Journal of Chromatographic Science 2013;51:26–32
doi:10.1093/chromsci/bms100 Advance Access publication June 12, 2012

Article

UPLC–MS-MS Method for Simultaneous Determination of Caffeine, Tolbutamide, Metoprolol, and Dapsone in Rat Plasma and its Application to Cytochrome P450 Activity Study in Rats

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Received 16 February 2012; revised 23 April 2012

A specific ultra-performance liquid chromatography tandem mass spectrometry method has been described for the simultaneous determination of caffeine, tolbutamide, metoprolol and dapsone in rat plasma, which are the four probe drugs of the four cytochrome P450 (CYP450) isoforms CYP1A2, CYP2C9, CYP2D6 and CYP3A4. The chromatographic separation was achieved using a Waters Acquity UPLC BEH HILIC C_{18} column (2.1 × 50 mm, 1.7 μm). The mobile phase consisted of acetonitrile and water (containing 0.1% formic acid) (15:85, v/v). The triple quadrupole mass spectrometric detection was operated by positive electrospray ionization. Phenacetin was chosen as internal standard. Plasma samples were extracted with dichloromethane–butanol (10:1, v/v). The recoveries ranged from 67.5% to 98.5%. The calibration curves in plasma were linear in the range of 2.5–1,000 ng/mL for caffeine and dapsone, 5–5,000 ng/mL for tolbutamide and 2.5–250 ng/mLfor metoprolol, with correlation coefficient (r^2) of 0.9936, 0.9966, 0.9990 and 0.9998, respectively. The method was successfully applied to pharmacokinetic studies of the four probe drugs of the four CYP450 isoforms and used to evaluate the effects of breviscapine on the activities of CYP1A2, CYP2C9, CYP2D6 and CYP3A4 in rats.

Introduction

The cytochrome P450 (CYP450) superfamily is a major drug metabolizing enzyme system that plays an important role in the metabolism or the biotransformation of many endogenous and exogenous substances (1). It is important to identify whether a drug affects the activities of CYP450 enzymes, to avoid possible drug–drug interactions and drug adverse reactions (2, 3). CYP1A2, 2C9, 2D6 and 3A4 are the major CYP450 isoforms that are responsible for metabolizing of more than 90% of drugs (4). Caffeine, tolbutamide, metoprolol and dapsone are probe drugs for CYP1A2, CYP2C9, CYP2D6 and CYP3A4 and are used to test the activities of these isoenzymes (5, 6). If the four probe drugs are administrated and determined at the same time, the activities of the four isoenzymes can be simultaneously evaluated. Compared to the traditional method of single probe, this "cocktail" method can be used to evaluate the activity of more than one isoform of CYP450 in one experiment and minimize the individual variability; meanwhile, it is more convenient and rapid.

Early publications have described methods for the simultaneous determination of multiple probe drugs using high-performance liquid chromatography (HPLC) (7, 8), but it is very difficult to achieve ideal separation, and it is time-consuming or requires gradient elution, which usually influences the baseline of the chromatogram and results (9), so it is very necessary to develop a rapid and reliable method.

To date, there is no documented record on the simultaneous determination of caffeine, tolbutamide, metoprolol and dapsone in a single-run process. In this study, we developed an ultra-performance liquid chromatography tandem mass spectrometry (UPLC–MS-MS) method for analysis of the four probe drugs in a single-run process, and the four drugs were simultaneously extracted from rat plasma with a single extraction solvent. The column was packed with C_{18} particles of 1.7 μm, which contribute to higher column performance, efficient separation and a short analysis time (10, 11). In this study, the total run time for each injection was 5 min.

The method established in this study was successfully applied to study the pharmacokinetics of the four probe drugs and to evaluate the effects of breviscapine on CYP450 isoforms. Breviscapine is a traditional herbal medicine, and is widely used in the treatment of cerebral thrombosis, cerebral hemorrhage, sequela, coronary disease, stenocardia and arrhythmia (12, 13). To date, many CYP450-mediated interactions have been reported between herbs and drugs (14–16). Clinically, breviscapine is often used in combination with substrates of CYP450 (17, 18), however, we do not know whether breviscapine has an effect of on CYP450, nor do we know the consequences of such an effect on the substrates. To this end, we have investigated the effects of breviscapine on CYP1A2, 2C9, 2D6 and 3A4 in rats. Our results show that breviscapine exhibits an induced effect on CYP1A2 and an inhibitory effect on CYP3A4, but no significant changes in CYP2C9 and CYP2D6 activities.

Experimental

Chemicals and materials

Caffeine, metoprolol and phenacetin were obtained from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Tolbutamide was purchased from Dr. Ehrenstorfer (Augsburg, Germany). Dapsone was purchased from Sigma Chemical Co. (St Louis, MO). Breviscapine injection (5 mg per milliliter) was obtained from Kunming Longjin Pharmaceutical Co. (Yunnan, China). Methanol and formic acid were of HPLC grade, acetonitrile was hypergrade for LC–MS, and all other reagents used were of

analytical grade. The mixture solution of the probe drugs was dissolved with normal saline, including proper polysorbate 80.

Instrumentation

Samples were analyzed by UPLC–MS-MS using a Waters Acquity (Waters, Milford, MA) UPLC, a Waters MS (Waters MS Technologies, Manchester, UK) and a micromass Quattro Micro API QqQ with an electrospray ionization (ESI) source. Instrument control and data acquisition was performed with MassLynxTM V 4.1 software (Waters).

Chromatographic conditions

The chromatographic separation was carried out using an Acquity UPLC–MS-MS and performed on a Waters Acquity UPLC BEH HILIC C18 column (2.1 × 50 mm, 1.7 μm). The column temperature was maintained at 40°C. The chamber temperature in the autosampler was kept at 10°C. The mobile phase consisted of acetonitrile and water (containing 0.1% formic acid) (15:85, v/v) at a flow rate of 0.25 mL/min, and the total run time for each injection was 5 min.

Mass spectrometric conditions

A Waters Micromass Quattro Micro API triple quadrupole tandem MS was equipped with an ESI source that was set to positive ion mode, with ionization conditions as follows: capillary voltage 3.3 KV, cone voltage 30 V, source temperature 120°C, and desolvation gas (nitrogen) heated at 350°C (650 L/h). Cone gas flow rate was 50 L/h. The collision energy was set at 24 eV for caffeine and dapsone, 32 eV for tolbutamide, 20 eV for metoprolol and 20 eV for phenacetin. Quantification was performed using multiple reaction monitoring (MRM) with the transitions of m/z 195 → 138, 271 → 91, 268 → 116, 249 → 92 and 180 → 110 for caffeine, tolbutamide, metoprolol, dapsone and phenacetin (internal standard; IS), respectively.

Sample preparation

Twenty microliters each of the four probe drugs were mixed in a tube and evaporated to dryness at 40°C under a gentle stream of nitrogen, then 20 μL of IS working solution and 100 μL of blank plasma were added to the tube and mixed for 3 min on a vortex shaker. The plasma sample was extracted with 2.0 mL dichloromethane–butanol (10:1, v/v). After vortexing for 3 min and centrifugation at 5,000 × g for 5 min, the organic phase was transferred to another tube and evaporated to dryness in a 40°C water bath under a gentle stream of nitrogen. The residue was reconstituted with 200 μL of mobile phase. Finally, an aliquot of 10 μL was injected into the UPLC–MS-MS system for analysis.

Preparation of calibration standards and quality control samples

Stock solutions of all analytes were prepared at the concentration of 1 mg/mL in methanol–water (5:5, v/v). Series of working solutions were obtained by diluting the stock solutions with water, storing at 4°C and bringing to room temperature before use.

Calibration standards and quality control (QC) samples were prepared by spiking the working solutions into 100 μL of blank plasma, and processing them as described previously. The final plasma concentrations of calibration samples were adjusted to 2.5, 5, 20, 50, 200, 500 and 1,000 ng/mL for caffeine and dapsone; 5, 10, 50, 200, 500, 2,500 and 5,000 ng/mL for tolbutamide; and 2.5, 5, 12.5, 25, 62.5, 125 and 250 ng/mL for metoprolol. QC samples were prepared at concentrations of 5, 200 and 1,000 ng/mL for caffeine and dapsone; 10, 500 and 5,000 ng/mL for tolbutamide; and 5, 62.5 and 250 ng/mL for metoprolol.

Method Validation

Specificity

The specificity of the assay was investigated by comparing the potential interferences at the retention times of the four probe drugs and the IS. The chromatogram of the blank plasma sample was compared with that obtained from the same batch spiked with the four probe drugs and IS to confirm the absence of endogenous interferences in the rat plasma sample.

Calibration curve and lower limit of quantification

The calibration curves of caffeine, tolbutamide, metoprolol and dapsone were prepared by the previously described method. Each curve was constructed by the analyte/IS peak area ratio as vertical coordinate, the concentrations of the calibration standards as horizontal ordinate. The lower limit of quantification (LLOQ) was the lowest concentration of the standard curve; it was set at ± 20% bias of nominal concentration.

Precision and accuracy

Three QC samples in five replicates were analyzed on the same day and on three consecutive days to evaluate the intra-day and inter-day accuracy and precision. The accuracy was evaluated as the relative error (RE), and the precision was evaluated as the relative standard deviation (RSD). The acceptable values of RE and RSD were set to not exceed 15%.

Recovery and matrix effect

The extraction recoveries of the analytes were evaluated by comparing the peak areas of the analytes extracted from plasma with peak areas of the same concentration of standard solutions. They were assayed by analysis of three concentrations (QC samples) in quintuplicate, respectively.

The matrix effects (ME) were examined by comparing the peak areas of the analytes between the pure standards dissolved with mobile phase and the extracted standards from the blank plasma spiked with the same concentrations of analytes. The ME was defined as (1− signal of the extracted samples/signal of pure standards) × 100%. The acceptable bias was set within ± 15%.

Stability

The stability of the samples in rat plasma under different conditions was investigated at three QC levels ($n = 3$). Freeze–thaw stability was evaluated after three freeze (−20°C) and thaw (room temperature) cycles. The long-term stability was evaluated after storage of the test samples at −20°C for three weeks, and short-term stability was for 3, 6 and 12 h at room temperature. To assess the stability of the processed samples, the samples were extracted and placed at 4°C for 12 h, and then compared with those of the same QC samples that had been analyzed immediately.

Application of the analytical method in CYP450 activity study

The described method was applied to pharmacokinetic studies of the four probe drugs and used to evaluate the effects of breviscapine on the activities of CYP1A2, CYP2C9, CYP2D6 and CYP3A4 in rats.

Wistar rats (200 ± 20 g, male) were supplied by the Animal Experimental Center of the Harbin Medical University, which was fully accredited by the Institutional Animal Care and Use Committee (IACUC). Rats were handled in a manner that met with all the recommendations formulated by the National Society for Medical Research and Guidelines for the Care and Use of Laboratory Animals.

The rats were randomly divided into control and breviscapine-treated groups, eight rats in each group, and administered 0.2 mL physiological saline and breviscapine injection by caudal vein for seven consecutive days, respectively; the next day, they were given caffeine (10 mg/kg), tolbutamide (10 mg/kg), metoprolol (20 mg/kg) and dapsone (10 mg/kg) by intraperitoneal injection, and tails were snipped near the tip to allow the collection of blood samples. Blood samples (0.3 mL) were obtained immediately before (0 h) and after the administration of the drugs at 0.17, 0.5, 0.83, 1.17, 1.5, 2, 3, 5, 8, 12 and 24 h. The blood samples were centrifuged at 5,000 × g for 10 min and plasma samples were separated and stored at −20°C until analysis.

Data analysis

Data were expressed as means ± standard deviation (SD) and analyzed by the Dunnett's test. The pharmacokinetics parameters of the four probe drugs were derived with a nonlinear regression iterative program, DAS 2.0 (Chinese Pharmacological Society) pharmacokinetic statistical software. $P < 0.05$ and $P < 0.01$ were considered to be statistically significant and very significant, respectively.

Results

Sample preparation

Extraction conditions were optimized by using several solvents, and dichloromethane–butanol (10:1, v/v) displayed better extraction recoveries (> 67%) for all of the four probe drugs, with no interfering endogenous peak at the retention times of the analytes.

Optimization of UPLC–MS-MS conditions

The positive ion electrospray mass scannning spectra of the probe drugs and IS after direct injection in mobile phase are presented in Figure 1. The observed scan mass spectra showed the prominent protonated molecular ions $[M + H]^+$ of m/z 195 for caffeine, 271 for tolbutamide, 268 for metoprolol, 249 for dapsone and 180 for phenacetin (IS), respectively, which were chosen as the parent ions for fragmentation in the MRM mode. Based on the mass scan spectra, the transitions m/z 195 → 138, 271 → 253, 268 → 191, 249 → 156 and 180 → 138 were selected, respectively, for quantitative measurement of caffeine, tolbutamide, metoprolol, dapsone and phenacetin (IS). To obtain maximum sensitivity of the MRM and optimizing conditions for quantitative determination, the MS parameters such as capillary voltage, cone voltage, collision energy, source temperature, desolvation gas temperature and nitrogen flow rate were optimized. The other MS parameters were adopted from the recommended values for the instrument.

Method validation

Specificity

Figure 2 shows the chromatographs of blank plasma (Figure 2A), a blank plasma sample spiked with the probe drugs (2.5 ng/mL for caffeine, metoprolol and dapsone, respectively; 5 ng/mL for tolbutamide) and IS (100 ng/mL) (Figure 2B), and a plasma sample after intraperitoneal administration of the probe drugs (Figure 2C). The retention times were approximately 1.15 min for caffeine, 3.10 min for tolbutamide, 2.31 min for metoprolol, 2.31 min for dapsone and 2.63 min for phenacetin (IS), respectively. No remarkable interferences were observed at the retention times of caffeine, tolbutamide, metoprolol, dapsone and IS.

Calibration curve and LLOQ

The ranges of linearity of the calibration curve were 2.5–1,000 ng/mL for caffeine and dapsone, 5–5,000 ng/mL for tolbutamide and 2.5–250 ng/mL for metoprolol. The calibration curves were obtained by using a weighting factor of $1/x^2$. The regression equations are shown in Table I.

The LLOQ concentrations of the analytes are also listed in Table I.

Precision and accuracy

Accuracy and precision data for all the analytes are summarized in Table II. None of the RE and RSD values of the intra-day and inter-day accuracy and precision exceeded 15%.

Recovery and matrix effect

The extraction recoveries of all the probe drugs from rat plasma are shown in Table II. The average recoveries were 81.6% for caffeine, 90.5% for tolbutamide, 86.0% for metoprolol, 84.4% for dapsone and 86.7% for IS.

All ME values were within ± 15%. The results indicated that there was no remarkable influence for the ionization of the analytes.

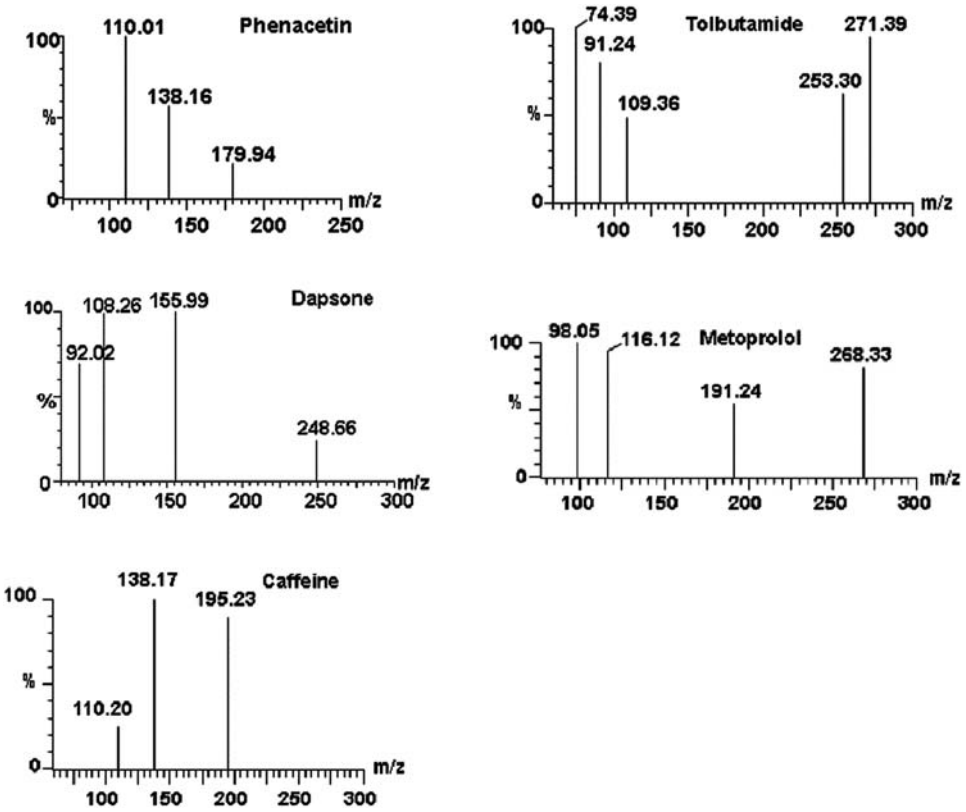

Figure 1. Positive ion electrospray mass scan spectra of the probe drugs (caffeine, tolbutamide, metoprolol and dapsone) and IS (phenacetin).

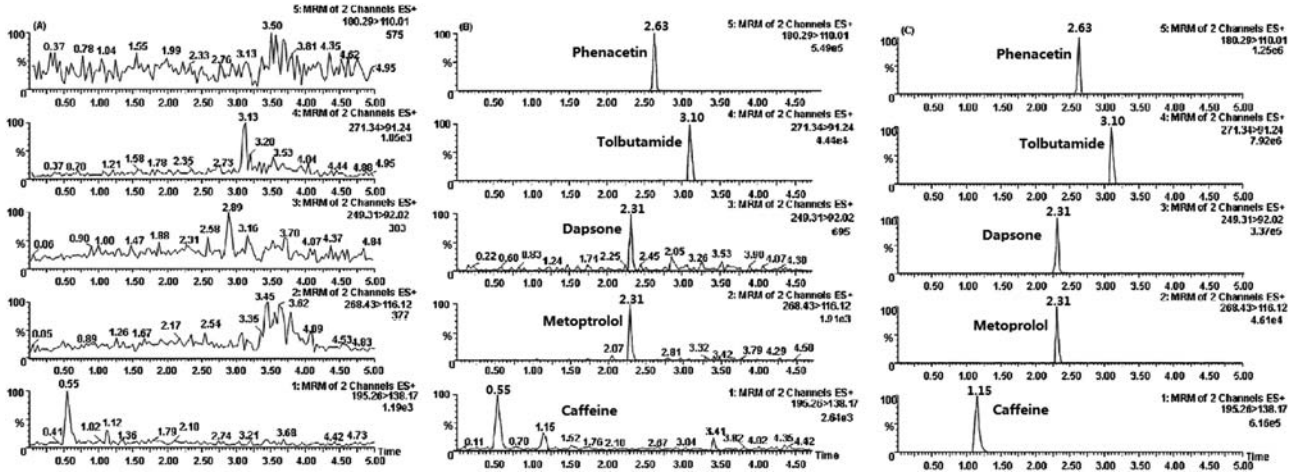

Figure 2. Chromatograms of analytes: blank plasma (A); a blank plasma sample spiked with the probe drugs at LLOQ level (2.5 ng/mL for caffeine, metoprolol and dapsone, respectively; 5 ng/mL for tolbutamide) and IS (100 ng/mL for phenacitin) (B); a plasma sample after intraperitoneal administration of the probe drugs (C).

Stability

The results of the stability tests indicated that all the samples and analytes were stable at the experimental conditions. The standard deviations were all below 10%.

Effects of breviscapine on CYP450 activities in rats

The mean plasma concentration–time profiles and pharmacokinetics parameters of the four probe drugs in the control group and the breviscapine group are shown in Figure 3 and Table III, respectively. According to the results, breviscapine induced the activity of CYP1A2 and accelerated the metabolism of caffeine, by decreased $t_{1/2}$ (24.73%, $P < 0.01$), C_{max} (47.88%, $P < 0.01$) and $AUC_{0-\infty}$ (59.41%, $P < 0.01$) in the breviscapine group compared to the control group. Conversely, the activity of CYP3A4 was inhibited after treatment with breviscapine, by increased $t_{1/2}$ (51.04%, $P < 0.05$), C_{max} (211.54%, $P < 0.01$) and $AUC_{0-\infty}$ (194.32%, $P < 0.01$) of dapsone. No significant differences were observed in the major pharmacokinetics

Table I
Calibration Curves of the Analytes in Rat Plasma, LLOQ

Analytes	Regression equation*	R^2	Linear range (ng/mL)	LLOQ (ng/mL)
Caffeine	$y = 3.0 \times 10^{-4}x - 4.50 \times 10^{-3}$	0.9936	2.5–1,000	2.5
Tolbutamide	$y = 4.0 \times 10^{-4}x + 1.06 \times 10^{-2}$	0.9990	5–5,000	5
Metoprolol	$y = 3.0 \times 10^{-4}x - 1.00 \times 10^{-4}$	0.9998	2.5–250	2.5
Dapsone	$y = 9.0 \times 10^{-6}x + 1.00 \times 10^{-5}$	0.9966	2.5–1,000	2.5

*y: peak area ratios of analytes to IS; x: concentrations of analytes in plasma (ng/mL).

Table II
Intra-Day and Inter-Day Precision and Accuracy ($n = 5$), Extraction Recovery ($n = 9$)

Analytes	Concentration (ng/mL)	Intra-day		Inter-day		Recovery	
		Precision (RSD%)	Accuracy (RE%)	Precision (RSD%)	Accuracy (RE%)	Mean ± SD (%)	RSD (%)
Caffeine	5	2.9	5.1	5.8	7.1	71.4 ± 3.5	4.9
	200	4.1	1.1	3.5	6.2	86.1 ± 4.9	5.8
	1,000	1.2	7.7	1.5	4.3	87.2 ± 1.7	1.9
Tolbutamide	10	6.9	8.8	7.5	6.7	86.2 ± 3.0	3.5
	500	0.9	8.3	3.7	1.8	87.6 ± 4.5	5.2
	5,000	0.6	2.3	3.6	3.5	97.8 ± 1.7	1.7
Metoprolol	5	7.8	6.8	5.8	7.5	77.3 ± 3.5	4.5
	62.5	4.4	2.4	3.5	1.6	89.5 ± 6.1	6.8
	250	3.1	2.1	3.4	4.3	91.3 ± 4.7	5.2
Dapsone	5	4.5	5.1	6.1	3.1	67.5 ± 6.7	9.9
	200	1.8	0.6	3.0	2.0	87.1 ± 1.6	1.8
	1,000	1.3	4.5	2.3	5.8	98.5 ± 0.8	0.8
IS	100					86.7 ± 2.6	3.0

parameters of metoprolol and tolbutamide between the breviscapine group and the control group, which meant that there were no effects of breviscapin on CYP2C9 and CYP2D6.

Discussion

Blood samples contain a significant amount of endogenous components that can interfere with the detection of analytes. A viable extraction protocol is needed to recover as much probe drugs from the samples with minimum interference. Liquid–liquid extraction is commonly used for sample preparation (19, 20), and was employed for the isolation of the four probe drugs from plasma samples in this study. Different extraction solvents were tested and extraction conditions were optimized during the method development. Diethyl ether, 4-methyl *tert*-butyl ether, dichloromethane, chloromethane, ethyl acetate and butanol were used, and the average extraction recoveries for the four probe drugs ranged from 9%–43%, 13%–32%, 17%–45%, 25%–38%, 18%–33% and 11%–23%, respectively, which were low and could not meet the demands. During the experiment, dichloromethane–butanol (10:1, v/v) displayed better extraction recoveries (>67%) for all of the four probe drugs, with no interfering endogenous peak at the retention times of the analytes. Therefore, dichloromethane–butanol (10:1, v/v) was proved to be a simple, rapid and efficient way to extract the four probe drugs from plasma in a single-run process.

When performing a series of routine analyses, it is important to consider speed, sensitivity and resolution, which means choosing appropriate mobile phases and column. The aim of this study was to develop and validate a simple, fast and reliable UPLC–MS-MS method to simultaneously measure the four probes of the CYP450 isoforms. The chromatographic separation was performed on a Waters Acquity UPLC BEH HILIC C_{18} column (2.1 × 50 mm, 1.7 μm), which contributes to an efficient separation and a short analysis time. The mobile phase played a critical role in achieving good chromatographic behavior. When the chromatographic conditions were optimized, it was found that acetonitrile resulted in lower background noise and better peak shape than methanol, therefore, acetonitrile was chosen as organic phase. In positive ion mode, the presence of a low amount of formic acid in the mobile phase can improve the sensitivity by promoting the ionization of the analytes and achieve symmetrical peak shapes, thus, 0.1% formic acid was added to the mobile phase.

UPLC–MS-MS, which was used for the subsequent quantitation of the probe drugs, showed faster analysis time than conventional HPLC or LC–MS and tremendously enhanced signal intensity (21, 22). It took only 5 min to finish analyzing a blood sample using the method established in this paper, which can save much time in experimental studies with hundreds of samples. In addition, the lower limit of determination (LLOD) for the four probe drugs was comparatively low, which is satisfactory for determining a lower plasma concentration in the last sampling time point, and the sample injection volume can be reduced, so a small plasma volume (100 μL) was employed for processing in this experiment.

This is the first work using UPLC–MS-MS for the simultaneous determination of caffeine tolbutamide, metoprolol and dapsone, the four probe drugs of CYP1A2, 2C9, 2D6 and 3A4. Both the extraction and the UPLC–MS-MS determination were in a single-run process. The method showed excellent sensitivity, reliability, specificity, accuracy and precision at the examined range of concentrations, and can be used for pharmacokinetic studies of the four probe drugs in rats. Changes in pharmacokinetics parameters of the probe drugs can reflect the changes of activities of CYP450 isoforms (23), so the method established in this paper can be applied to evaluate the effects of drugs on activities of the four CYP450 isoforms.

Conclusion

In this study, a simplified, rapid and selective analytical UPLC–MS-MS method was developed for the simultaneous quantification in rat plasma of caffeine, tolbutamide, metoprolol and dapsone, which are the four probe drugs of CYP1A2, 2C9, 2D6 and 3A4. The method can be used for pharmacokinetic studies of the four probe drugs in rats and can be applied to evaluate the effects of drugs on activities of the four CYP450 isoforms. The method was successfully applied to evaluate the effects of breviscapine on the activities of the four CYP450 isoforms in rats. This is the first report that breviscapine induces the activity of CYP1A2 and inhibits the activity of CYP3A4, but had no effects on the activities of CYP2C9 and CYP2D6. Given that CYP1A2 and CYP3A4 are responsible for the metabolism and disposition of a large number of currently used drugs, the potential herb-drug interactions of breviscapine preparations with drugs that are substrates of CYP1A2 and CYP3A4 may be important.

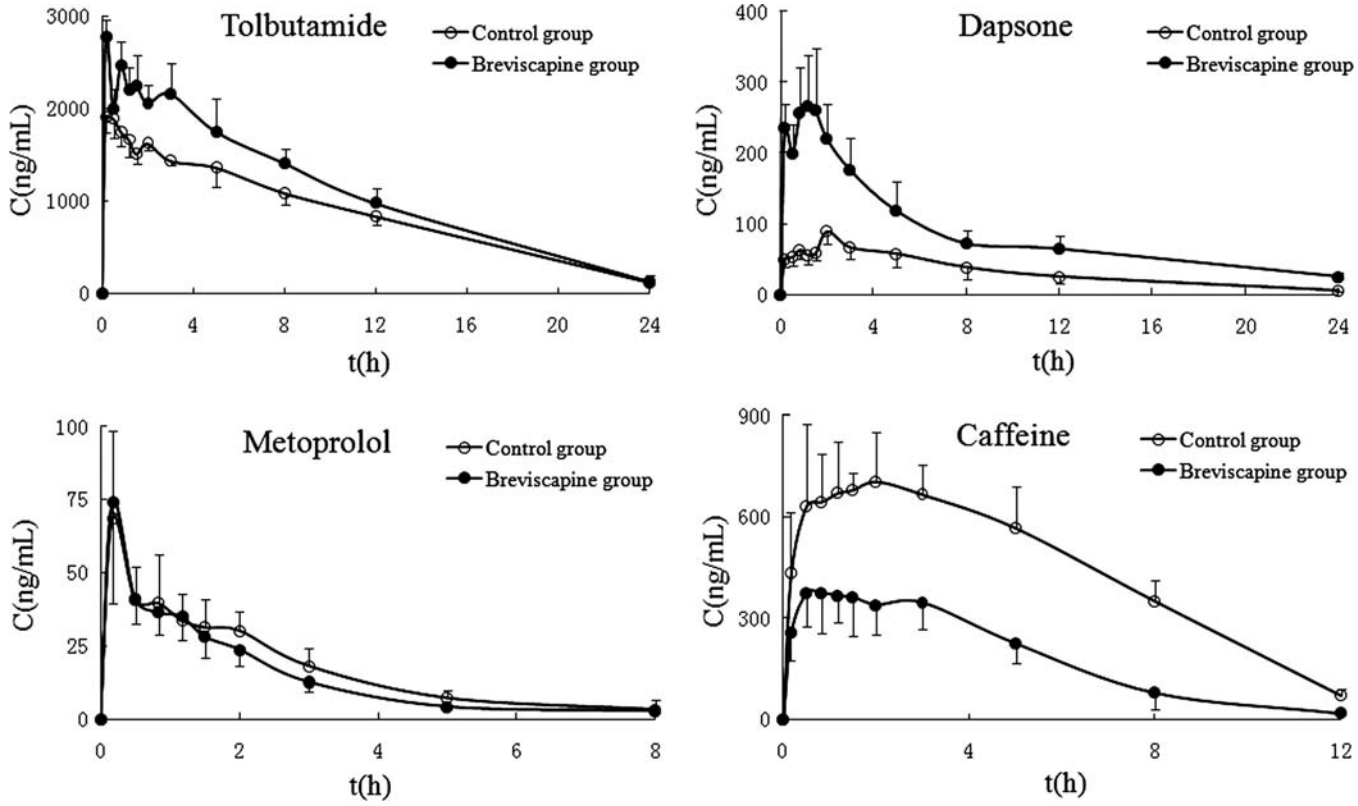

Figure 3. Mean plasma concentration–time profiles of the four probe drugs in the control group and the breviscapine group.

Table III
Pharmacokinetics Parameters of the Four Probe Drugs (Mean ± s, $n = 8$)

	Caffeine		Tolbutamide		Metoprolol		Dapsone	
	Control group	Breviscapine group	Control group	Breviscapine group	Control group	Breviscapine group	Control group	Breviscapine group
$t_{1/2}$/h	2.83 ± 0.45	2.13 ± 0.18†	5.39 ± 0.92	4.75 ± 3.64	2.09 ± 1.45	2.03 ± 0.23	6.76 ± 1.95	10.21 ± 2.84*
t_{max}/h	2.25 ± 0.99	1.25 ± 0.45*	1.06 ± 0.77	1.03 ± 1.13	0.28 ± 0.27	0.17 ± 0.01	1.67 ± 1.01	0.67 ± 0.59*
C_{max}/ ng/mL	794.25 ± 142.15	413.98 ± 96.94†	2,055.41 ± 526.65	3,439.85 ± 1,218.49*	71.40 ± 29.50	74.05 ± 34.42	106.09 ± 15.52	330.51 ± 67.45†
AUC_{0-t}/ng · h/mL	5,365.62 ± 593.68	2,241.94 ± 514.53†	20,939.0 ± 5,643.9	26,552.6 ± 10,080.9	127.65 ± 49.90	119.59 ± 20.19	786.07 ± 180.86	2,055.39 ± 405.73†
$AUC_{0-\infty}$/ng · h/mL	5,671.58 ± 564.60	2,302.35 ± 520.55†	25,989.4 ± 8,604.9	35,031.8 ± 14,809.3	152.75 ± 39.06	136.53 ± 31.42	997.68 ± 256.26	2,936.42 ± 191.45†

*$P < 0.05$.
†$P < 0.01$ compared with that of the control.

Funding

This work was supported by Natural Science Foundation of Heilongjiang [D201044] and National Natural Science Foundation of China [81173659].

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