RAPID COMMUNICATIONS IN MASS SPECTROMETRY
Rapid Commun. Mass Spectrom. 2004; **18**: 2921–2933
Published online in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/rcm.1704

Rapid determination of five probe drugs and their metabolites in human plasma and urine by liquid chromatography/tandem mass spectrometry: application to cytochrome P450 phenotyping studies

Ophelia Q. P. Yin[1,2]*, Sherry S. L. Lam[1], Cindy M. Y. Lo[1] and Moses S. S. Chow[1,2]

[1]School of Pharmacy, The Chinese University of Hong Kong, Shatin, N.T., Hong Kong
[2]Drug Development Centre, The Chinese University of Hong Kong, Shatin, N.T., Hong Kong

Received 5 August 2004; Revised 1 October 2004; Accepted 1 October 2004

A liquid chromatography/mass spectrometry method, for rapid determination of five cytochrome P450 (CYP) probe drugs and their relevant metabolites in human plasma and urine, is described. The five specific probe substrates/metabolites, caffeine/paraxanthine (CYP1A2), tolbutamide/4-hydroxytolbutamide/carboxytolbutamide (CYP2C9), omeprazole/5-hydroxyomeprazole (CYP2C19), debrisoquine/5-hydroxydebrisoquine (CYP2D6) and midazolam/1′-hydroxymidazolam (CYP3A), together with the internal standards (phenacetin and paracetamol), in plasma and urine, were extracted using solid-phase extraction. The chromatography was performed using a C_{18} column with an isocratic mobile phase consisting of acetonitrile and 0.1% formic acid in water (70:30). The triple-quadrupole mass spectrometer was operated in both positive and negative modes, and multiple reaction monitoring was used for quantification. The method was validated over the concentration ranges 0.05–5 μg/mL for caffeine and paraxanthine, 0.02–2 μg/mL for tolbutamide, 0.1–20 μg/mL for 4-hydroxytolbutamide, carboxytolbutamide, debrisoquine and 5-hydroxydebrisoquine, 5–2500 ng/mL for omeprazole and 5-hydroxyomeprazole, and 1–100 ng/mL for midazolam and 1′-hydroxymidazolam. The intra- and inter-day precision were 0.3–13.7% and 1.9–14.3%, respectively, and the accuracy ranged from 93.5–107.2%. The lower limit of quantification varied between 1 and 100 ng/mL. The present method provides a robust, fast and sensitive analytical tool for the five-probe drug cocktail, and has been successfully applied to a clinical phenotyping study in 16 subjects. © **2004 John Wiley & Sons, Ltd.**

The cytochrome P450 (CYP) system is primarily comprised of drug-metabolizing enzymes involved in oxidative reactions. Among the various CYP isozymes, CYP1A2, 2C9, 2C19, 2D6 and 3A are the major isoforms responsible for the metabolism of more than 90% of marketed drugs.[1,2] To determine the real-time activity of the CYP isozymes, specific probe drugs can be employed.[3] Recently, the use of multiple probe drugs, i.e. a 'cocktail' approach, has become popular in pharmacogenetic studies as this provides a high-throughput approach in evaluating CYP isozyme activities.

A number of cocktails (i.e., five- or six-drug cocktails) have been described in the literature. These include the 'Pittsburgh cocktail', 'GW cocktail', 'Cooperstown cocktail' and 'Karolinska cocktail' (Table 1).[4–10] So far most of the analytical methods for these cocktails are tedious and usually require a separate high-performance liquid chromatography (HPLC), gas chromatography (GC) or liquid chromatography/mass spectrometry (LC/MS) technique for each probe drug and its metabolite.[4,5,8–10] Recently, Scott *et al.*[6] were the first to report a fast gradient LC/MS method for the simultaneous determination of CYP substrates and metabolites in their 'GW cocktail'. However, this cocktail has several practical limitations. First of all, the use of diclofenac as a CYP2C9 marker is undesirable due to its variable absorption in humans.[11] Secondly, the use of mephenytoin is inconvenient as this drug is no longer commercially available in many parts of the world, besides its sedation side effect occurring especially in poor metabolizers.[12] Thirdly, chlorzoxazone, a probe drug in the cocktail, can significantly inhibit the CYP3A-mediated first-pass metabolism of midazolam in the gut and its use for the present purpose is not recommended.[7]

**Correspondence to*: O. Q. P. Yin, School of Pharmacy, Faculty of Medicine, The Chinese University of Hong Kong, Shatin, N.T., Hong Kong.
E-mail: qpyin@cuhk.edu.hk
Contract/grant sponsor: Innovation and Technology Commission of the Government of Hong Kong SAR; contract/grant number: ITS/174/00.
Contract/grant sponsor: Research Grants Council of Hong Kong SAR; contract/grant number: CUHK4180/02M.

Copyright © 2004 John Wiley & Sons, Ltd.

Table 1. Reported five- and six-drug cocktails for CYP phenotyping

Reference	Probe drug (CYP isozyme)	Specimen	Assay method
Frye *et al.*[4]	Caffeine (CYP1A2)	Plasma	HPLC
	Mephenytoin (CYP2C19)	Urine	HPLC
	Debrisoquine (CYP2D6)	Urine	HPLC
	Chlorzoxazone (CYP2E1)	Plasma	HPLC
	Dapzone (CYP3A)	Urine	HPLC
Damkier and Brosen[5]	Caffeine (CYP1A2)	Plasma	HPLC
	Tolbutamide (CYP2C9)	Urine	HPLC
	Mephenytoin (CYP2C19)	Urine	GC
	Sparteine (CYP2D6)	Urine	GC
	Cortisol (CYP3A)	Urine	HPLC
Scott *et al.*[6]	Caffeine (CYP1A2)	Plasma	
Palmer *et al.*[7]	Mephenytoin (CYP2C19)	Urine	
	Diclofenac (CYP2C9)	Urine	LC/MS
	Debrisoquine (CYP2D6)	Urine	
	Chlorzoxazone (CYP2E1)	Plasma	
	Midazolam (CYP3A)	Plasma	
Streetman *et al.*[8]	Caffeine (CYP1A2)	Urine	HPLC
Chainuvati *et al.*[9]	Warfarin + vitamin K (CYP2C9)	Plasma	LC/MS
	Omeprazole (CYP2C19)	Plasma	HPLC
	Dextromethorphan (CYP2D6)	Urine	HPLC
	Midazolam (CYP3A)	Plasma	LC/MS
Christensen *et al.*[10]	Caffeine (CYP1A2)	Plasma	HPLC
	Losartan (CYP2C9)	Urine	HPLC
	Omeprazole (CYP2C19)	Plasma	HPLC
	Debrisoquine (CYP2D6)	Urine	HPLC
	Quinine (CYP3A)	Plasma	HPLC
The cocktail proposed in this work	Caffeine (CYP1A2)	Plasma	
	Tolbutamide (CYP2C9)	Urine	
	Omeprazole (CYP2C19)	Plasma	LC/MS
	Debrisoquine (CYP2D6)	Urine	
	Midazolam (CYP3A)	Plasma	

To overcome the above-mentioned limitations, we have recently developed a five-drug cocktail, consisting of caffeine, tolbutamide, omeprazole, debrisoquine and midazolam, for phenotyping of CYP1A2, 2C9, 2C19, 2D6 and CYP3A isozyme activity, respectively. We choose tolbutamide for CYP2C9 and omeprazole for CYP2C19 because both drugs have been demonstrated to be reliable markers, and are convenient to administer as well as readily available.[13,14] Chlorzoxazone at present is the only available *in vivo* probe for CYP2E1. In view of its significant inhibitory effect on midazolam as well as the minor contribution of CYP2E1 to CYP-mediated drug clearances, we chose not to include chlorzoxazone in the present cocktail. To support the clinical application of this cocktail, a general analytical method for the determination of all the substrates and their metabolites has been developed. In this paper we describe the development and validation of a robust, fast and sensitive LC/MS method for the simultaneous extraction and analysis of the five probe drugs and their metabolites (Fig. 1) in human plasma and urine. The method has also been successfully applied to a clinical study in 16 healthy subjects.

EXPERIMENTAL

Chemicals and reagents

Caffeine, paraxanthine, debrisoquine sulfate, tolbutamide and 4-hydroxytolbutamide were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Carboxytolbutamide, midazolam and 1′-hydroxymidazolam were obtained from Ultrafine Chemicals (Manchester Science Park, UK). Omeprazole was purchased from USP Science (Rockville, MD, USA) and its metabolite 5-hydroxyomeprazole was kindly supplied by AstraZeneca R&D (Molndal, Sweden). 4-Hydroxydebrisoquine was purchased from ICN Biomedicals (Irvine, CA, USA). Phenacetin and paracetamol (used as internal standards) were obtained from Sigma Chemical Co. All the above standard compounds possess purity of better than 99%. HPLC-grade acetonitrile and methanol were obtained from Labscan (Asia) Co. Ltd. (Bangkok, Thailand). All other reagents were purchased from BDH Laboratory Supplies (Dorset, UK) and were of analytical grade. Deionized water was prepared using a Barnstead-Nanopore® water purification system and used throughout the study. Oasis® HLB solid-phase extraction (SPE) cartridges (1 mL, 30 mg) were supplied by Waters (Milford, MA, USA).

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 debrisoquine and 4-hydroxydebrisoquine which were 2 mg/mL in water. The stock solutions of the internal standards, phenacetin and paracetamol, were prepared at 1 and 2.5 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).

Copyright © 2004 John Wiley & Sons, Ltd.

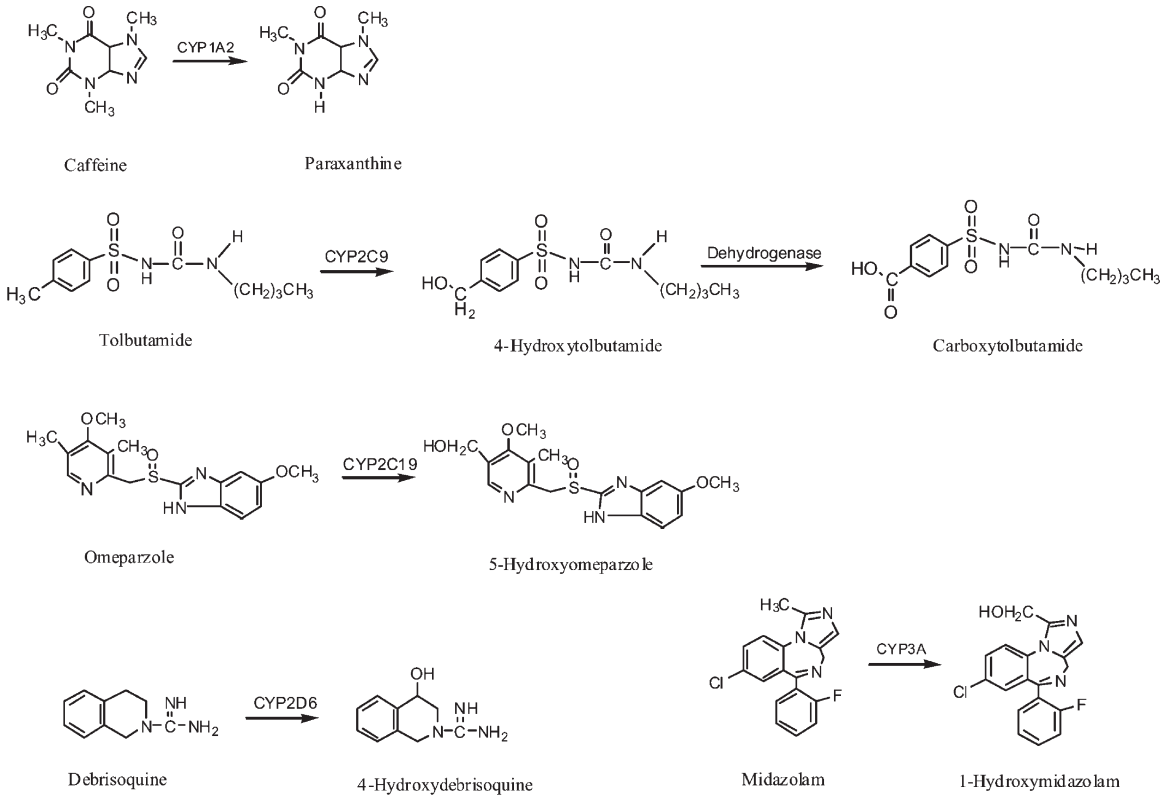

Figure 1. Probe drugs used in the study and their *in vivo* metabolic conversions.

Standard calibration samples (consisting of 10 different concentrations over a sufficient range for each case) were prepared by spiking the blank human plasma or urine with the working solution of each analyte. Quality control (QC) samples were prepared in a similar manner at four different concentrations, i.e. at the lower limit of quantification (LLOQ), and low, medium and high concentrations. For caffeine and paraxanthine the calibration standard and QC samples were prepared with 5% bovine serum albumin (BSA) instead of using pooled 'blank' plasma, due to presence of trace amounts of caffeine and paraxanthine which are usually present in the commercially available 'blank' plasma.[15,16]

Sample preparation

Plasma samples

10 μL of internal standard solution (10 μg/mL phenacetin) was added to 0.5 mL plasma. The mixture was then loaded onto an Oasis® HLB cartridge, which was conditioned with 1 mL methanol and 1 mL water. After washing with 1 mL water and 1 mL 5% methanol in water, the cartridge was eluted with 2 × 1 mL methanol. The eluate was collected

Table 2. MRM transitions and fragmentation parameters for the analytes and internal standards

Analyte	Polarity	Precursor (*m/z*)	Product (*m/z*)	DP (V)	FP (V)	CE (eV)	CEP (V)	CXP (V)
Caffeine	+ve	195	138	60	220	19	9	30
Paraxanthine	+ve	181	124	59	250	29	9	16
Omeprazole	+ve	346	198	56	120	19	9	30
5-Hydroxyomeprazole	+ve	362	214	56	210	23	13	32
Midazolam	+ve	326	291	105	78	48	15	47
1-Hydroxymidazolam	+ve	342	324	85	310	41	15	50
Tolbutamide	−ve	269	170	71	230	21	16	10
4-Hydroxytolbutamide	−ve	285	186	81	250	25	20	10
Carboxytolbutamide	−ve	299	92	66	330	43	38	6
Debrisoquine	+ve	176	134	56	370	27	2	18
5-Hydroxydebrisoquine	+ve	192	132	61	310	30	25	15
Phenacetin (IS)	+ve	180	110	70	290	26	8	16
Paracetamol (IS)	−ve	150	107	86	170	21	16	8

DP: declustering potential; FP: focusing potential; CE: collision energy; CEP: cell entrance potential; CXP: cell exit potential.

Copyright © 2004 John Wiley & Sons, Ltd.

RCM

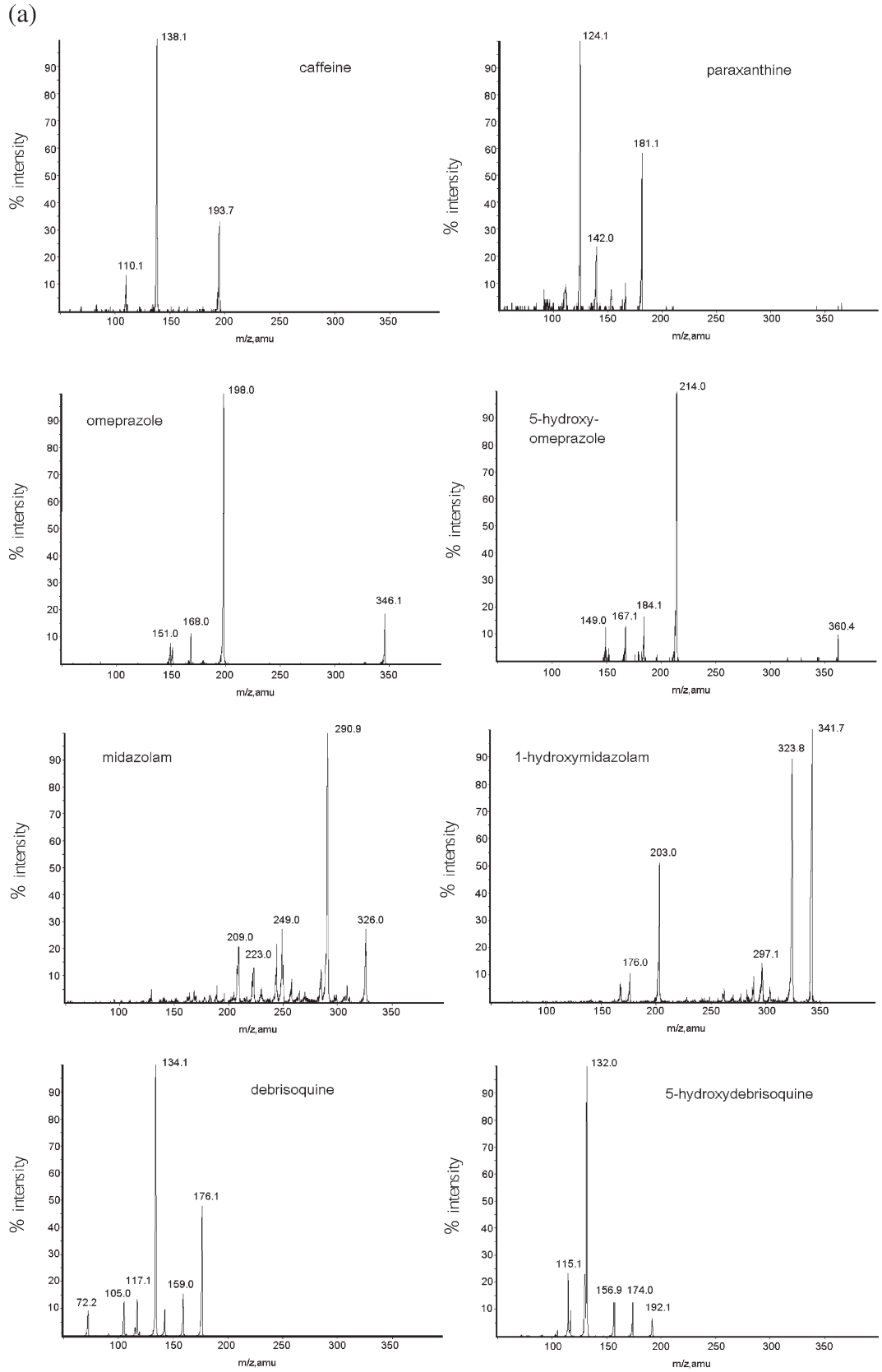

Figure 2. Product ion mass spectra of the probe drugs, metabolites and internal standards: (a) positive ion analytes and (b) negative ion analytes and internal standards.

Copyright © 2004 John Wiley & Sons, Ltd.

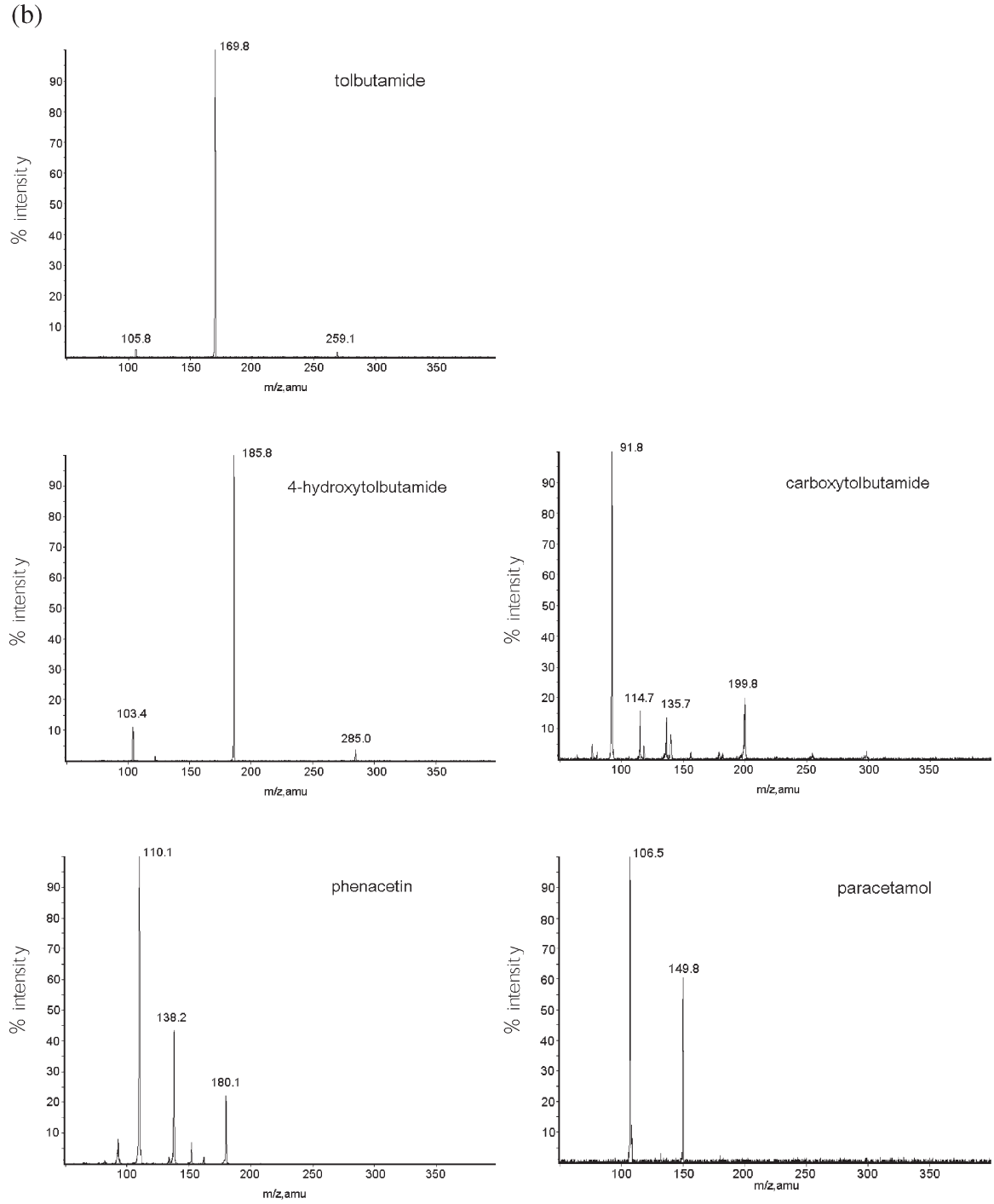

Figure 2. Continued.

and evaporated to dryness at 25°C under a stream of nitrogen. The residue was reconstituted with 150 μL mobile phase, vortex-mixed briefly, and transferred to a clean autosampler vial. A 25-μL aliquot was subsequently injected into the LC/MS/MS system.

Urine samples

100 μL urine were diluted with 400 μL water, followed by the addition of 10 μL of 25 μg/mL phenacetin solution (IS for debrisoquine and its metabolite) and 4 μL of 2.5 mg/mL paracetamol solution (IS for debrisoquine and its metabolite). The mixture was acidified with 10 μL of concentrated phosphoric acid, loaded onto an Oasis® HLB cartridge and then extracted using the same procedure as described above. 5 μL of the reconstituted aliquot were injected for LC/MS/MS analysis.

Liquid chromatography

A Perkin-Elmer LC system (Perkin-Elmer, Norwalk, CT, USA), equipped with two series 200 micropumps and an autosampler, was used. Chromatography was performed on a Waters symmetry C_{18} guard cartridge (3.9 mm × 20 mm, 3.5 μm; Waters, Milford, USA). The mobile phase consisted of acetonitrile and 0.1% formic acid in water (70:30) at a flow rate of 0.3 mL/min. The total run time is 3.5 min per sample.

Copyright © 2004 John Wiley & Sons, Ltd.

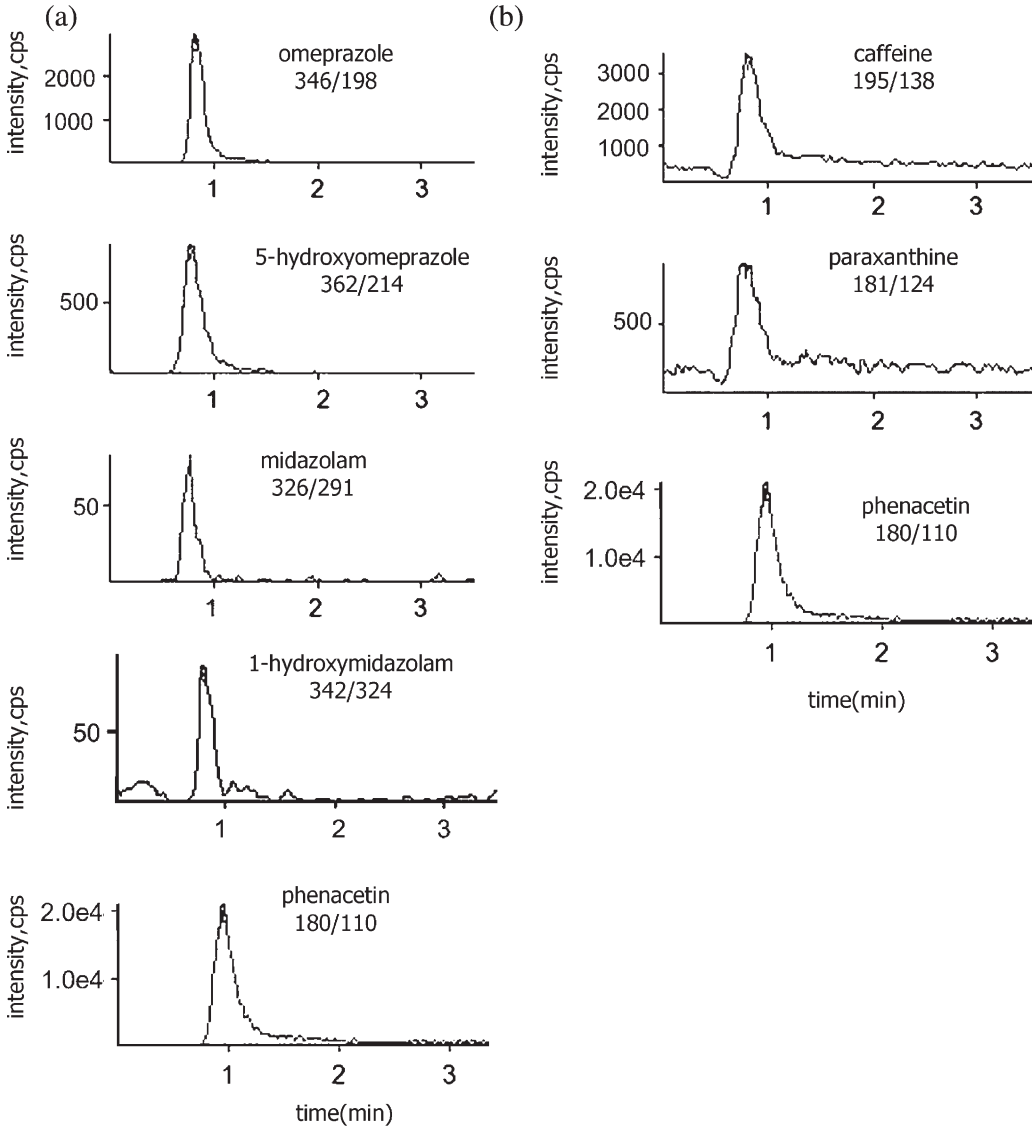

Figure 3. MRM chromatograms: (a) of a QC plasma sample containing omeprazole (20 ng/mL), 5-hydroxyomeprazole (20 ng/mL), midazolam (2 ng/mL), 1′-hydroxymidazolam (2 ng/mL), and phenacetin (200 ng/mL) and (b) of a QC sample (prepared in 5% BSA) containing caffeine (250 ng/mL), paraxanthine (250 ng/mL) and phenacetin (200 ng/mL).

Mass spectrometry

A Perkin-Elmer Sciex API-2000 triple-quadrupole mass spectrometer, equipped with an electrospray ionization (ESI) source, was used for the mass analysis and detection. The ESI was performed in either positive mode (5500 V) or negative mode (4500 V), with the main working parameters set as follows: nebulizer gas (Gas1) 45 psi; auxiliary gas (Gas2) 80 psi; curtain gas 40 psi; and turboionspray temperature 400°C. Multiple reaction monitoring (MRM) was used for drug quantification. The MRM transitions and fragmentation conditions selected for individual analytes are shown in Table 2. The mass peak widths were 0.7 Da for Q1 and Q3. Data acquisition and analysis were performed using a Macintosh computer with the aid of MassChrom software (version 1.1, Sciex).

Method validation

Validation of the assay was conducted according to FDA guidelines, with respect to the linearity, specificity, sensitivity, extraction recovery, precision and accuracy.[17] The matrix effect as well as analyte stability in plasma and urine were also determined. Four QC samples (at LLOQ, low, medium and high concentration levels) were utilized for these tests, using the assay procedure described in the previous section.

Linearity and sensitivity

Calibration standards were prepared and analyzed in triplicate in three independent runs. Calibration curves were constructed using the analyte/IS peak area ratio versus the analyte concentration, and were fitted by linear least-squares regression analysis with or without weighting (weighting factor of $1, 1/x, 1/x^2$, x = concentration). The model with lowest total bias and the most constant bias across the concentration range was considered to be the best fit. 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

Copyright © 2004 John Wiley & Sons, Ltd.

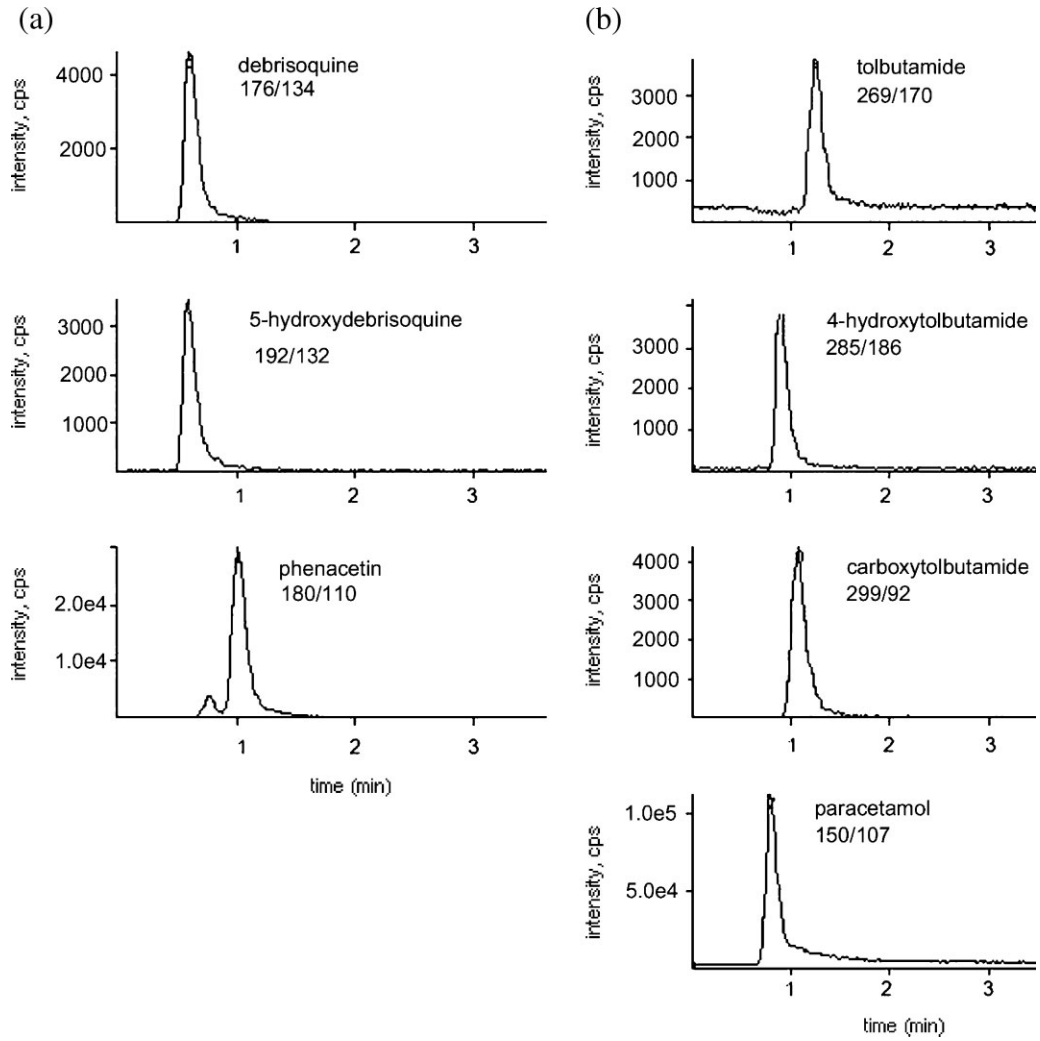

Figure 4. MRM chromatograms of a QC urine sample (a) for positive ion analytes: debrisoquine (200 ng/mL), 5-hydroxyldebrisoquine (200 ng/mL) and phenacetin (2.5 μg/mL); and (b) for negative analytes: tolbutamide (50 ng/mL), 4-hydroxytolbutamide (200 ng/mL), carboxytolbutamide (200 ng/mL) and paracetamol (100 μg/mL).

Table 3. Calibration curves, extraction recoveries and sensitivity of the assay

Analyte	Matrix	Calibration range (ng/mL)	r^2	Recovery (n = 9)[b] (%)	LLOQ (ng/mL)
Caffeine[a]	Plasma	50–5000	0.9995 ± 0.0002	87.4 ± 5.6	50
Paraxanthine[a]	Plasma	50–5000	0.9962 ± 0.0049	83.3 ± 7.5	50
Omeprazole	Plasma	5–2500	0.9958 ± 0.0032	87.4 ± 7.0	5
5-Hydroxyomeprazole	Plasma	5–2500	0.9962 ± 0.0016	83.1 ± 7.0	5
Midazolam	Plasma	1–100	0.9963 ± 0.0022	96.8 ± 9.9	1
1-Hydroxymidazolam	Plasma	1–100	0.9965 ± 0.0015	106.4 ± 13.0	1
Tolbutamide	Urine	20–2000	0.9987 ± 0.0006	92.0 ± 5.4	20
4-Hydroxytolbutamide	Urine	100–20 000	0.9980 ± 0.0010	80.1 ± 6.8	100
Carboxytolbutamide	Urine	100–20 000	0.9953 ± 0.0006	100.0 ± 3.3	100
Debrisoquine	Urine	100–20 000	0.9980 ± 0.0018	100.1 ± 3.9	100
5-Hydroxydebrisoquine	Urine	100–20 000	0.9953 ± 0.0022	101.0 ± 3.5	100

[a] Calibration standards and QC samples of caffeine and paraxanthine were prepared in 5% BSA.
[b] For each analyte, the recovery experiment was performed with three QC concentrations (low, medium and high), with triplicate determinations for each concentration.

deviation of ±20% was permitted. The LLOQ was determined based on the criteria that (1) the analyte response at LLOQ is 5 times the baseline noise, and (2) that the analyte response at LLOQ can be determined with precision of ≤20% and accuracy of 80–120%.

Recovery

The extraction recoveries for the plasma and urine samples were determined by the following procedure: (1) Blank plasma or urine was processed by SPE and the eluate was evaporated to dryness; the dry extract was then

Copyright © 2004 John Wiley & Sons, Ltd.

Table 4. Intra- and inter-day precision and accuracy

Analyte	Matrix	Nominal conc. (ng/mL)	Measured conc. (ng/mL)	Accuracy (%bias)	Intra-day precision (%RSD)	Inter-day precision (%RSD)
Caffeine[a]	Plasma	50	50.8	1.6	9.4	9.1
		250	258.5	3.4	4.7	8.5
		1250	1230.9	−1.5	7.7	9.0
		3750	3757.8	0.2	0.5	5.2
Paraxanthine[a]	Plasma	50	50.4	0.8	13.7	14.3
		250	242.3	−3.1	13.5	13.1
		1250	1180.3	−5.6	6.9	7.0
		3750	3776.9	0.7	4.7	6.4
Omeprazole	Plasma	5	4.8	−4.0	4.9	7.5
		20	21.2	6.0	3.4	4.2
		100	106.3	6.3	2.8	2.6
		1000	980.4	−2.0	2.0	1.9
5-Hydroxyomeprazole	Plasma	5	4.7	−6.0	6.5	7.8
		20	20.3	1.5	3.3	3.4
		100	103.0	3.0	3.4	3.8
		1000	934.5	−6.5	6.2	7.2
Midazolam	Plasma	1.0	1.0	0.0	11.8	14.0
		2.0	2.0	0.0	9.4	11.9
		7.5	7.4	−1.3	7.2	9.8
		75	74.7	−0.4	0.4	8.8
1-Hydroxymidazolam	Plasma	1.0	1.0	0.0	9.6	11.3
		2.0	2.0	0.0	10.8	10.9
		7.5	7.5	0.0	6.9	8.8
		75	71.9	−4.1	3.4	3.5
Tolbutamide	Urine	20	19.9	−0.5	3.3	5.3
		50	49.3	−1.4	7.9	8.1
		200	207.8	3.9	8.6	7.7
		1500	1535.4	2.4	0.4	6.0
4-Hydroxytolbutamide	Urine	100	98.8	−1.2	8.2	6.6
		200	198.6	−0.7	6.0	5.6
		2000	2031.1	1.6	4.9	4.3
		15 000	15 614.4	4.1	0.3	4.2
Carboxytolbutamide	Urine	100	98.8	−1.2	8.2	6.6
		200	192.1	−4.0	4.6	6.9
		2000	2038.6	1.9	2.6	5.3
		15 000	15 471.6	3.1	0.4	6.3
Debrisoquine	Urine	100	96.0	−4.0	7.2	7.1
		200	203.0	1.5	4.3	4.8
		2000	2040.2	2.0	2.9	3.9
		15 000	14 554.9	−3.0	0.5	5.8
5-Hydroxydebrisoquine	Urine	100	97.6	−2.4	8.5	9.4
		200	214.3	7.2	5.7	6.5
		2000	2018.2	0.9	3.9	4.5
		15 000	14 287.0	−4.8	0.8	11.5

[a] Calibration standards and QC samples of caffeine and paraxanthine were prepared in 5% BSA.

dissolved in an analyte standard solution, the final result thus representing 100% recovery. (2) QC samples spiked with the analyte were processed by SPE in a similar manner; their responses were determined and then compared to those from the blank samples spiked post-extraction, from step (1) above. For each analyte, the recovery experiment was performed with three QC concentrations (low, medium and high), with triplicate determinations at each concentration.

Precision and accuracy

QC samples in five replicates were analyzed on the same day to determine the intra-day precision and accuracy, and on each of three separate days to determine inter-day precision and accuracy. Precision was calculated as the relative standard deviation (RSD), whereas accuracy was assessed as the percentage bias from the nominal concentration (% bias). The acceptable intra- and inter-day precision and bias were set at $\leq$15%, except at the LLOQ where they were set at $\leq$20%.

Specificity and matrix effect

To determine assay specificity, six different lots of blank human plasma or urine were analyzed to investigate the potential interferences at the LC peak region for each analyte and IS. The experiment was performed in duplicate.

The matrix effect was evaluated in the following three ways: (1) To assess the 'absolute' matrix effect, i.e. the potential ion suppression/enhancement due to the matrix components in plasma or urine, six different lots of each of

Copyright © 2004 John Wiley & Sons, Ltd.

blank plasma and urine were extracted by SPE and spiked with each analyte (at low and high QC concentrations) or IS, separately. The corresponding peak areas were then compared to those of the aqueous standards at equivalent concentrations. For each lot of blank matrix the experiment was performed in duplicate. The ion suppression was calculated as $100 \times (A_{st} - A_{extr})/A_{st}$, where A_{extr} is the peak area of individual analyte or IS from the post-extraction spiked sample (blank plasma/urine extracted by SPE and then spiked with individual analyte or IS) and A_{st} is the peak area of individual analyte or IS from direct injection of the aqueous standard. (2) To assess the co-elution effect, i.e. the potential ion suppression effect of co-eluting analytes of interest, 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 of combined IS and analytes. The experiments were preformed in triplicate at the medium QC concentration for each analyte. (3) To assess the lot-to-lot matrix variation, six different lots of blank plasma/urine were used to prepare the QC samples at both low and high concentrations. For each lot, duplicate samples were analyzed at each concentration. The RSD of the peak area ratio of each analyte versus IS among the six lots was calculated as an indicator of the inter-lot matrix variability.

Stability

The stability testing was determined in three ways, using triplicate QC samples: (1) For storage stability, the QC samples were prepared and stored at −80°C for 2 months. All samples were subsequently thawed and analyzed together with calibration samples that were freshly prepared. (2) For freeze/thaw stability testing, the QC samples were determined after three freeze (−80°C) and thaw (23°C) cycles, and analyzed together with freshly prepared calibration samples. (3) To assess the injector stability of the processed samples, the QC samples were extracted and placed in the autosampler at 23°C for 24 h, and then injected into the LC/MS system for analysis. The measured concentrations were then compared to those of the same QC samples that had been analyzed immediately after processing. For each of the above stability tests, the experiments were performed at three QC concentrations (low, medium and high), with triplicate determinations for each concentration. The percent deviation in concentration was used as an indicator of stability. The analyte was considered stable when the percent deviation was within 15%.

RESULTS

Mass spectrometry and chromatography

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. Figure 2 shows the full-scan product ion MS/MS spectra of each analyte. Generally, the most abundant product ion of each analyte was selected for MRM monitoring (Table 2).

The MS/MS settings were adjusted to maximize the response of each of the precursor-product ion combinations. Figure 3 shows the extracted ion chromatograms of a spiked QC plasma sample containing omeprazole, 5-hydroxyomeprazole, midazolam and 1′-hydroxymidazolam, as well as those of a QC sample (prepared in 5% BSA) containing caffeine and paraxanthine. The extracted ion chromatograms of a spiked QC urine sample are shown in Fig. 4.

Linearity and sensitivity

The assay was linear over the concentration ranges 0.05–5 μg/mL for caffeine and paraxanthine, 5–2500 ng/mL for omeprazole and 5-hydroxyomeprazole, 1–100 ng/mL for midazolam and 1′-hydroxymidazolam, 0.02–2 μg/mL for tolbutamide, and 0.1–20 μg/mL for 4-hydroxytolbutamide, carboxytolbutamide, debrisoquine and 4-hydroxydebrisoquine. 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 ($r^2 \geq 0.995$; Table 3). Using the present method, the lower limit of quantification (LLOQ) varied between 1–100 ng/mL for the drugs studied (Table 3).

Recovery

The SPE method used in this study yielded a mean recovery of better than 80% for all analytes (Table 3). The extraction recovery was found to be consistent for each analyte over its calibration range, suggesting that the extraction efficiency of this method is independent of concentration in the ranges studied.

Precision and accuracy

Precision and accuracy data for each analyte are summarized in Table 4. The precision, presented as RSD, ranged from 0.3–13.7% and 1.9–14.3% for intra-day and inter-day determinations, respectively. The accuracy, presented as percentage bias against the nominal concentration, ranged from −6.5% to 7.2%.

Specificity and matrix effect

Except for the trace amounts of caffeine and paraxanthine, other analyte and IS peaks were free of interference for all six different lots of plasma/urine. (An earlier eluting endogenous peak was observed in the urine sample, but it was well separated from the IS phenacetin.) The extracted ion chromatograms of a representative 'blank' plasma and urine sample are shown in Figs. 5(a) and 5(b), respectively. The concentrations of caffeine and paraxanthine, although detected in commercially available 'blank' plasma and reported in other studies, should not alter the specificity of the assay procedure.[15,16,18]

For all the analytes in urine, the degree of ion suppression ranged from 1.89–16.9%, suggesting a minimal matrix effect on the ionization of these compounds under these conditions. For the analytes in plasma, the mean signal suppression was 25.4, 36.9 and 38.7% for omeprazole, 5-hydroxyomeprazole and phenacetin, respectively, and less than 20% for all other analytes. The matrix effect was consistent in all plasma/urine samples tested. As shown in Table 5, the lot-to-lot variation was less than 13% for all analytes, demonstrating the absence

Copyright © 2004 John Wiley & Sons, Ltd.

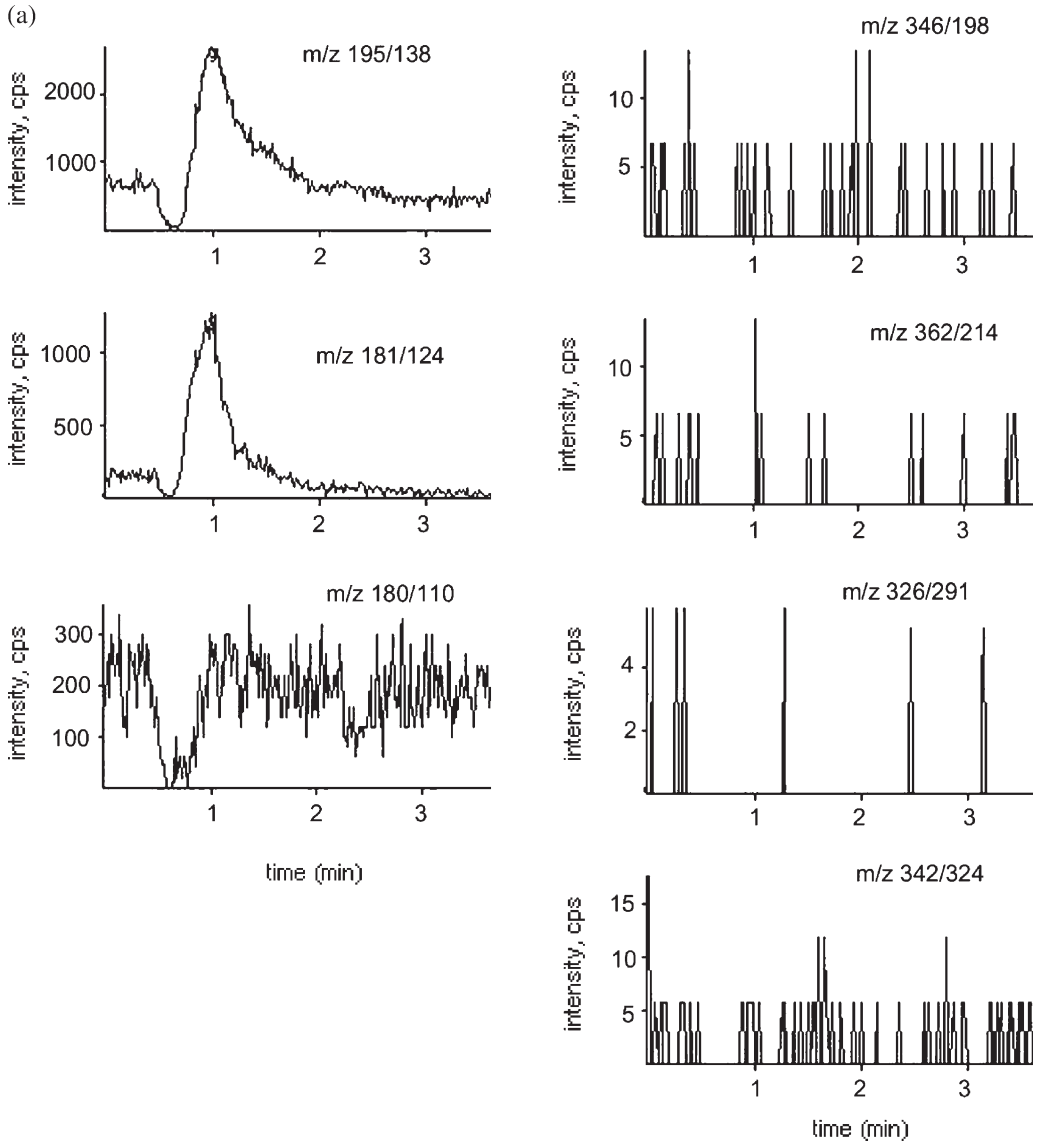

Figure 5. MRM chromatograms of: (a) a 'blank' plasma sample (contains traces of caffeine and paraxanthine) and (b) a blank urine sample.

of significant inter-source variability in the matrix effect. The 13 co-eluting compounds in the plasma or urine did not cause significant mutual enhancement or suppression of the MS/MS response for each analyte.

Stability

All analytes were found to be stable in the plasma and urine samples when stored at −80°C for 2 months or after three freeze/thaw cycles. No significant degradation was observed in the analyte concentrations when extracts were kept in the autosampler at 23°C for up to 24 h. The mean deviations in the concentrations of the analytes were −6.9% to 7.6%.

Application to clinical study

The assay method was applied to a clinical cocktail study in 16 healthy subjects. This study was approved by The Joint Chinese University of Hong Kong-New Territories East Cluster (CUHK-NTEC) Clinical Research Ethics Committee. All subjects provided written informed consent to participate in the study. After an overnight fast of 10 h, the subjects received a single oral dose of 100 mg caffeine, 500 mg tolbutamide, 40 mg omeprazole, 10 mg debrisoquine and 3.75 mg midazolam. Two blood samples were collected at 2 and 3 h, and urine samples at 0–6 and 6–12 h post-dosing. All samples were stored at −80°C and analyzed within 2 months.

Ratios of the plasma concentrations of paraxanthine to caffeine, 5-hydroxyomeprazole to omeprazole, and 1′-hydroxymidazolam to midazolam, at 2 or 3 h post-dosing, were used to represent the activities of CYP1A2, 2C19 and 3A, respectively. The CYP2C9 activity was determined using the tolbutamide urinary metabolic ratio, calculated as the sum of the amounts of 4′-hydroxytolbutamide and carboxytolbutamide divided by the amount of tolbutamide recovered in 6–12 h urine. The activity of CYP2D6 was assessed by the debrisoquine urinary metabolic ratio, calculated as the ratio of amounts of 4-hydroxydebrisoquine to debrisoquine recovered in 0–6 h urine. The phenotypic ratios representing the specific CYP isozyme activities, thus determined in a

Copyright © 2004 John Wiley & Sons, Ltd.

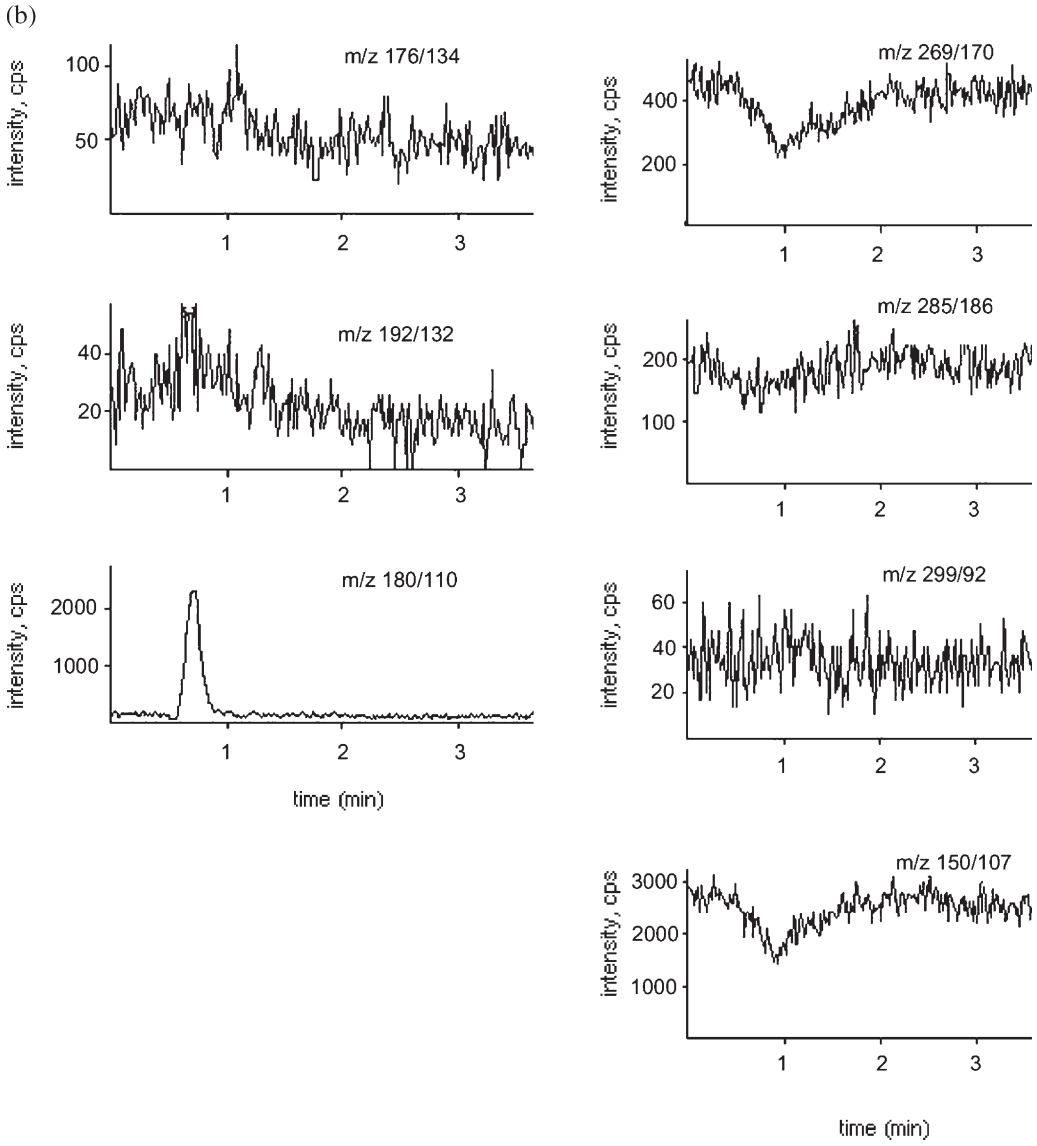

Figure 5. Continued.

Table 5. Co-elution effect and inter-lot matrix variation

Analyte	Matrix	Peak area ratio[a] (individual/combined) (n = 3)	Inter-lot variations[b] (RSD%) Low QC (n = 12)	High QC (n = 12)
Caffeine	Plasma	1.04	—	—
Paraxanthine	Plasma	1.02	—	—
Omeprazole	Plasma	1.05	8.4	8.1
5-Hydroxyomeprazole	Plasma	1.00	12.7	11.1
Midazolam	Plasma	0.97	9.9	6.3
1-Hydroxymidazolam	Plasma	1.03	11.0	6.9
Tolbutamide	Urine	1.03	8.9	6.7
4-Hydroxytolbutamide	Urine	1.04	12.6	9.4
Carboxytolbutamide	Urine	0.94	5.3	6.8
Debrisoquine	Urine	1.03	12.9	8.9
5-Hydroxydebrisoquine	Urine	1.04	11.4	9.2
Phenacetin (IS)	Plasma	0.99	—	—
Phenacetin (IS)	Urine	0.98	—	—
Paracetamol (IS)	Urine	0.94	—	—

[a] Co-elution effect was evaluated using pooled blank plasma/urine.
[b] Inter-lot variation was evaluated using six different lots of blank plasma/urine (the inter-lot variation was not assessed for caffeine/paraxanthine due to their presence in blank plasma).

Copyright © 2004 John Wiley & Sons, Ltd.

Table 6. Phenotypic indices determined in a study subject

CYP isozyme	Phenotypic index	Ratio
1A2	Paraxathine/caffeine	0.52
2C9	(4-Hydroxytolbutamide+ carboxytolbutamide)/tolbutamide	1384
2C19	5-Hydroxyomeprazole/omeprazole	0.45
2D6	4-Hydroxydebrisoquine/debrisoquine	0.31
3A	1′-Hydroxymidazolam/midazolam	0.91

study subject, are shown in Table 6. Genotyping of the subject suggested that this was a carrier of CYP2C9*1/*1, 2C19*1/*2 and 2D6*1/*10. These genotypes are consistent with the observed phenotype ratios determined from the cocktail study.

DISCUSSION

An important general limitation in the use of multiple probe drug cocktails for pharmacogenetic studies is the complicated and tedious assay methodology of the probe drugs/metabolites. Thus, simultaneous determination of all the probe drugs and metabolites using a 'high-throughput' system is most desirable. In this study we used a solid-phase extraction procedure which enables simultaneous extraction of all 13 analytes (including IS) from plasma/urine, followed by chromatography with tandem mass spectrometry detection. The method yielded a mean recovery of better than 80% for all the analytes, with good sensitivity, precision and accuracy for the five different probe drug/metabolite pairs. These results show that our method is capable of generating the analytical data in a 'high-throughput' manner.

In the present assay, urinary tolbutamide and its metabolites were found to exhibit much better ionization efficiency in the negative ion mode, whereas urinary debrisoquine and its metabolite were found to achieve better responses in the positive ion mode. Thus two IS for the positive and negative ion analytes were used, and the extract of each urine sample was injected twice, once for the detection of tolbutamide/4-hydroxytolbutamide/carboxytolbutamide, and the other for debrisoquine/5-hydroxydebrisoquine. These components can alternatively be analyzed in a single chromatographic run with use of a modified mobile phase that can separate all analytes and with the use of polarity switching for the relevant MRM transitions. We conducted a preliminary investigation of this polarity switching approach and confirmed its feasibility, but decided to not use it since our primary purpose was to develop a general method to achieve high-throughput analysis, i.e. one that allows consecutive runs without changing the mobile phase between plasma and urine assays. Our current method permits the most efficient allocation of instrument time. Since a chromatographic run using the current LC condition (for both plasma and urine samples) only requires 3.5 min, repeat injections of a urine extract only requires doubling the analysis time and yields the same precision and accuracy.

In this study, an isocratic elution system was used; this choice was based on our initial observation of peak splitting associated with a fast gradient elution system. Since potential matrix effects are a concern with the fast isocratic system, we extensively investigated the potential ionization suppression effect due to the undetected matrix components in plasma/urine as well as to the co-eluting analytes of interest. Although ionization suppression was indeed observed for some analytes (i.e. omeprazole, 5-hydroxyomeprazole and phenacetin in plasma), the effect was consistent over the entire QC concentration ranges of the analytes as well as for different lots of samples. In addition, we did not find a significant change in the ionization response of each analyte by the co-eluting analytes. Furthermore, the inter-lot matrix variation was less than 13% for all analytes and was consistent with the inter- and intra-day precision data. Thus, despite the matrix effects that were observed, the present analytical method is reliable.

Our current LC/MS method significantly improves the efficiency of use of our cocktail for pharmacogenetic studies. Compared to other clinically validated cocktails reported in the literature, the present LC/MS method is much simpler and faster as it requires only a single extraction plus one injection for a plasma sample and two injections for a urine sample. Although the chromatographic turnaround time for each sample was set for 3.5 min in practice, based on the retentions of the analytes shown in Figs. 3 and 4, a 2-min run time would be feasible. This should result in higher throughput for this method. In addition to its application to *in vivo* CYP enzyme studies, this method is also potentially applicable (with slight modification) to *in vitro* CYP enzyme studies which are currently in progress in our laboratory.

CONCLUSIONS

A robust, fast and sensitive LC/MS/MS method has been developed for the simultaneous determination of five CYP probe drugs and their metabolites in human plasma and urine. The cocktail method is suitable for clinical pharmacogenetic studies, and should represent an improvement over the existing cocktail methods due to its combination of high-throughput and multi-analyte characteristics.

Acknowledgements

This study was supported in part by grants from the Innovation and Technology Commission of the Government of Hong Kong SAR (ITS/174/00) and the Research Grants Council of Hong Kong SAR (CUHK4180/02M). We are grateful to AstraZeneca for providing the 5-hydroxyomeprazole standard.

REFERENCES

1. Chang GWM, Kam PCA. *Anaethesia* 1999; **54**: 42.
2. Rodrigues AD. *Biochem. Pharmacol.* 1999; **57**: 465.
3. Streetman DS, Bertino JS Jr, Nafziger AN. *Pharmacogenetics* 2000; **10**: 187.
4. Frye RF, Matzke GR, Adedoyin A, Porter A, Branch RA. *Clin. Pharmacol. Ther.* 1997; **62**: 365.

Copyright © 2004 John Wiley & Sons, Ltd.

5. Damkier P, Brosen K. *Clin. Pharmacol. Ther.* 2000; **68**: 199.
6. Scott RJ, Palmer J, Lewis IA, Pleasance S. *Rapid Commun. Mass Spectrom.* 1999; **13**: 2305.
7. Palmer JL, Scott RJ, Gibson A, Dickens M, Pleasance S. *Br. J. Clin. Pharmacol.* 2001; **52**: 555.
8. Streetman DS, Bleakley JF, Kim JS, Nafziger AN, Leeder JS, Gaedigk A, *et al*. *Clin. Pharmacol. Ther.* 2000; **68**: 375.
9. Chainuvati S, Nafziger AN, Leeder JS, Gaedigk A, Kearns GL, Sellers E, *et al*. *Clin. Pharmacol. Ther.* 2003; **74**: 437.
10. Christensen M, Andersson K, Dalen P, Mirghani RA, Muirhead GJ, Nordmark A, *et al*. *Clin. Pharmacol. Ther.* 2003; **73**: 517.
11. Morin S, Loriot MA, Poirier JM, Tenneze L, Beaune PH, Funck-Brentano C, *et al*. *Eur. J. Clin. Pharmacol.* 2000; **56**: 793.
12. Zhang Y, Blouin RA, McNamara P, Steinmetz J, Wedlund PJ. *Br J Clin Pharmacol* 1991; **31**: 350.
13. Lee CR, Pieper JA, Frye RF, Hinderliter AL, Blaisdell JA, Goldstein JA. *J. Clin. Pharmacol.* 2003; **43**: 84.
14. Yin OQP, Tomlinson B, Chow AHL, Waye MMY, Chow MSS. *J. Clin. Pharmacol.* 2004; **44**: 582.
15. Denero CP, Brown CR, Wilson M, Jacob P, Benowitz NL. *Clin. Pharmacol. Ther.* 1990; **48**: 277.
16. Holland DT, Godfredsen KA, Page T, Connor JD. *J. Chromatogr.* 1998; **707**: 105.
17. US Department of Health and Human Services Food and Drug Administration, Guidance for Industry: Bioanalytical Method validation. US Department of Health and Human Services: Beltsville, MD, 2001.
18. Frye RF, Stiff DD, Branch RA. *J. Liq. Chromatogr. Rel. Technol.* 1998; **21**: 1161.

Copyright © 2004 John Wiley & Sons, Ltd.