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Simultaneous determination of oxymatrine and its active metabolite matrine in dog plasma by liquid chromatography–mass spectrometry and its application to pharmacokinetic studies

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Abstract

A simple, rapid and reliable method was developed for the quantification of oxymatrine (OMT) and its metabolite matrine (MT) in beagle dog plasma using a liquid–liquid extraction procedure followed by liquid chromatography–electrospray ionization mass spectrometric (LC–ESI-MS) analysis. Extend-C18 column (2.1 mm i.d. × 50 mm, 5 μm) with a C18 guard column (2.1 mm i.d. × 12.5 mm) was used as the analytical column. Linear detection responses were obtained for OMT concentration ranging from 5 to 4000 ng/ml and for MT concentration ranging from 5 to 2000 ng/ml. The precision and accuracy data, based on intra- and inter-day variations over 5 days, were lower than 5%. The limit of quantitation for OMT and MT were 2 and 1 ng/ml, respectively, and their recoveries were greater than 90%. Pharmacokinetic data of OMT and its active metabolite MT obtained with this method following a single oral dose of 300 mg OMT capsules to six beagle dogs was also reported for the first time.

Keywords: Oxymatrine; Matrine; Liquid chromatography–mass spectrometry; Dog plasma; Metabolite; Pharmacokinetics

1. Introduction

Oxymatrine(OMT) and matrine (MT) are the major quinolizidine alkaloids from the root of *Sophora flavescens* Ait. (kushen), but also from *Sophora subprostrata* (shandougen), and from the above ground portion of *Sophora alopecuroides* [1,2]. Chemical structures of two constituents are shown in Fig. 1. They have been extensively used in China for treatment of viral hepatitis, cancer, cardiac diseases (such as viral myocarditis), and skin diseases (such as psoriasis and eczema) [3–5].

In China, OMT, called kushensu, is frequently used in clinical practice, especially for oral administration. When taken orally, OMT can be reduced to the more absorbable MT by intestinal bacteria in the gastrointestinal tract and liver, which might have pharmacological and toxicological implications in clinical practice [6,7]. However, up to now, plasma concentration–time profiles of OMT and its metabolite MT after the oral administration of OMT were not reported. Several methods for the determination of OMT or MT in plasma have been described in the literature. These methods include HPLC/UV[8–10], gas chromatography–mass spectrometry (GC–MS) [11], high-performance capillary electrophoresis (HPCE) [12], fluorescence quenching method [13]. HPLC/UV methods were frequently used to determine OMT or MT concentrations in the blood, which ranged from 0.5 to 50 μg/ml. However, these HPLC/UV methods are not sensitive enough for detecting metabolite MT in the plasma of dogs within an hour after an oral dose of 300 mg OMT capsules. Although an GC–MS method could be used to detect matrine in the plasma at low ng/ml concentrations, a wide range melting point of matrine-type alkaloids make it difficult to apply capillary gas chromatography for their separation.

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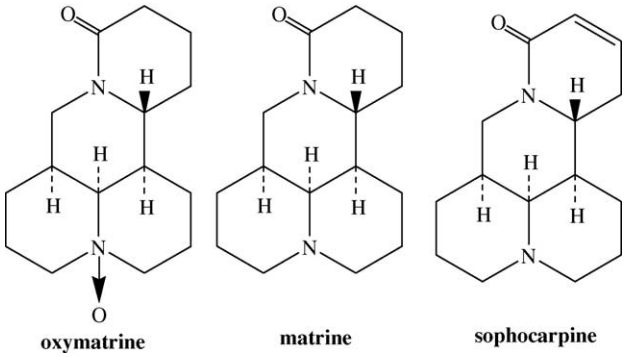

Fig. 1. Chemical structures of oxymatrine (OMT), matrine (MT) and sophocarpine (internal standard).

HPCE method lack quantitative precision and reproducibility. Fluorescence quenching method are too complex in the samples pretreatment. Therefore, we developed a simple, sensitive high performance liquid chromatography–mass spectrometry (LC–MS) method for the simultaneous determination of OMT and MT in beagle dog plasma and study the pharmacokinetic of OMT and its active metabolite MT in dogs following oral administration of OMT capsules at a therapeutic dose for the first time.

2. Experimental

2.1. Chemicals and reagents

OMT, MT, and sophocarpine (>99% purity) were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). OMT capsules (100 mg/capsule, >98.5% purity) were purchased from Jiangsu Chia Tai-Tian Qing Pharmaceutical Co. Ltd. (Lianyungang, China). Acetonitrile was of HPLC grade (Fisher, USA). All other chemicals were of analytical reagent grade. Distilled, deionized water was produced by a Milli-Q Reagent Water System (Millipore, MA, USA).

2.2. Equipment and chromatographic conditions

All analytical procedures were performed on a Shimadzu 2010A LC–MS system (Shimadzu Company, Japan), with ESI probe (Q-array-Octapole-Quadrupole mass analyzer). Shimadzu 10ADvp Pump, Shimadzu 10ATvp Autosampler. Nitrogen, the carrier gas was supplied by the Gas Supplier Center of Nanjing University, China. Separation was carried out by an Extend-C18 Column (2.1 mm i.d. × 50 mm, 5 μm, Agilent Technologies, USA) with a C18 guard column (2.1 mm i.d. × 12.5 mm, Agilent Technologies, USA), maintained at 40 °C. The mobile phase was acetonitrile-water containing 10 mmol/l ammonium acetate (15:85) at the flow rate of 0.2 ml/min. The following optimized MS conditions were selected: gas flow: 1.5 l/min; curve dissolution line (CDL) temperature: 250 °C; block temperature: 200 °C. Detector voltage: 1.65 kV, probe voltage: 4.5 kV, CDL voltage fixed as in Tuning. Mass Vacuum was obtained by Turbo molecular pump. LC–MS Solution Version 2.04 working on Windows 2000 operating system was used for data processing. The injection volume was 5 μl. The ESI ion source was set in the positive ion polarity mode for acquiring all mass spectrometry data. The selective ion monitoring (SIM) was used, and protonated molecules $[M+H]^+$ were detected at *m*/*z* 265.1 for OMT, *m*/*z* 249.2 for MT and 247.3 for the internal standard (sophocarpine, molecular structure seen in Fig. 1).

2.3. Animals and blood sampling

Six beagle dogs (three males, three females), weighing 10.1 ± 0.3 kg (mean ± SD), were utilized in the studies. Animals were provided by the experimental animal center of China Pharmaceutical University. The animals were housed in stainless steel metabolic cages equipped with an automated watering valve. They were cared for according as the regulations of the animal committee under a constant temperature at (22 ± 1°C), humidity at (50 ± 20%), 12 h light/12 h dark cycle and 10–15 air changes per hour. Their healthy status was checked after a quarantine period of 2 weeks. Diet was prohibited for 6 h before the experiment while water was taken freely. They were given a single 300 mg (three capsules) oral dose of OMT. Blood samples (about 1 ml) were collected in 1.5 ml heparinized polythene tubes at 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 6, 8, 10, 12 and 24 h after dosing.

2.4. Sample preparation

2.4.1. Standard solution, calibration curve and quality control samples

The standard stock solution of 400 μg/ml of OMT and 200 μg/ml of MT were prepared in doubly distilled water. A series of standard working solutions with concentrations in the range of 5–4000 ng/ml for OMT and 5–2000 ng/ml for MT were obtained by further dilution of the standard stock solution with the mobile phase. The internal standard stock solution of 200 μg/ml of SC was prepared in methanol. Internal standard working solution (2 μg/ml) was prepared by diluting internal standard stock solution with the mobile phase. All solutions were stored at 4 °C. A series of standard working solutions were evaporated to dryness at 35 °C under a gentle stream of nitrogen. The residue was reconstituted in 0.2 ml of blank plasma to prepare the calibration standards containing 4000, 2000, 1000, 500, 200, 100, 50, 20, 10 and 5 ng/ml for OMT and 2000, 1000, 500, 200, 100, 50, 20, 10 and 5 ng/ml for MT.

Quality control (QC) samples were prepared in the same way as calibration standards with blank plasma, and QC sample concentrations were 20, 200, 1000 ng/ml. QC samples were stored at −80 °C until analysis.

2.4.2. Sample extraction

Each collected blood sample was immediately centrifuged at 1500 × *g* for 10 min at 4 °C (Himac CT 13R, Hitachi Co, Japan). The resulting plasma (0.2 ml) and the internal standard working solution 20 μl (2 μg/ml) were added to a 5 ml polythene tube. They were vortex-mixed for 30 s and then mixed with 3 ml chloroform using vortex for 3 min. The centrifuge tube was centrifuged at 1100 × *g* for 8 min. The organic layer 2.5 ml was transferred to another tube and evaporated to dryness at 35 °C under a gentle stream of nitrogen. The residue was reconstituted in 0.2 ml mobile phase, and centrifuged at 21000 × *g* at 4 °C for 15 min.The supernatant (80 μl) was transferred to a MS vial for LC–MS analysis. The same sample handling process was used for recovery and precision determinations in plasma.

2.5. Validation of the method

2.5.1. Linearity

Linearity of calibration was tested by extraction and assayed ($n = 5$). Calibration curves in the concentration range of 0.005–4.0 μg/ml for OMT and of 0.005–2.0 μg/ml for MT were constructed by plotting the peak-area ratios of analyte/internal standard versus OMT or MT concentration in beagle dog plasma. Weighted ($1/\text{conc}^2$) least-squares linear regression analysis was used to determine the slope, intercept and correlation coefficient. The concentration of OMT or MT in plasma was determined from the peak-/area ratios by using the equations of linear regression obtained from the calibration curves.

2.5.2. Recovery

To determine the recovery of OMT and MT by the liquid–liquid extraction method, plasma samples were spiked with OMT or MT at concentrations of 0.02, 0.2 and 1.0 μg/ml. The resulting peak-area ratios (analyte:internal standard) were compared with that of the standards prepared in mobile phase to provide the recovery values.

2.5.3. Precision and accuracy

Intra-day accuracy and precision (each $n = 5$) were evaluated by analysis of QC samples at different times during the same day. Inter-day accuracy and precision were determined by repeated analysis of QC samples over five consecutive days ($n = 1$ series per day). The concentration of each sample was determined using calibration standards prepared on the same day. Accuracy of the method was determined by the relative error (RE), which was calculated by the equation: (mean of determined concentration–actual concentration/actual concentration) × 100%. Precision was determined by the coefficients of variation (CV).

2.5.4. Stability

The effect of three freeze-thaw cycles, the compound stability for 4 h at room temperature in plasma were evaluated by repeated analysis ($n = 3$) of QC samples. Long-term stability in plasma was also tested by assaying frozen QC samples after storage at −80 °C for 6 months. The amount of them in the plasma samples was determined using a newly prepared calibration curve. Stability was expressed as a percentage of nominal concentration.

2.6. Assay application

The present method was used to determine concentration-time profiles of OMT and MT in beagle dog plasma after oral administration of OMT capsules. Pharmacokinetic parameters were estimated by non-compartmental analysis using 3p87 software package(version 1.1 PK software, Chinese Pharmacological Association).The elimination half-life ($t_{1/2\beta}$) was $0.693/k_{el}$, where k_{el}, the elimination rate constant, was calculated by linear regression from the terminal linear portion of plasma concentration-time curve. Maximum drug plasma concentrations (C_{max}) and time to reach the maximum concentrations (T_{max}) were taken directly from the observed data. The area under concentration versus time curve (AUC) was calculated using the trapezoidal rule. The mean residence time (MRT) was the ratio of area under the first moment versus time curve (AUMC) to AUC.

3. Results and discussion

3.1. Chromatography and mass spectrum

Fig. 2a–c show the mass spectra of OMT, MT and internal standard (I.S.), respectively. It is clear that the analytes and I.S. formed predominantly protonated molecules $[M + H]^+$ in full scan spectra, and protonated molecules $[M+H]^+$ were detected at *m*/*z* 265.1 for OMT, *m*/*z* 249.2 for MT and 247.3 for I.S. No sodium or other solvent adducts or dimmers were observed.

As shown in Fig. 3, the retention times of OMT, MT and SC (I.S.) were approximately 1.3, 2.8 and 3.4 min, respectively. There were no endogenous plasma components interfering with them. The overall chromatographic run time was finished within 5 min. Ion suppression was investigated and was not detected in the assay.

3.2. Calibration curves

OMT or MT was dissolved in mobile phase and diluted to give a series of standard solutions for the calibration curves of the drug in beagle dog plasma. The linear regression analysis of OMT or MT was constructed by plotting the peak-area ratio of OMT or MT to the internal standard (*y*) versus analyte concentration (ng/ml) in spiked plasma samples (*x*). The calibration curves were constructed in the range 0.005–4.0 μg/ml for OMT and 0.005–2.0 μg/ml for MT. The regression equation of these curves and their correlation coefficients

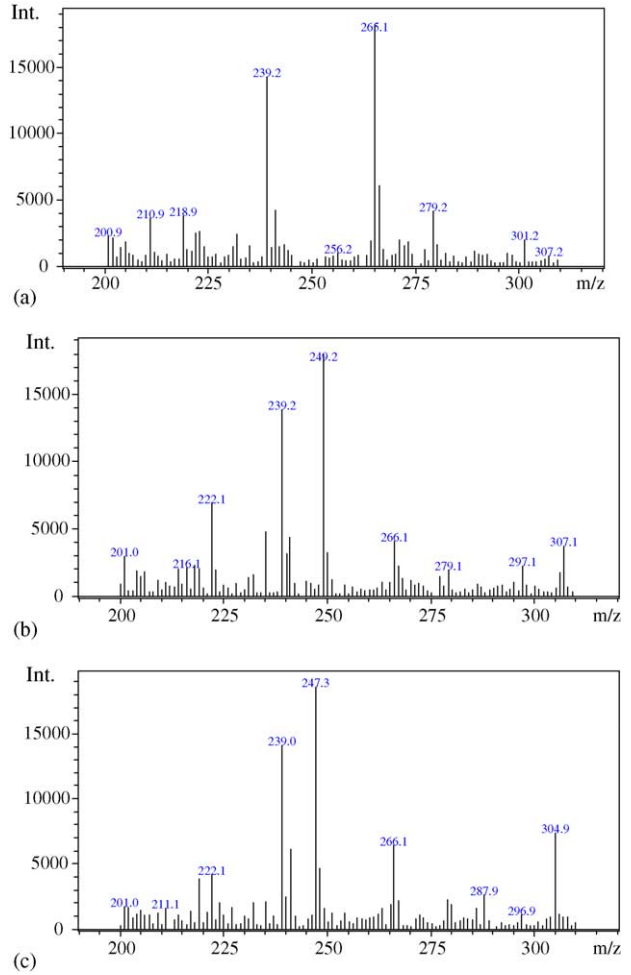

Fig. 2. Mass spectra of (a) oxymatrine, (b) matrine, (c) sophocarpine (internal standard).

(r) were calculated as follows: OMT, $y = 0.0039x - 0.0174$ ($r = 0.9996$); MT, $y = 0.0061x - 0.0046$ ($r = 0.9991$); it showed good liner relationships between the peak areas and the concentrations. Signals three and ten times higher than the peak noise height were regarded as the detection and quantification limit. The detection and quantification limit of these two constituents were 0.6 and 0.3 ng/ml for OMT and MT, 2 and 1 ng/ml for OMT and MT, respectively.

3.3. Recovery

The recovery was assessed by comparing the peak-area ratios (analyte:internal standard) obtained from spiked plasma samples of different analyte concentrations to the peak-area ratios for the samples containing the equivalent amounts of the analyte and internal standard directly dissolved in mobile phase. The recoveries of OMT and MT from beagle dog plasma were shown in Table 1. The mean recoveries of OMT and MT were more than 90% ($n = 5$).

3.4. Accuracy and reproducibility

Analytical accuracy and precision data were shown in Tables 2 and 3. The reproducibility of the method was defined by examining both intra- and inter-day variance. The intra-day and inter-day CV data of OMT and MT assays at low to high concentrations was less than 4% and 5%, respectively. Assay accuracy, assessed by RE, was found to range from −3.6% to 2.0%.

3.5. Stability

All stability results are shown in Table 4. OMT and MT were stable for at least 4 h at room temperature in plasma samples; the mean recoveries from the nominal concentration were 108.0%, 97.9% and 98.9%, respectively, and 95.5%, 99.8% and 99.6%, respectively, at 20, 200 and 1000 ng/ml. OMT and MT were stable in plasma samples when stored at −80 °C for a 6-month period, and following three freeze-thaw cycles. Their mean recoveries from the nominal concentration were more than 92%.

The sensitivity of this method for determining concentrations of OMT and MT in beagle dog plasma is higher than that of the reported methods (the detection limit of OMT and MT ≥ 1 ng/ml) [8–12,14]. The method described above is also outstanding with respect to simplicity in the samples pretreatment and short run time. These results indicated that the assay was simple, accurate, sensitive and reproducible.

3.6. Application

In order to estimate the metabolism of OMT in beagle dog, the plasma concentrations of OMT and MT following oral administration of 300 mg OMT capsules were analyzed. The plasma concentration-time profiles of OMT and MT are shown in Figs. 4 and 5. Estimated pharmacokinetic parameters are shown in Table 5. As shown in Figs. 3 and 4, both OMT (about 223 ng/ml) and MT (about 19 ng/ml) were detected at 0.25 h after administration, indicating that the reduction of OMT rapidly occurred. In this study, the time to reach the maximum plasma concentrations (T_{max}) following oral administration of OMT capsules was about 2.9 h. However, in previous studies [10], The T_{max} of MT following oral administration of MT capsules in dogs was about 1.4 h. These results suggested MT detected in dog blood plasma following oral administration of OMT capsules was from the reduction of OMT, and the delay of T_{max} was likely due to the time required for the reduction to MT in some organs and the gastrointestinal transit to caecum where a large amounts of bacteria exist. The elimination half-life ($t_{1/2\beta}$) and the mean residence time of metabolite MT were longer than those of OMT, indicating that elimination of MT was slower than parent drug in beagle dogs. The AUC_{0-t} value for metabolite MT following oral administration of OMT capsules (7.07 mg h/l) was about 1.2 times as large as that of OMT (5.86 mg h/l), suggesting most of OMT is reduced to MT in beagle dogs,

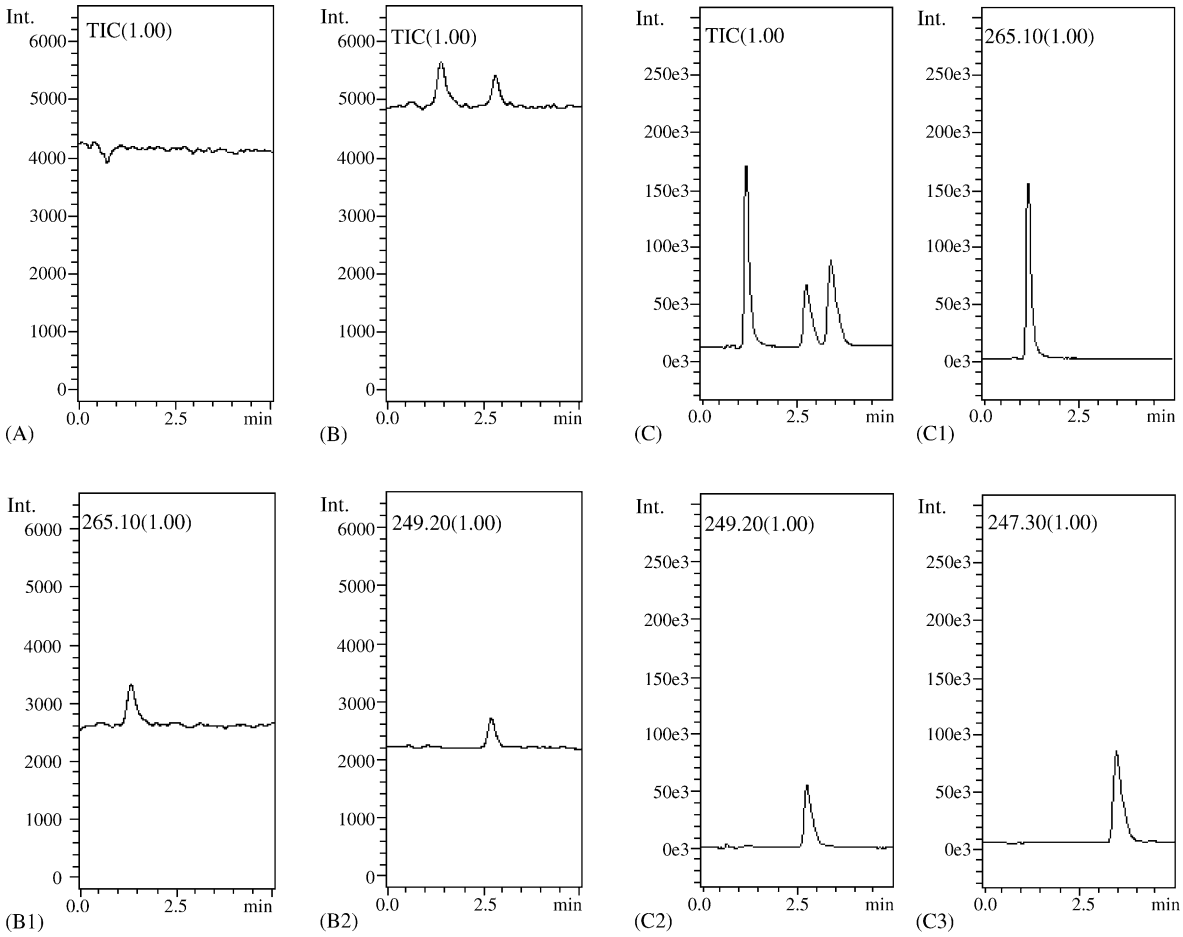

Fig. 3. Chromatograms of oxymatrine and matrine in dog plasma: (A) blank dog plasma; (B) blank plasma spiked with OMT (2 ng/ml) and MT (1 ng/ml), (B1) OMT (extracted ion *m*/*z* 265.1), (B2) MT (extracted ion *m*/*z* 249.2); (C) a dog plasma sample 0.5 h after oral administration of 300 mg OMT capsules. (C1) OMT (extracted ion *m*/*z* 265.1), (C2) MT (extracted ion *m*/*z* 249.2), (C3) internal standard (extracted ion *m*/*z* 247.3, 200 ng/ml).

Table 1
Recoveries of OMT and MT in beagle dog plasma ($n = 5$)

Constituent	Spiked concentration (ng ml^{-1})	Measured concentration (ng ml^{-1})	Recovery (%)	Mean ± SD (%)
OMT	20	18.1 ± 0.7	90.5 ± 3.5	90.7 ± 0.3
	200	181.2 ± 7.3	90.7 ± 3.7	
	1000	910.5 ± 8.4	91.1 ± 0.8	
MT	20	18.2 ± 0.3	91.0 ± 1.4	91.7 ± 2.0
	200	187.8 ± 2.4	93.9 ± 1.2	
	1000	901.7 ± 17.2	90.2 ± 1.7	

Table 2
Intra-day assay variations of OMT and MT in beagle dog plasma

Constituent	Spiked concentration (ng ml^{-1})	Measured concentration(ng ml^{-1})	Intra-day	
			RE (%)	CV (%)
OMT	20	20.2 ± 0.8	1.2	3.9
	200	196.6 ± 50.3	−1.7	2.7
	1000	995.1 ± 23.8	−0.5	2.4
MT	20	20.4 ± 0.3	2.0	1.5
	200	199.1 ± 5.8	−0.5	2.9
	1000	993.2 ± 28.0	−0.7	2.8

Mean ± SD ($n = 5$).

Table 3
Inter-day assay variations of OMT and MT in beagle dog plasma.

Constituent	Spiked concentration (ng ml^{-1})	Measured concentration (ng ml^{-1})	Inter-day	
			RE(%)	CV(%)
OMT	20	20.0 ± 0.9	0	4.6
	200	192.8 ± 4.2	−3.6	2.2
	1000	995.0 ± 33.0	−0.5	3.3
MT	20	19.9 ± 0.6	−0.7	3.0
	200	200.2 ± 7.1	0.1	3.5
	1000	1004.1 ± 32.4	0.4	3.2

Mean ± SD (n = 5).

Table 4
Stability of oxymatrine and matrine in plasma

	Oxymatrine nominal concentration (ng/ml)			Matrine nominal concentration (ng/ml)		
	20	200	1000	20	200	1000
Three freeze-thaw cycles						
Measured concentration (ng/ml)	19.3 ± 2.4	200.2 ± 7.8	987.5 ± 22.9	19.8 ± 2.1	202.8 ± 9.7	978.1 ± 28.6
Accuracy (%)	96.5	100.1	98.8	99.0	101.4	97.8
Room temperature (4 h)						
Measured concentration (ng/ml)	21.6 ± 3.4	195.8 ± 4.7	989.1 ± 17.3	19.1 ± 1.9	199.6 ± 3.8	995.7 ± 22.5
Accuracy (%)	108.0	97.9	98.9	95.5	99.8	99.6
Stored at −80 °C for 6 months						
Measured concentration (ng/ml)	20.3 ± 1.2	184.4 ± 5.1	941.3 ± 27.9	20.7 ± 2.7	191.3 ± 8.9	982.9 ± 15.3
Accuracy (%)	101.5	92.2	94.1	103.5	95.7	98.3

Accuracy (%): measured concentration/nominal concentration × 100%.

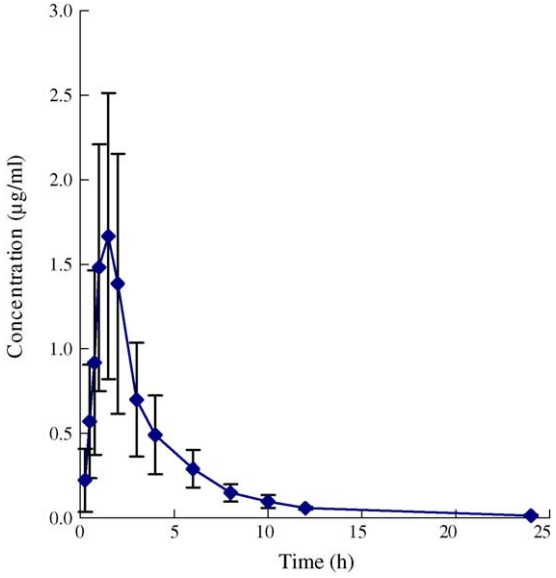

Fig. 4. Plasma concentration–time profiles of oxymatrine following oral administration of 300 mg oxymatrine capsules to beagle dogs. Each point represents the mean ± SD of six experiments.

and its active metabolite MT seem to play an important role in the pharmacological action of orally administered OMT. Furthermore, additional studies are needed to clarify whether the pharmacological action and toxicity of OMT following oral administration will vary for its extensive metabolism.

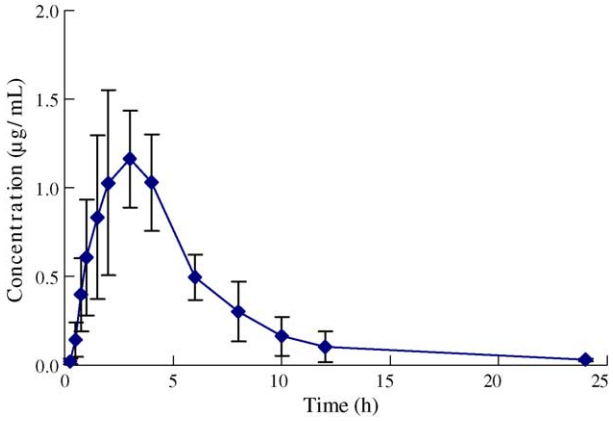

Fig. 5. Plasma concentration–time profiles of metabolite matrine following oral administration of 300 mg oxymatrine capsules to beagle dogs. Each point represents the mean ± SD of six experiments.

Table 5
Mean pharmacokinetic parameters of OMT and its metabolite MT after single oral dose of 300 mg OMT capsules to beagle dogs

Parameter	OMT	MT
C_{max} (μg/ml)	1.81 ± 0.87	1.36 ± 0.23
T_{max} (h)	1.3 ± 0.4	2.9 ± 1.0
$t_{1/2\beta}$ (h)	4.4 ± 0.8	4.8 ± 0.8
MRT (h)	4.7 ± 0.7	6.1 ± 0.9
AUC_{0-t} (mg h/l)	5.86 ± 2.23	7.07 ± 1.79
$AUC_{0-\infty}$ (mg h/l)	5.95 ± 2.24	7.27 ± 1.83

Mean ± SD (n = 6).

In conclusion, this paper describes a simple, rapid, sensitive, accurate and precise procedure for the determination of OMT and MT, suitable for the analysis of large numbers of plasma samples. The assay was validated to meet the requirements of pharmacokinetic studies. The extensive metabolism of OMT following oral dosing would be used as a suitable reference in clinical application.

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