4	−7.3	10.6
		5000	4884.4	−2.3	5.6	5175.4	3.5	11.4

Moreover, it should be ensured that untreated and deglucuronized urine samples do not differ in their analytical behavior, i.e. due to thermal decomposition of the analytes during incubation at 37 °C.

2.7. Study on the potential in vivo interactions between probes in the cocktail

To assess the potential *in vivo* interaction between the individual probes present in the cocktail, potential metabolic interference of each CYP isozyme was investigated by comparing the phenotypic indices of individual administration of probe substrate versus co-administration of the five cocktail-probe drugs in 36 rats. Rats were divided into six groups randomly (n = 6) and administrated orally with phenacetin (5 mg/kg), midazolam (2 mg/kg), mephenytoin (0.5 mg/kg), dextromethorphan (4 mg/kg), tolbutamide (2 mg/kg) and cocktail consisted of the above five probe drugs, respectively. Blood samples were collected at 0.5 and 1 h, while urine samples at 0–6 and 6–12 h post-dosing. Before determination, 0.500 mL of each diluted urine sample at 0–6 h was incubated with 250 μL of 20 mM acetic acid (pH 4.75) and 250 μL of a 5000-units/mL β-glucuronidase solution (in 20 mM ammonium formate, pH 4.75) in a water bath at 37 °C for 12 h to deconjugation. The plasma and urine samples were then processed in the manner described above and analyzed with standard and QC samples.

2.8. Verification of the in vivo cocktail system by known CYP enzyme inducers

Phenobarbital and rifampicin are known CYP enzyme inducers. To test the effectiveness of the analytical method, rats were randomly divided into three groups with six each, including blank control group, phenobarbital positive control group (for CYP1A2, 2C9, 2D6 and 3A) and rifampicin positive control group (for CYP2C19). After oral administration of blank solution, phenobarbital (40 mg/kg) and rifampicin (50 mg/kg), respectively for seven consecutive days, rats were administrated orally with the cocktail solution consisted of phenacetin (5 mg/kg), midazolam (2 mg/kg), mephenytoin (0.5 mg/kg), dextromethorphan (4 mg/kg) and tolbutamide (2 mg/kg). Procedures of sample collection and preparation as well as determination were the same as mentioned in the above sections.

2.9. Data analysis

As reference and our previous experiment shown, ratio of the plasma concentrations of paracetamol to phenacetin at 0.5 h (unpublished data) and 1′-hydroxymidazolam to midazolam at 1 h postdose were used to represent the activities of CYP1A2 and CYP3A, respectively [27]. The CYP2C9 activity was determined using the tolbutamide urinary metabolic ratio, calculated as the sum of the amount of 4-hydroxytolbutamide and carboxytolbu-

Table 5
Stability of samples ($n = 3$)

Analyte	Spiked Concentration (ng/mL)	3 months at −20 °C, RE (%)	Three freeze-thaw cycles, RE (%)	24 h at 4 °C in the autosampler, RE (%)[a]	24 h at room temperature, RE (%)	12 h at 37 °C incubated with β-glucuronidase
Phenacetin	5	2.2	0.4	10.4	−2.7	–
	100	6.6	6.3	12.3	9.4	–
	1000	−2.5	−6.8	−6.1	−1.7	–
Paracetamol	5	5.6	6.9	4.1	−2.2	–
	100	7.2	6.1	4.5	0.7	–
	1000	−3.0	−4.0	−5.8	−4.1	–
Midazolam	10	10.0	1.9	1.2	−10.9	–
	50	−1.7	−10.5	−3.9	−5.4	–
	200	−9.4	−9.1	−9.4	−10.6	–
1′-Hydroxymidazolam	1	7.4	8.1	6.4	7.0	–
	20	11.1	6.6	7.8	13.4	–
	200	−4.5	−7.9	−6.0	−3.5	–
Dextromethorphan	10	−1.2	−1.8	−6.0	−2.3	−4.8
	100	−3.8	8.3	0.3	−5.5	−2.0
	500	−8.9	8.5	9.8	3.3	4.2
Dextrorphan	100	2.7	−10.7	−12.3	3.8	−1.2
	1000	8.6	12.2	10.5	3.1	2.9
	5000	14.0	1.2	9.2	9.9	9.1
4′-Hydroxymephenytoin	100	−0.4	7.4	1.3	5.8	8.5
	1000	−14.2	−3.5	−4.3	−6.3	−7.5
	5000	−11.5	−6.4	−9.5	−0.6	−8.6
Tolbutamide	10	3.2	8.0	2.2	6.0	–
	100	4.7	9.2	−5.6	1.0	–
	1000	−4.2	−9.5	−2.1	−1.3	–
4-Hydroxytolbutamide	100	3.3	0.6	2.0	−0.7	–
	1000	5.3	5.3	0.4	−2.0	–
	10000	5.2	−1.7	3.5	11.6	–
Carboxytolbutamide	50	−5.4	−5.7	−9.8	3.8	–
	500	−9.1	−7.1	−5.7	−2.9	–
	5000	−7.0	−10.6	−8.2	8.5	–

[a] RE (%) = (measured concentration − freshly prepared concentration)/freshly prepared concentration × 100%.

tamide divided by the amount of tolbutamide recovered in 6–12 h urine [11]. The total urinary recovery of 4′-hydroxymephenytoin in 0–6 h urine was used as the phenotypic measure of CYP2C19 activity [27]. The activity of CYP2D6 was accessed by the dextromethorphan urinary metabolic ratio, calculated as the ratio of amount of dextromethorphan to dextrorphan recovered in 0–6 h urine [28–30].

2.10. Statistical analysis

All analyses for comparing the phenotypic indices determined from probe drug alone with probe drug combination were performed with the SPSS software system version 11.0 (SPSS Inc., Chicago) by use of Student's *t*-test. The level of significance was set at $P < 0.05$.

3. Results and discussion

3.1. Probe substrate selection

Diclofenac was considered first as a CYP2C9 substrate. However, the use of diclofenac as a CYP2C9 marker is undesirable according to previous literatures because of its reported variable absorption *in vivo* [10,31]. Additionally, tolbutamide is assessed as the recommended substrate probe for CYP2C9 [19,32]. Therefore, tolbutamide was chosen to replace diclofenac as CYP2C9 substrate.

Although there are some problems of using mephenytoin as a probe drug such as its possible sedation, rapid *S*-mephenytoin metabolism and urinary stability of individual enantiomers, *S*-mephenytoin is still considered as the most specific and accurate substrate for CYP2C19. Literature reports demonstrated that *S*-mephenytoin 4′-hydroxylation was successfully employed for determining the polymorphism of CYP2C19 and reflected the CYP2C19 poor metabolizer phenotype representing 2–5% of the Caucasian population but 13–23% of Oriental populations [14,33–35]. Thus in this study we still chose *S*-mephenytoin as the *in vivo* phenotypic probe for CYP2C19. There are two mephenytoin standard metrics used for the estimation of CYP2C19 activity, i.e. urinary recovery of 4′-hydroxymephenytoin and the *S*/*R* ratio of unchanged mephenytoin in urine [27,32]. The *S*/*R* ratio is affected by instability of a *S*-mephenytoin cysteine conjugate, exclusively found in the urine of extensive metabolizers, resulting in an increase in *S*-mephenytoin concentrations during sample storage in the freezer [36,37]. Therefore, we selected the urinary recovery of 4′-hydroxymephenytoin as the metric of choice in this experiment [27,38,39].

In most of the published literature reports, caffeine has been widely used as the substrate of CYP1A2 [32]. The ratio of paraxanthine to caffeine measured in plasma or saliva 4–7 h after caffeine intake reflects most properly the CYP1A2 activity and serves as a criterion standard [40]. However, data showed that the *N*-acetyltransferase 2 (NAT 2) phenotype seems to affect paraxanthine clearance (CL), and body mass as well as other factors affect the ratio of paraxanthine to caffeine [41,42]. Additionally, the availability of caffeine is a problem due to its controlled drug nature. Phenacetin has been the most reliable probe drug for CYP1A2 *in*

Table 6
Phenotyping values of individual and cocktail probe administration

CYP isozyme	Phenotypic index	Results[a]		*P*-Value
		Alone	Cocktail	
1A2	Paracetamol/phenacetin[b]	0.34 (0.30–0.37)	0.38 (0.29–0.43)	0.257
3A	1′-Hydroxymidazolam/midazolam[c]	0.79 (0.72–0.93)	0.84 (0.80–0.87)	0.338
2C19	Urinary recovery of 4′-hydroxymephenytoin (ng)[d]	4485 (2993–6788)	4115 (3110–4808)	0.554
2D6	Dextromethorphan/dextrorphan[d]	0.39 (0.19–0.46)	0.37 (0.22–0.54)	0.815
2C9	(4-Hydroxytolbutamide + carboxytolbutamide)/tolbutamide[e]	156 (139–165)	190 (146–283)	0.705

[a] Results expressed as median (range).
[b] Determined from plasma at 0.5 h postdose.
[c] Determined from plasma at 1 h postdose.
[d] Determined from 0 to 6 h urine collection.
[e] Determined from 6 to 12 h urine collection.

Table 7
Comparison of phenotypic indices obtained from the blank control and inducer positive controls

CYP isozyme	Phenotypic index	Results [a]		*P*-Value
		Blank control group	Inducer positive control group	
1A2	Paracetamol/phenacetin [b]	0.30 (0.26–0.35)	2.75 (1.82–3.97)	$3.02e^{-5}$*
3A	1′-Hydroxymidazolam/midazolam [c]	0.96 (0.80–1.36)	1.39 (1.13–1.63)	0.007*
2C19	Urinary recovery of 4′-hydroxymephenytoin (ng) [d]	4099 (2749–6711)	8054 (4872–10480)	0.046*
2D6	Dextromethorphan/dextrorphan [d]	0.38 (0.28–0.51)	0.24 (0.12–0.32)	0.031*
2C9	(4-Hydroxytolbutamide + carboxytolbutamide)/tolbutamide [e]	207 (182–279)	282 (220–363)	0.019*

*$P < 0.05$.
[a] Results expressed as median (range).
[b] Determined from plasma at 0.5 h postdose.
[c] Determined from plasma at 1 h postdose.
[d] Determined from 0 to 6 h urine collection.
[e] Determined from 6 to 12 h urine collection.

vitro and has been found to be almost exclusively metabolized by CYP1A2 to its metabolite paracetamol [43–45]. Phenacetin and its metabolites paracetamol were also successfully used to evaluate the *in vivo* activity of CYP1A2 [24–26]. Thus we performed a preliminary study and found a significant correlation between phenacetin CL and caffeine CL ($r = 0.994$, $P = 0.016$; unpublished data). Moreover, significant correlation with the phenacetin CL was found for the plasma concentration ratio of paracetamol/phenacetin measured 0.5 h after phenacetin intake ($r = 0.735$, $P = 0.015$; unpublished data). Therefore, phenacetin was selected as CYP1A2 substrate in the current study.

Finally, studies have suggested that debrisoquine may not correlate well with the CYP2D6 probe sparteine *in vivo* [16,17]. Besides, debrisoquine have the potential of causing significant hypotension, further limiting its usefulness [18]. Thus in our study we chose dextromethorphan as CYP2D6 probe drug since it was assessed as the recommended substrate probe for CYP2D6 [32,46].

3.2. Method development

Analytes were at first characterized by MS^2 scan and MS–MS product ions to ascertain their precursor ions and to select product ions for use in MRM mode, respectively. Data shows that a full-scan mass spectrum of each analyte was acquired in both positive and negative ion modes using ESI. Except for tolbutamide and its two metabolites (4-hydroxytolbutamide and carboxytolbutamide), which responded better in negative ion mode, all other compounds responded better in positive ion mode. The full-scan product ion MS/MS spectra of each analyte are shown in Fig. 2. To get the richest relative abundance, MS/MS parameters of each of the analytes, such as sheath gas, the auxiliary gas and collision energy, were adjusted to optimize MS conditions and increase response of each of the precursor–product ion combinations. Table 1 shows the MS/MS transitions and fragmentation parameters of each of the analyte.

Our primary purpose was to develop a general method to allow consecutive runs without changing the mobile phase between plasma and urine assays. Therefore, the effect of the methanol versus 20 mM ammonium formate ratio on the peak resolutions was investigated and optimized to produce best sensitivity, efficiency and peak shapes. Formic acid 0.1% was added as a volatile modifier in the mobile phase to provide an acidic pH. In order to obtain an identical mobile phase under different operation modes, 0.1% formic acid was used in both ESI positive and negative ion modes. Although the use of a modified mobile phase can save run time to some extent, it can hardly avoid strong matrix effects of some analytes (i.e. 1′-hydroxymidazolam). Taking all these into consideration, methanol and 0.1% formic acid in 20 mM ammonium formate (75:25) were used for the mobile phase throughout the study. Under the optimized chromatographic conditions, the retention times were 6.46 min for phenacetin, 5.51 min for paracetamol, 7.92 min for midazolam, 7.31 min for 1′-hydroxymidazolam, 4.86 min for brodimoprim, 5.87 min for 4′-hydroxymephenytoin, 5.07 min for dextromethorphan, 4.76 min for dextrorphan, 4.95 min for ofloxacin, 5.38 min for tolbutamide, 4.16 min for 4-hydroxytolbutamide, 3.66 min for carboxytolbutamide and 4.38 min for meloxicam.

Sample preparation is a critical step for accurate and reliable LC–MS/MS assays. Thus different extraction procedures, including protein precipitation (PPT), liquid–liquid extraction (LLE) and solid-phase extraction, were investigated and compared during the method development. The divergent chemical nature of these analytes precludes their simultaneous isolation from a plasma or urine sample by traditional LLE procedures. Additionally, the results demonstrated that 4′-hydroxymephenytoin was not detectable in all protein precipitation samples. PPT with methanol also showed a severe ion enhancement for 1′-hydroxymidazolam at low concentrations. However, SPE with Oasis® HLB cartridges displayed a better extraction recovery (≥75%; except for midazolam, 67.0%;

Table 2) and higher response for each analyte than PPT with methanol. Therefore, SPE with Oasis® HLB cartridges was used for the sample preparation throughout the study.

3.3. Method validation

3.3.1. Specificity and matrix effect

LC-MS/MS method has high selectivity since only selected ions produced from selected precursor ions are monitored. Comparison of the chromatograms of the control blank and the spiked rat plasma/urine (Figs. 3 and 4) indicated no significant interference at the expected retention times of analytes and ISs from endogenous substances in plasma/urine.

Except for midazolam and dextromethorphan, the mean values of absolute matrix effect ranged from −7.3 to 15.9%, suggesting a minimal matrix effect on the ionization of these compounds under the experimental conditions (Table 3). Although absolute matrix effect was indeed observed for midazolam and dextromethorphan, which were 22 and 27% (mean value), respectively, the effect was consistent over the entire QC concentration ranges of the two analytes. In addition, the 13 analytes in the plasma or urine did not cause significant mutual enhancement or suppression of the MS/MS response for each analyte. Thus, despite the absolute matrix effects that were observed, the present analytical method is reliable.

3.3.2. Linearity and sensitivity

The assay was linear over the concentration ranges 2.5–2500 ng/mL for phenacetin, 2.5–2500 ng/mL for paracetamol, 5–500 ng/mL for midazolam, 0.5–500 ng/mL for 1′-hydroxymidazolam, 5–1000ng/mL for dextromethorphan, 0.05–10 μg/mL for dextrorphan and 4′-hydroxymephenytoin, respectively, 5–2000 ng/mL for tolbutamide, 0.05–20 μg/mL for 4-hydroxytolbutamide and 0.025–10 μg/mL for carboxytolbutamide. The best-fit line of the calibration curve for each analyte was obtained by using a weighting factor of $1/x^2$. Excellent correlation coefficients were obtained ($r^2 \geq 0.9904$; Table 2). Using the present method, the lower limit of quantification varied between 0.5 and 50 ng/mL for the drugs studied (Table 2).

3.3.3. Precision and accuracy

Precision and accuracy data for each analyte are summarized in Table 4. The precision, presented as R.S.D., ranged from 4.3 to 12.4% and 1.5 to 14.8% for intra-day and inter-day determination, respectively. The accuracy, presented as percentage bias against the nominal concentration, ranged from −9.1 to 8.3% and −10.0 to 9.2% for intra-day and inter-day determination, respectively.

3.3.4. Recovery

Table 2 shows the extraction recoveries of all the selected analytes from plasma or urine following SPE method. The SPE method used in this study yielded a mean recovery of greater than 80% for all analytes, except for midazolam (67.0%) and carboxytolbutamide (75.0%). The extraction recovery was found to be consistent for each analyte over the calibration ranges, suggesting that the extraction efficiency of the method is reliable over the studied concentration ranges.

3.3.5. Stability

There was no significant degradation under the conditions described in this study (Table 5). All analytes were found to be stable either in plasma or in urine when stored at −20 °C for 3 months or after three freeze–thaw cycles. All analytes were also stable when the extracted samples were kept in the autosampler at 4 °C or at room temperature for 24 h. The urine samples processed without β-glucuronidase incubation did not influence LC–MS/MS analysis. Stability for each analyte was consistent over its calibration range. The mean deviation for all measured analytes were from −14.2 to 14.0%.

3.4. Study on the potential in vivo interactions between probes in the cocktail

Statistic analysis demonstrated that there was no significant difference between individual administration of probe drug and co-administration of the cocktail. The ability of the five probe drugs to measure the activity of their corresponding enzymes is not affected by their co-administration at test doses (Table 6). Consequently, the validation of such a cocktail serves as an important step in making enzyme phenotyping a more practical tool in preclinical research.

3.5. Verification of the in vivo cocktail system by known CYP enzyme inducers

The results showed that comparing with blank control, phenobarbital significantly induced CYP1A2 ($P = 3.02e^{-5*}$), 2C9 ($P = 0.019^*$), 2D6 ($P = 0.031^*$) and 3A ($P = 0.007^*$); rifampicin induced CYP2C19 ($P = 0.046^*$) significantly (Table 7). Therefore, phenobarbital and rifampicin can be chosen as positive induction controls for evaluation of CYP1A2, 2C9, 2C19, 2D6 and 3A induction potential of drug candidates.

4. Conclusion

A LC-MS/MS method has been developed for the simultaneous evaluation of the activities of five cytochrome P450s in rat plasma and urine. Administration of this probe-substrate cocktail can provide comprehensive information about CYP functionality. Detailed validation following FDA guideline indicated that the developed method had high sensitivity, reliability, specificity and good accuracy. This is the first work combining these five probe drugs for these five CYPs. The high-throughput screening cocktail method was further verified with known CYP enzyme inducers. Thus, the described method can be adapted for preclinical screening of new drug candidates.

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