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Short communication

Establishment of a liquid chromatographic/mass spectrometry method for quantification of tetrandrine in rat plasma and its application to pharmacokinetic study

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ABSTRACT

A rapid and sensitive liquid chromatography-tandem mass spectrometric method (LC/MS/MS) for the determination of tetrandrine in rat plasma has been developed, fully validated and successfully applied to pharmacokinetic study in Sprague–Dawley (SD) rats after a single oral administration. Sample preparation involves a liquid–liquid extraction with *n*-hexane–dichlormethane (65:35, containing 1% 2-propanol isopropyl alcohol, v/v). Tetrandrine and brodimoprim (internal standard) were well separated by LC with a Dikma C_{18} column using acetonitrile–methanol–ammonium formate aqueous solution (20 mM) containing 0.3% formic acid (20:30:50, v/v/v) as mobile phase. Detection was performed on a triple quadrupole mass spectrometer in multiple reaction monitoring mode. The ionization was optimized using ESI(+) and selectivity was achieved using MS/MS analysis, *m/z* 623.0 → 381.0 and *m/z* 339.0 → 281.0 for tetrandrine and I.S., respectively. The present method exhibited good linearity over the concentration range of 5–2000 ng/mL for tetrandrine in rat plasma with a lower limit of quantification of 5 ng/mL. The intra- and inter-day precision were 2.0–9.2% and 4.5–9.4%, and the intra- and inter-day accuracy ranged from −7.6 to 10.3% and −6.0 to 5.3%, respectively. No endogenous compounds were found to interfere with the analysis, and tetrandrine was stable during the whole assay period. The method was successfully applied to a pharmacokinetic study after an intragastric administration (i.g.) of tetrandrine to SD rats with a single dose of 50 mg/kg. The results confirm that the assay is suitable for the pharmacokinetic study of tetrandrine.
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1. Introduction

Tetrandrine (Fig. 1) is an important bisbenzylisoquinoline alkaloid isolated from the bulbous root of *Stephania tetrandra* S Moore of the *Menispermaceae* family [1,2]. This plant is popularly known in Chinese as "hanfangji" and is one Chinese herbal medicine traditionally used for the treatment of edema, rheumatic disorders, and inflammatory diseases [3]. As a major active component, tetrandrine possesses a remarkable pharmacological profile [4–6]. In the past several decades, a number of studies have demonstrated tetrandrine being effective in the treatment of arrhythmia, hypertension, silicosis, and so on [7–9]. Tetrandrine has also been reported to possess bioactivities of anti-inflammatory, antiallergic, antioxidant, antifibrogenetic, as well as immunomodulation and inhibition of platelet aggregation [9,10]. Besides its pharmacological effects, tetrandrine was also reported for its liver toxicity in dogs when administered in high dose for a relatively long period [11]. Although plenty of work on the pharmacology has been reported, pharmacokinetic and toxicokinetic properties of tetrandrine have seldom been studied in recent years, which is essential to its further research for clinical use.

As for analysis, a variety of analytical methods have been reported in quantitative determination of tetradrine in biological matrix, including TLC [12], CE [13,14], HPLC [15,16], HPTLC [17], ion pair-HPLC [18], micella-fluorometry [19] and so on. Most of these methods required laborious sample pretreatment and long analysis time, thus was not convenient for large numbers of samples in pharmacokinetic and toxicokinetic research. For more rapid, convenient and sensitive LC/MS/MS method, it was only developed for quantitative determination of tetrandrine in plants or medicinal preparations [20,21]. Till now, no LC/MS/MS method for quantitative determination of tetrandrine in biological matrix has been developed.

The purpose of this study was to develop and validate a more rapid, selective, efficient and sensitive LC/MS/MS method for the quantitative determination of tetrandrine in rat plasma. The

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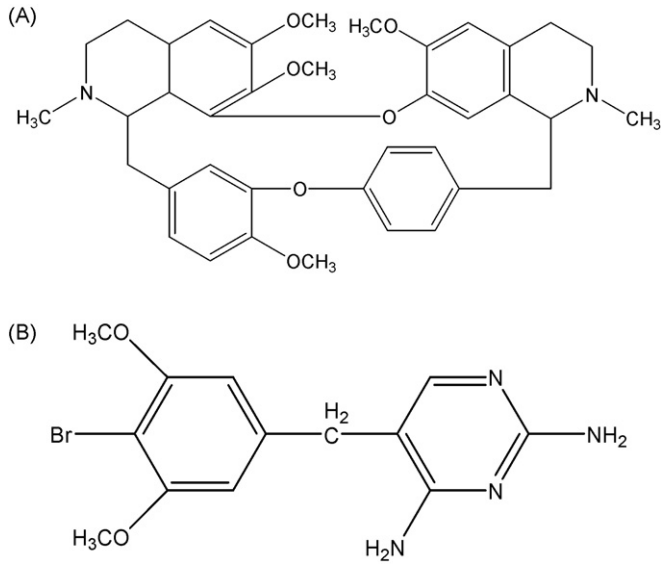

Fig. 1. Chemical Structure of (A) tetrandrine ($C_{38}H_{42}O_6N_2$, M_w = 622.73 Da) and (B) brodimoprim (I.S.) ($C_{13}H_{15}BrN_4O_2$, M_w = 339.19 Da).

present method was successfully applied to pharmacokinetic study after a single oral dose of 50 mg/kg tetrandrine in SD rats.

2. Experimental

2.1. Chemicals and regents

Tetrandrine standard was kindly provided by Prof. Lou Jianshi (Tianjin Medical University, Tianjin, China) with purity better than 99.5%. Tetradrine raw material was purchased from Xi'an Shanchuan Biotechology Co.Ltd. (Xi'an, China) with purity better than 99.2%. Brodimoprim (Fig. 1) (used as internal standard, I.S.) was supplied by National Key Laboratory of Pharmacokinetics and Pharmacodynamics, Tianjin Institute of Pharmaceutical Research (Tianjin, China) with purity better than 99.0%. HPLC-grade acetonitrile and methanol were obtained from Tianjin Concord Tech Reagent Co. Ltd. (Tianjin, China). Deionized water was prepared using a SYZ550 quartz pure water distiller (Tianjin Xinzhou Tech Co. Ltd., Tianjin, China) and used throughout the study. Ammonium formate and other chemicals and solvents used were all of analytical grade.

2.2. LC/MS/MS conditions and quantifications

The thermo electron LC system (San Jose, CA, USA) consisted of a Surveyor quaternary narrowbore LC pump, a Surveyor autosampler, as well as a tempered tray and a column oven. A thermo electron TSQ quantum triple quadrupole tandem mass spectrometer (San Jose, CA, USA), equipped with an electrospray ionization (ESI) source, was used for the mass analysis and detection. Chromatography was performed on a Dikma Diamonsil-C_{18} analytical column (4.6 mm × 150 mm i.d., particle size 5 μm, Dikma Technologies, Beijing, China) at 35 °C. The mobile phase was composed of acetonitrile–methanol–ammonium formate aqueous solution (20 mM) containing 0.3% formic acid (20:30:50, v/v/v) at a flow rate of 0.3 mL/min. Under these conditions, tetrandrine and I.S. were eluted at approximately 4.79 and 6.90 min, respectively, with no significant interference observed at these two retention times in the mass chromatograms of blank rat plasma.

All measurements were carried out using the positive ion mode. Mass spectrometer conditions were optimized to obtain maximum sensitivity. The ion spray voltage was set at 4000 V, and the capillary temperature was set at 280 °C with ultra-high-purity nitrogen as the curtain gas (40.0 L/min) and collision gas (10.0 L/min). Multiple reaction monitoring (MRM) was used for drug quantification. The mass peak widths were 0.7 Da for both Q1 and Q3. $[M+H]^+$ at *m/z* 623 was for tetrandrine, and $[M+H]^+$ at *m/z* 339 for the I.S. Peak area ratios of tetrandrine to I.S. was calculated, and the calibration curve was established with the ratios as *Y*-axis, while the corresponding nominal concentrations of tetrandrine as *X*-axis.

2.3. Stock solutions and quality control samples

The primary stock solutions of tetrandrine and brodimoprim were prepared at 1.0 mg/mL in methanol, respectively. The stock solutions were stored at −20 °C, and were stable for at least 6 months. A set of seven standard working solutions ranging from 5 to 2000 ng/mL were prepared by further dilution of the standard stock solutions with methanol. I.S. working solution (100 ng/mL) was prepared by diluting I.S. stock solution with methanol. All the stock solutions were stored at −20 °C, and were stable for at least 6 months. The calibration standards were prepared by spiking blank drug-free rat plasma with appropriate amounts of working solutions. Quality control (QC) samples were prepared in a similar manner at three concentrations (10, 100 and 1000 ng/mL).

2.4. Preparation for plasma samples

The extraction regent was composed of *n*-hexane–dichloromethane (65:35, containing 1% 2-propanol isopropyl alcohol, v/v). A 100 μL aliquot of rat plasma, spiked with I.S. working solution (100 μL), 1M sodium hydroxide (NaOH) (100 μL), methanol (100 μL) was vortex mixed for 30 s and extracted with the extraction regent mentioned before (3 mL) for another 1.5 min vortex mixing. After centrifuging at 4000 rpm for 10 min, 2 mL of the upper organic phase was transferred into a 10 mL glass tube and evaporated to dryness under a steam of nitrogen. The residues were then reconstituted with 100 μL mobile phase, vortex-mixed briefly, and a 10 μL aliquot was injected into the LC/MS/MS system for analysis.

2.5. Method validation

Calibration standards were prepared and analyzed in triplicate in three independent runs. The calibration curve was constructed using tetrandrine/I.S. peak area ratios vs tetrandrine concentration consisting of seven concentration levels (5, 10, 50, 100, 500, 1000 and 2000 ng/mL of tetrandrine in rat plasma). 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. To determine assay specificity, six different lots of blank plasma samples from SD rats were analyzed to investigate the potential interferences at the LC peak region for tetrandrine and I.S., each in duplicate.

Precision was calculated as the relative standard deviation (R.S.D.), and accuracy was assessed as the percentage bias from the nominal concentration (% bias). QC samples (at three different plasma concentrations of tetrandrine) 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 absolute recovery of plasma sample was determined by comparing the analytical results of extracted plasma samples at three QC concentrations (10,

100 and 1000 ng/mL) with pure standards without extraction at the same concentration.

The sample solution stability testing was determined in the following four ways: (1) For long-term storage stability, the QC samples were prepared and stored at −20 °C for 4 months. (2) For freeze and thaw stability testing, the QC samples were determined after three freeze (−20 °C) and thaw cycles. (3) For the short-term storage stability, QC samples were extracted and placed in the autosampler at 4 °C for a period of 24 h, and then injected for analysis. (4) The stability of tetradrine plasma sample at room temperature was also determined by placing the spiked plasma samples at room temperature for 24 h, the samples were then extracted and injected for analysis. All samples were analyzed together with calibration samples that were freshly prepared. The measured concentrations were then compared to those of the same QC samples that had been analyzed immediately after processing. The percent deviation in concentration was used as an indicator of stability. The analyte was considered stable when the percent deviation was within 15%.

The matrix effect was evaluated by assessing the 'absolute' matrix effect, i.e. the potential ion suppression/enhancement due to the matrix components in plasma. Blank rat plasma was extracted by liquid–liquid extraction mentioned above and then spiked with tetrandrine (at three QC concentrations) or I.S., separately. The corresponding areas were then compared to those of the standard solutions at equivalent concentrations. The result of matrix effect was calculated as $100 \times (A_{st} - A_{extr})/A_{st}$, where A_{extr} is the peak area of tetrandrine or I.S. from the post-extraction spiked sample (extracted blank plasma sample spiking with tetrandrine or I.S.), while A_{st} is the peak area of tetrandrine and I.S. from direct injection of the standard solution.

2.6. Pharmacokinetic study

The assay method was applied to a clinical pharmacokinetic study in SD rats. SD rats (Certificate No. SCXK 2005-0001), male and female, weighting 190–230 g were purchased from Institute of Radiation Medicine, Chinese Academy of Medical Sciences, Tianjin, China. After an overnight fast (12 h) with access tap water, the rats were orally given a single dose of tetrandrine solution (50 mg/kg) formulated in 1% CMC-Na (sodium carboxymethylcellulose) solution (m/v). The rats were further fasted for 2 h after i.g. administration with free access to water. About 0.5 mL of blood samples were collected from epicanthic vein into heparinized tubes before (0 h) and at 0.25, 0.5, 1, 2, 3, 4, 6, 8, 12, 24, 48 and 72 h after dosing. The blood samples were centrifuged at 12,000 rpm for 10 min at room temperature, then the separated plasma was stored at −20 °C and analyzed within 2 months. Main pharmacokinetic parameters were determined for each rat by DAS (Drug and Statistics) software (edited by Chinese Mathematical Pharmacology Society).

3. Results and discussion

3.1. LC/MS/MS method

The full-scan positive ion turboionspray product ion mass spectra showed that the precursor ions of tetrandrine and I.S. were the protonated molecules, $[M+H]^+$, of *m/z* 623.0 and *m/z* 339.0, respectively. After collision-induced dissociation, the most abundant ion in the product ion mass spectrum was at *m/z* 623.0 → *m/z* 381.0 for tetrandrine at a collision energy of 44.0 eV, and *m/z* 339.0 → *m/z* 281.0 for I.S. at a collision energy of 35.0 eV for the identification and quantification in SRM mode. Fig. 2 showed the full-scan product ion MS/MS spectra of tetrandrine and I.S. The most suitable collision energy was determined by observing the response obtained vs selectivity response for the fragment ion for the analyte. The best collision energies set were 44.0 eV for tetrandrine and 35.0 eV for I.S. obtaining fragments *m/z* 381.0 and *m/z* 281.0 from each protonated compound.

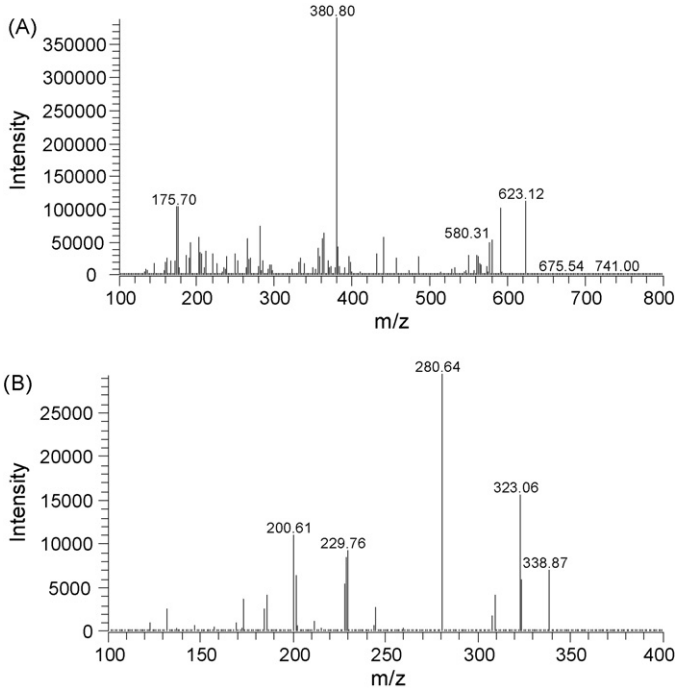

Fig. 2. Product ion mass spectra of tetrandrine (A) and I.S. (B).

The mobile phase was composed of acetonitrile–methanol–ammonium formate aqueous solution (20 mM) containing 0.3% formic acid (20:30:50, v/v/v), the addition of formic acid was to get better peak shape and to increase sensitivity. In the previous reports [8,22,23], ethyl acetate, dichloromethane and chloroform were used for the liquid–liquid extraction of tetrandrine from plasma sample, and usually the plasma samples were extracted twice to get higher recovery. In the present study, we found that compared with the above reagents, the combination of *n*-hexane and dichloromethane could not only increase recovery, but also reduce matrix effect, thus *n*-hexane-dichloromethane (65:35, containing 1% 2-propanol isopropyl alcohol, v/v) was chosen as extraction reagent. Since tetrandrine is a bisbenzylisoquinoline alkaloid, making the matrix being alkaline before extraction is an important way to get higher sensitivity. Thus 1M NaOH was added to plasma sample before extraction, as a result the sensitivity of tetrandrine has been improved significantly.

3.2. Method validation

3.2.1. Linearity and LLOQ

Linearity for determining tetrandrine in rat plasma are summarized in Table 1. The calibration curves ($n = 3$) showed good linearity over the concentration range of 5–2000 ng/mL ($r^2 > 0.99$). The best-fit line of the calibration curves for tetrandrine was obtained by using a weighing factor of $1/x^2$. Using the present method, the LLOQ was established at a concentration of 5 ng/mL for tetrandrine (R.S.D. < 20%), which is sufficient to support its pharmacokinetic and toxicokinetic studies.

Table 1
Linearity for determining tetrandrine in rat plasma

Nominal concentration (ng/mL)	Run-1			Run-2			Run-3		
	Concentration found (mean ± S.D.) (ng/mL)	Precision[a] (%)	Accuracy[b] (%)	Concentration found (mean ± S.D.) (ng/mL)	Precision[a] (%)	Accuracy[b] (%)	Concentration found (mean ± S.D.) (ng/mL)	Precision[a] (%)	Accuracy[b] (%)
5	5.2 ± 0.3	3.3	4.5	5.2 ± 0.2	4.6	3.3	5.0 ± 0.2	4.8	0.8
10	9.1 ± 0.2	2.1	−9.3	9.3 ± 0.8	9.1	−6.8	10.1 ± 1.0	10.5	0.8
50	52.4 ± 1.4	2.6	4.8	51.8 ± 1.6	3.2	3.6	53.1 ± 1.3	2.4	6.2
100	93.3 ± 4.8	5.1	−6.7	95.0 ± 4.8	5.0	−5.0	99.2 ± 5.7	5.7	−0.8
500	473.1 ± 37.0	7.8	−5.4	477.3 ± 7.2	1.5	−4.5	462.4 ± 8.7	1.9	−7.5
1000	1045.8 ± 41.5	4.0	4.6	1042.0 ± 11.7	1.1	4.2	951.7 ± 35.8	3.8	−4.8
2000	2148.6 ± 105.3	4.9	7.4	2105.1 ± 20.2	1.0	5.2	2141.1 ± 79.9	3.7	7.0

[a] Expressed as R.S.D.%: (S.D./mean) × 100.
[b] Calculated as (% bias): (the difference between mean concentration found with nominal concentration/nominal concentration) × 100.

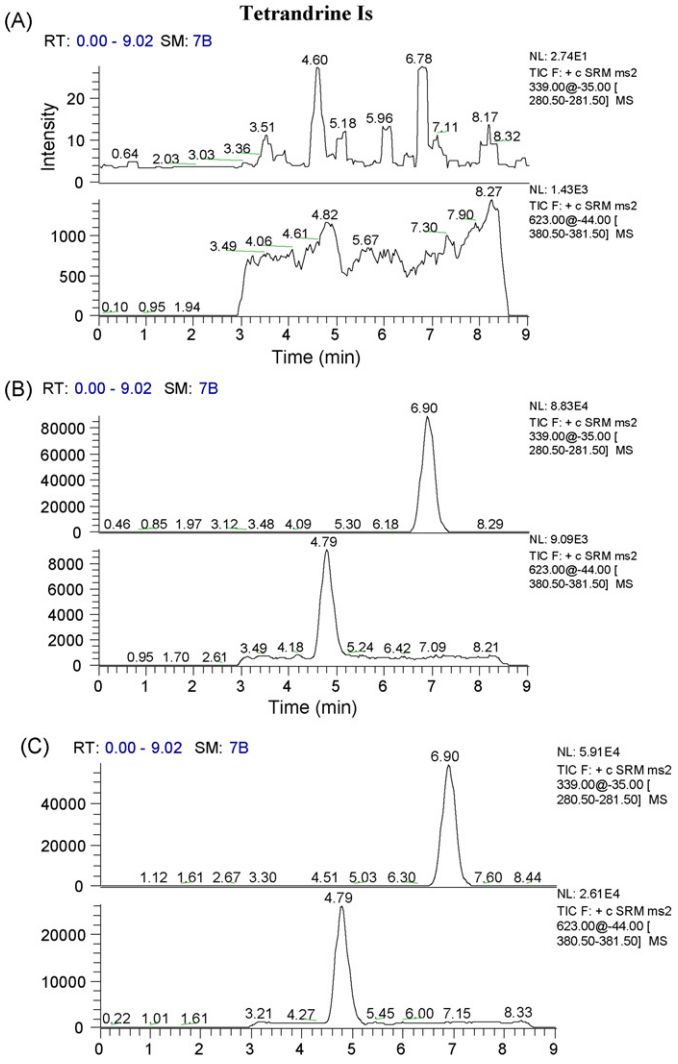

Fig. 3. Typical SRM chromatograms of: (A) blank rat plasma, (B) plasma spiked with tetrandrine (LLOQ, 5 ng/mL) and I.S. (100 ng/mL), and (C) rat plasma obtained 15 min after a single oral administration of 50 mg/kg tetrandrine spiked with I.S.

3.2.2. *Specificity and sensitivity*

Typical chromatograms of blank plasma, spiked plasma as well as plasma sample in clinical study are presented in Fig. 3, showing the retention times of 4.79 and 6.90 min for tetrandrine and the I.S., respectively. As shown in the figure, no endogenous peaks were observed in the chromatogram of blank plasma.

3.2.3. *Recovery*

The liquid–liquid extraction method used in this study yielded a mean absolute recovery of 81.4 ± 1.1% for tetrandrine at the three concentration levels. The extraction recovery was found to be consistent over its calibration range, suggesting that the extraction efficiency of the current method is independent of the concentrations in the ranges studied. The absolute recovery of I.S. was 77.7% at the concentration used in this study (100 ng/mL) and was found to be steady throughout the study.

3.2.4. *Accuracy and precision*

The intra- and inter-day precision and accuracy at low, medium and high concentration levels of tetrandrine in plasma are summarized in Table 2. The precision, presented as R.S.D., ranged from

Table 2
Accuracy and precision for determining tetrandrine in rat plasma

Nominal concentration (ng/mL)	Intra-day			Inter-day		
	Concentration found (mean ± S.D.) (ng/mL)	Precision[a] (%)	Accuracy[b] (%)	Concentration found (mean ± S.D.) (ng/mL)	Precision[a] (%)	Accuracy[b] (%)
5	5.2 ± 0.3	5.5	3.7	5.1 ± 0.2	4.5	2.7
10	11.0 ± 0.6	5.1	10.3	10.5 ± 1.0	9.4	5.3
100	92.4 ± 1.9	2.0	−7.6	94.0 ± 7.2	7.6	−6.0
1000	1029.8 ± 94.6	9.2	3.0	1005.3 ± 84.0	8.4	0.5

[a] Expressed as R.S.D.%: (S.D./mean) × 100.
[b] Calculated as (% bias): (the difference between mean concentration found with nominal concentration/nominal concentration) × 100.

2.0 to 9.2% and 4.5 to 9.4% for intra- and inter-day determinations, respectively. The accuracy, presented as percentage bias against the nominal concentration, ranged from −7.6 to 10.3% and −6.0 to 5.3% for intra- and inter-day determinations, respectively. The above result showed that the present method has a satisfactory accuracy, precision and reproducibility.

3.2.5. Stability

Results of the stability tests for tetrandrine are summarized in Table 3. Tetrandrine was found to be stable in the plasma samples through three freeze–thaw cycles, and after being stored at −20 °C for 4 months or being stored at room temperature for 24 h. No significant degradation was observed in tetrandrine concentrations when extracted plasma samples were kept at 4 °C in the autosampler for up to 24 h. The percentage deviations were found to range from −8.7 to 2.6%, −6.8 to 8.2%, −1.2 to 11.3%, −7.2 to 8.1%, respectively after three freeze–thaw cycles, stored at 4 °C in the autosampler and at room temperature for 24 h, as well as stored at −20 °C for 4 months.

3.2.6. Matrix effect

For tetrandrine at three QC concentrations in rat plasma, the degree of ion suppression ranged from −9.7% to 2.2%, suggesting a minimal matrix effect on the ionization of tetrandrine under these conditions. The matrix effect was consistent in all plasma samples tested.

3.3. Pharmacokinetic study

The LC/MS/MS method developed in this study yielded satisfactory results for the determination of tetrandrine in rat plasma and has been successfully applied to the pharmacokinetic study of tetrandrine after a single oral administration (50 mg/kg) to six SD rats. The profiles of the plasma tetrandrine concentration vs time are shown in Fig. 4. Plasma concentrations of tetrandrine were within the calibration curve range and remained above the LLOQ (5 ng/mL) for the entire study. There was no statistical difference in plasma concentrations of tetrandrine between male and female rats at the same time points, proving that there was no sexual difference in pharmacokinetics of tetrandrine in SD rats. The main pharmacokinetic parameters were presented in mean ± S.D. as follows: $AUC_{(0-72)}$ (μg h/L) was 6279.2 ±2411.5, ($AUC_{(0\rightarrow\infty)}$) (μg h/L) was 7002.7 ± 2528.0, C_{max} (μm/L) was 237.1 ± 95.9, T_{max} (h) was 6.0 ± 1.8, $T_{1/2}$ (h) was 20.6 ± 3.7, K_e (1/h) was 0.034 ± 0.006. The application to pharmacokinetic study showed that the established bioanalytical method was suitable for pharmacokinetic study [24].

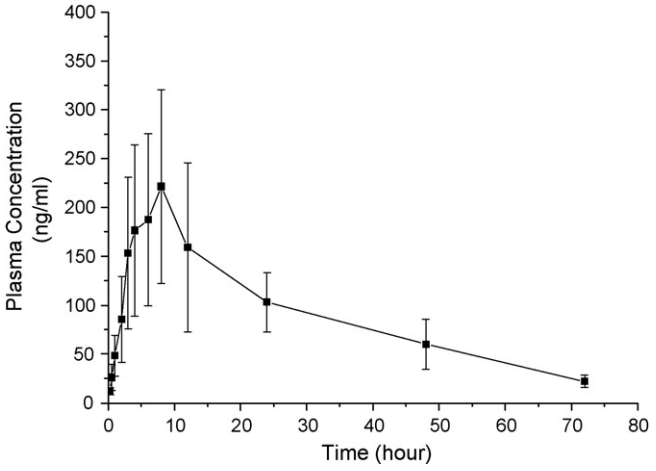

Fig. 4. Mean (±S.D.) plasma concentration–time profiles of tetrandrine after single oral administration of 50 mg/kg to six SD rats.

Table 3
Stability of tetrandrine in rat plasma under different storage conditions

Storage condition	Concentration added (ng/mL)	Concentration found (mean ± S.D., ng/mL)	Percent deviation[a] (100%)
Three freeze–thaw cycles	10	10.3 ± 0.8	2.6
	100	91.3 ± 5.8	−8.7
	1000	964.6 ± 64.1	−3.5
At 4 °C in the autosampler, 24 h	10	10.8 ± 0.3	8.2
	100	93.2 ± 4.1	−6.8
	1000	953.2 ± 92.2	−4.7
At room temperature, 24 h	10	10.3 ± 0.4	2.8
	100	98.8 ± 4.4	−1.2
	1000	1113.0 ± 32.1	11.3
At −20 °C, 4 months	10	10.2 ± 0.2	2.2
	100	92.8 ± 1.3	−7.2
	1000	1081.1 ± 48.8	8.1

[a] Percent deviation (%) = (the difference between mean concentration found with concentration added/concentration added) × 100; $n = 5$.

4. Conclusion

In this study, LC/MS/MS method has been developed for the quantitative determination of tetrandrine in biological matrix for the first time. A liquid–liquid extraction procedure was used to extract tetrandrine and I.S. from rat plasma, followed by chromatography with tandem mass spectrometry detection. The method yielded good sensitivity, precision and accuracy, as well as recovery for tetrandrine. Compared to the other clinically validated analytical methods for tetrandrine reported in previous literature, the present LC/MS/MS method is much simpler and faster, with higher sensitivity and efficiency, which facilitates its application in further pharmacokinetic and toxicokinetic studies of tetrandrine.

Acknowledgments

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