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Ultra-performance liquid chromatography–tandem mass spectrometric method for the determination of strychnine and brucine in mice plasma

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ABSTRACT

A selective, simple and efficient method-ultra-performance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS) was developed for determination of two toxic alkaloids, namely strychnine and brucine in mice plasma. The UPLC separation was carried out using a 1.7 μm BEH C_{18} column (50 mm × 2.1 mm) with a mobile phase consisting of methanol:0.1% formic acid (25:75, v/v), hence providing high efficiency, high resolution and excellent peak shape for the analytes and internal standard. The method was validated over the range of 2.48–496.4 ng/ml for strychnine and 2.64–528 ng/ml for brucine, respectively. Intra- and inter-day accuracy ranged from 95.0% to 107.9% for strychnine, 93.4% to 103.3% for brucine, and the precisions were within 13.8%. The extraction recoveries of both the two alkaloids exceed 81.9%. With a simple and minor sample preparation procedure and short run-time (<3 min), the proposed method was applicable for the pharmacokinetic and toxicological analysis of strychnine and brucine *in vivo*.

1. Introduction

Semen Strychni, the seed of *Strychnos nux-vomica L.* (Loganiaceae), is an important medicinal plant in Asia. It has been effectively used in Chinese folk medicine to alleviate inflammation, joint pains, allergic symptoms and treat nervous diseases [1]. However, for extremely toxic the seeds are forbidden to use directly and must be processed. Phytochemical analysis has revealed that alkaloids are the main bioactive ingredients in this species. Strychnine (Str) and brucine (Bru) (Fig. 1), the most abundant alkaloids in Semen Strychni, have important effects on the pharmacological and toxic properties [1]. On the one side, Str and Bru possess analgesic, anti-inflammatory and anti-tumor effects [2,3]; on the other side, through inhibiting inhibitory neurotransmitter, the toxic alkaloids can result in motor disturbance, increase muscle tone, hyperactivity of sensory, while high dose can induce convulsions of the central nervous system and finally death through respiratory or spinal paralysis or by cardiac arrest [3]. Several cases of Str poisoning have been reported [4–6]. The 50% lethal dose (LD_{50}) of Str in mice is reported to be 3.27 mg/kg (i.g.) and 1.53 mg/kg (i.p.), LD_{50} of Bru for mice is 233 mg/kg (i.g.) and 69 mg/kg (i.p.) [7]. In humans, the reported LD of orally ingested Str ranges from 50 to 100 mg/kg [8]. Thus, the determination of the two alkaloids is extremely important in toxicological and forensic analysis.

In recent years, many methods have been proposed for determination of Str and Bru in biological matrix, for example, CE [9], GC–MS [10–12], HPLC-UV [5,6,13], HPLC–MS [14] and LC–MS/MS [15,16]. However, some shortcomings exist in these methods such as long analytical time, complex procedures, low selectivity and sensitivity. The published LC–MS/MS method [15] is developed for screening many kinds of toxical alkaloids but lacked of applications to practical samples. In this work, a fast and new UPLC–MS/MS method is developed for simultaneous determination of Str and Bru in mice plasma.

Recently, ultra-performance LC (UPLC) has introduced and quickly adopted in quantitative analysis of biological matrix. The van Deemter equation indicates that, as the particle size decreases to less than 2.5 μm, there is a significant improvement in efficiency that will not reduce with increased LC flow rates. Compared with conventional HPLC columns, UPLC, by utilizing 1.7 μm particle, greatly increased the separation throughput and efficiency, resulting in LC peaks as narrow as or less than 2 s [17]. By using multiple reaction monitoring (MRM) as the MS detection, UPLC–MS/MS method can offer a more sensitive and selective detection, thus it is suitable for the research of pharmacokinetics and metabolic kinetics under toxical dose for strychnine and brucine.

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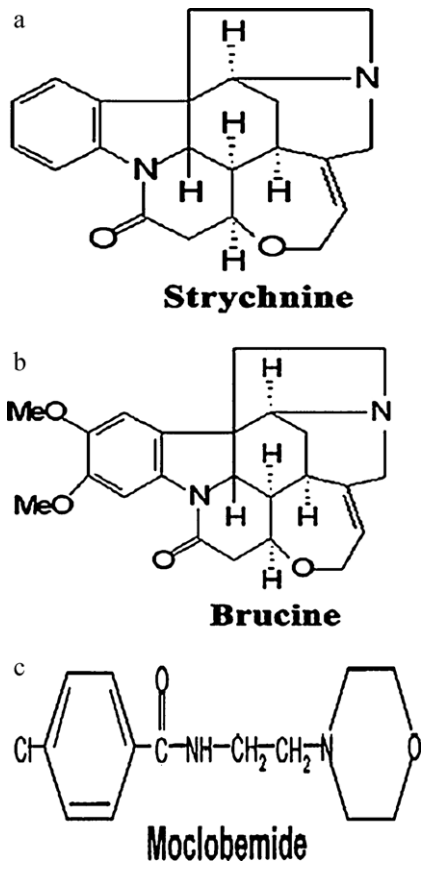

Fig. 1. Chemical structures of Str, Bru and moclobemide (IS).

2. Experimental

2.1. Chemical reagents and animals

Reference standards of Str, Bru and moclobemide (internal standard, IS) were obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China); HPLC grade methanol was purchased from Merck (Darmstadt, Germany); HPLC grade formic acid and tert-butyl methyl ether were purchased from Tedia (Fairfield, OH, USA); other chemicals were all of analytical grade; water was purified by redistillation and filtered through 0.22 μm membrane filter before using. Processed Semen Strychni was kindly provided by Baijia Pharmaceutical Company (Changsha, China). Male Kunming mice (Certificate No. SCXK-Xiang-2009-0004) were provided by the Slac experimental animal corporation (Shanghai, China). The study was approved by the Animal Ethics Committee of the Central South University (Approval No. 2009-0219).

2.2. Standard and working solutions

Individual standard stock solutions of Str (496.4 μg/ml), Bru (105.6 μg/ml) and IS (220.8 μg/ml) were prepared by accurately weighting the required amounts into separate volumetric flasks and dissolving in methanol. Further dilutions were made from these stocks to obtain a series of standard working solutions: Str (4.96, 0.496, 0.0496 μg/ml) and Bru (5.280, 0.528, 0.0528 μg/ml). IS stock solution was further diluted with methanol to prepare the working solution containing 44.0 ng/ml of moclobemide. The stock solutions and working solutions were all stored at 4 °C until use.

2.3. Calibration standards and quality control samples

The calibration standards were prepared by spiking blank plasma (1.0 ml) with appropriate amounts of above working solutions to yield final concentrations of 2.48–496.4 ng/ml for Str and 2.64–528 ng/ml for Bru. Quality control (QC) samples at low, middle and high concentrations of Str (4.96, 37.23 and 397.1 ng/ml), Bru (5.28, 39.6 and 422.4 ng/ml) were prepared with mice plasma for the determination of inter-day, intra-day accuracy and precision, room temperature and freeze–thaw stability.

2.4. Sample preparation

Plasma samples were kept in plastic vials at −70 °C until analysis. 100 μl of plasma sample, 20 μl of NaOH (0.1 mol/l) and 50 μl of IS solution (44.0 ng/ml) were added to a vitric tubu. The mixture was vortexed for 30 s, then extracted with 2 ml tert-butyl methyl ether by thoroughly vortexed for 2 min, followed by centrifugation at 3000 rpm for 5 min. The upper organic layer was transferred to another vitric tube and dried under a mild stream of N_2 at 38 °C. The residue was reconstituted in 100 μl mobile phase. A 2 μl aliquot of each supernatant was injected into the UPLC–MS/MS system for analysis.

2.5. Liquid chromatography

Liquid chromatography was performed on ACQUITY UPLC system (Waters, Milford, MA, USA) with autosampler and column oven. Chromatographic separations were performed on a Waters Acquity UPLC™ BEH C_{18} column (50 mm × 2.1 mm, i.d., 1.7 μm particle size). The column temperature was maintained at 40 °C. The mobile phase consisted of methanol:0.1% formic acid (25:75, v/v). The flow rate was 0.25 ml/min, the injection volume was 2 μl, and the duration of the run was 3 min.

2.6. Mass spectrometry

The MS instrument consisted of a Waters Micromass Quattro Micro™ triple-quadrupole system (Manchester, UK). Ionization was performed in the ESI positive mode. Quantification was performed using multiple reaction monitoring (MRM) mode. The transitions of m/z 335 → 184, m/z 395 → 324 and m/z 269 → 182 were selected for the quantification of Str, Bru and IS, respectively. The MS spectra and MS/MS spectra for Str, Bru and IS were shown in Fig. 2. The optimized ionization conditions were as follows: capillary voltage 4.0 kV, cone voltage 45 kV, source temperature 120 °C and desolvation temperature 400 °C. Nitrogen was used as desolvation and cone gas with the flow rate at 750 and 50 l/h, respectively. Argon was used as the collision gas at a flow rate of 0.15 ml/min. The collision energies for Str, Bru and IS were 35 eV, 32 eV and 18 eV, respectively. The dwell time was 0.05 s. Data acquisition was carried out by MassLynx 4.1 software.

2.7. Method validation

For quantitative bio-analytical procedures, the following essential parameters should be evaluated: selectivity, sensitivity, accuracy, precision, recovery and stability [18]. Matrix effects were evaluated as well.

Selectivity was assessed by comparing chromatograms of blank plasma obtained from mice without dosing with those of blank plasma spiked with Str, Bru, IS and plasma samples after administration of Semen Strychni. To evaluate linearity, calibration standards in plasma at seven concentration levels ranged from 2.48 to 496.4 ng/ml for Str and 2.64 to 528 ng/ml for Bru were prepared. Calibration curves in plasma were generated by plotting the peak

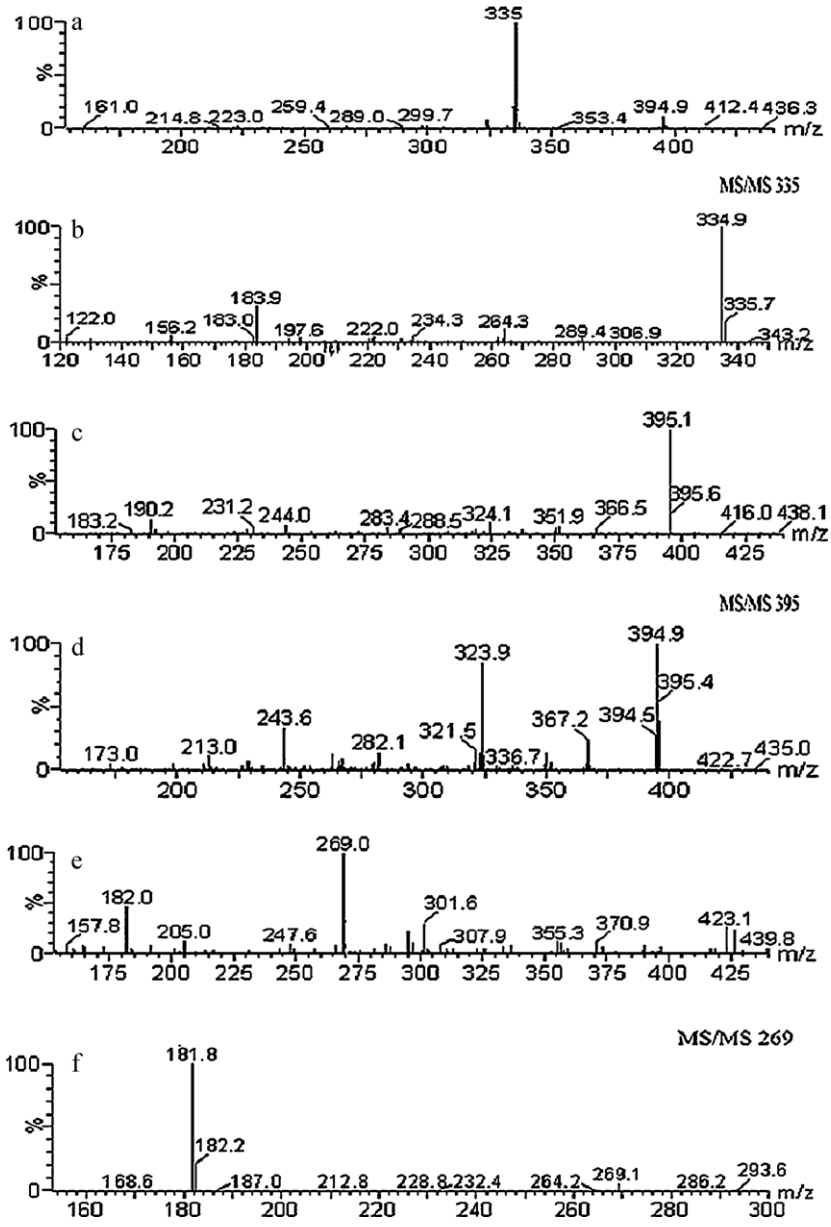

Fig. 2. The MS spectra for (a) Str, (c) Bru and (e) IS and MS/MS spectra for (b) Str, (d) Bru and (f) IS.

area ratio (y) of Str and Bru to IS versus nominal concentrations (x) of the two analytes by $1/x$ weighted least square linear regression. The lower limit of quantification (LLOQ) was defined as the lowest concentration of the calibration standards for which an acceptable accuracy within ±20% and a precision below 20% were obtained [19].

Intra-day accuracy and precision was evaluated by analysis of the QC samples for Str and Bru with five determinations per concentration in the same day. The inter-day accuracy and precision was measured on 3 consecutive days. Precision was expressed as relative standard deviation (RSD).

Three sets (A–C) of solutions were prepared for the evaluation of recovery and ionization suppression or enhancement. Set A was prepared to evaluate the MS/MS response of working standard solutions injected in mobile phase. Set B used blank mice plasma after extraction and dried, then the residue was reconstituted in 100 μl set A. Set C was composed of plasma samples spiked with working solutions after liquid–liquid extraction procedure. Five replicates of each set were used for determination of extraction recovery and matrix effect at three concentrations (Str: 4.96, 37.23, 397.1 ng/ml and Bru: 5.28, 39.6, 422.4 ng/ml). The matrix effect (ME) and the effect of extraction recovery (ER) were evaluated by comparing results from analysis of three sets of samples as follows. The recovery and matrix effect of IS were determined in a similar way.

$$\mathrm{ME}(\%) = \frac{B}{A} \times 100$$

A—mean peak area of Set A and B—mean peak area of Set B;

$$\mathrm{ER}(\%) = \frac{C}{A} \times 100$$

C—mean peak area of Set C.

The stability of QC plasma samples kept at room temperature for 12 h was evaluated. For the freeze–thaw stability, QC plasma samples were stored at −20 °C and thawed at room temperature at three cycles. All stability evaluations were based on back-calculated concentrations.

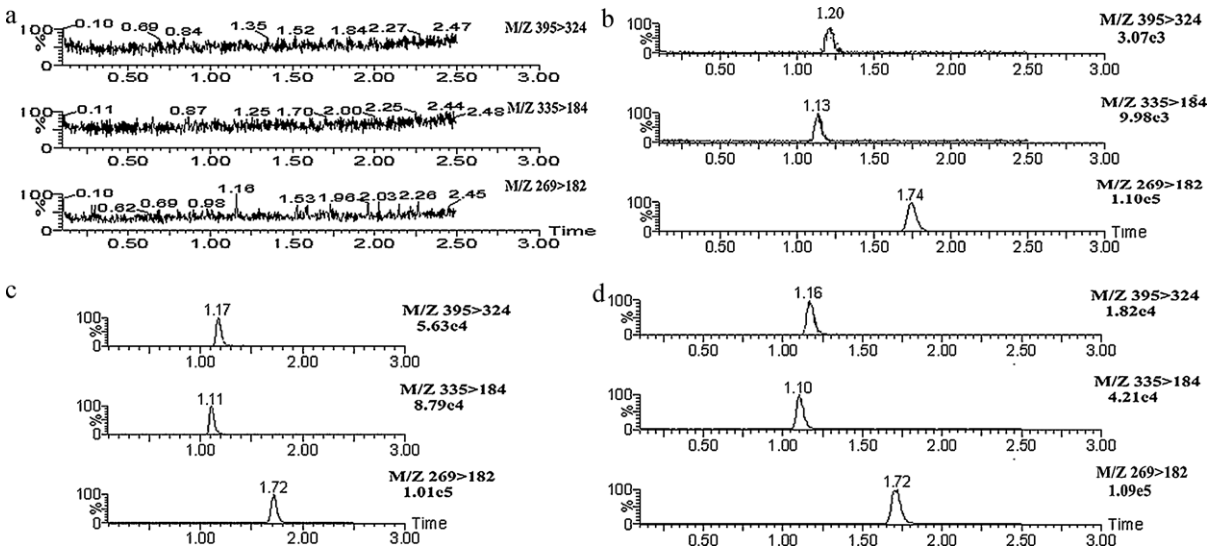

Fig. 3. (a) Representative chromatograms of extracted blank mice plasma sample. (b) Chromatograms of the LLOQ plasma spiked with 2.48 ng/ml of Str and 2.64 ng/ml of Bru. (c) Chromatograms of the plasma sample spiked with 37.23 ng/ml of Str and 39.6 ng/ml of Bru. (d) Representative chromatograms of mice plasma after 0.5 h of administrated Semen Strychni.

2.8. Method application

Twenty-seven male mice, weighting 29–32 g, were housed under controlled environmental conditions (temperature 22–25 °C, humidity 45–65%). Dosing solutions were prepared by suspending accurately weighted amount of Semen Strychni in sodium carboxymethyl cellulose (1%, w/v, water). Each mouse was given an oral administration Semen Strychni 120 mg/kg and collected blood samples from ophthalmic venous plexus at 0, 0.25, 0.5, 1, 2, 3, 5, 8 and 12 h. Plasma samples were obtained by centrifugation at 6000 rpm for 10 min. All these samples were stored at −70 °C until analysis.

3. Results and discussion

3.1. Ultra-performance liquid chromatography and mass spectrometry

In our experiment, a 50 mm column at a flow rate of 0.25 ml/min was used to obtain the chromatograms. Very narrow chromatographic peaks (about 9 s) were generated by UPLC. Compared with LC–MS/MS method [16] which generated peaks over 30 s, UPLC obviously resulted in an increase in chromatographic efficiency and sensitivity.

When combined with the high specificity of tandem mass spectrometry, UPLC–MS/MS has been demonstrated to be a powerful platform, which improves assay sensitivity, selectivity and throughput [17]. The published LC–MS method [14] had a high background near the signal of LLOQ in blank rat plasma. Our studies compared MRM mode with select ion monitoring (SIM) mode. The results revealed that some endogenous substances in plasma interfered the determination of analytes when using SIM mode. While with MRM mode, a pure background had been obtained with less interference.

Different mobile phases were evaluated. The formic acid (FA) in mobile phase could get a better peak shape compared with ammonium acetate. Str and Bru were alkaloids, so FA could not only regulate the PH of mobile phase but also promote ionization of the analytes in the ESI^+ mode. The reagent tert-butyl methyl ether for plasma extraction had a high recovery and less harm to environment contrast with other reagents such as the mixture of *n*-hexane, dichloromethane and isopropanol [14].

3.2. Selectivity

The selectivity of the method was examined by analyzing blank mice plasma extract against plasma spiked with the standard solution. As shown in Fig. 3a, no significant direct interference in the blank plasma was observed from endogenous substances in drug-free plasma at the retention time of the analytes. Other typical chromatograms were given in Fig. 3b–d. Observed retention times for Str, Bru and IS were 1.10–1.13, 1.16–1.23 and 1.72–1.75 min, respectively. Thus the method was not only selective but also faster and simpler than any other reported method.

3.3. Linearity and sensitivity

The seven-point calibration curves were found to be linear over the concentration of 2.48–496.4 ng/ml for Str and 2.64–528 ng/ml for Bru. The linear equations for Str and Bru were $y = 1.19e^{-2}x + 7.62e^{-3}$ ($r^2 = 0.9938$) and $y = 9.93e^{-3}x + 1.15e^{-3}$ ($r^2 = 0.9968$), respectively. The LLOQ for Str and Bru was 2.48 ng/ml and 2.64 ng/ml, respectively.

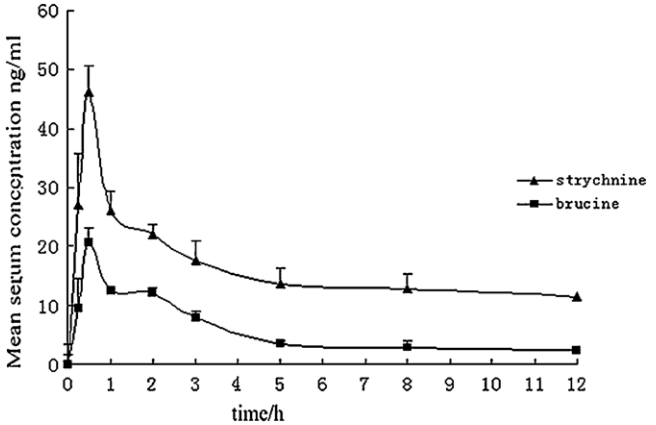

Fig. 4. Mean plasma concentration–time curve of Str and Bru in mice ($n = 3$) obtained after intragastric administration of 120 mg/kg Semen Strychni ($n = 3$, mean ± SD).

Table 1
Intra-day and Inter-day precision and accuracy of strychnine and brucine.

Analytes	Concentration added (ng/ml)	Intra-day precision ($n = 5$)			Inter-day precision ($n = 15$)		
		Concentration found (mean ± SD (ng/ml))	Precision (%)	Mean accuracy (%)	Concentration found (mean ± SD (ng/ml))	Precision (%)	Mean accuracy (%)
Str	4.96	5.17 ± 0.40	7.8	104.1	5.01 ± 0.34	6.8	100.9
	37.23	40.18 ± 1.12	2.8	107.9	40.08 ± 3.69	9.2	107.7
	397.1	377.34 ± 10.07	2.7	95	382.71 ± 44.83	11.7	96.4
Bru	5.28	5.26 ± 0.46	8.8	99.7	5.40 ± 0.74	13.8	102.3
	39.6	37.18 ± 1.47	3.9	93.9	40.93 ± 4.47	10.9	103.3
	422.4	402.04 ± 9.51	2.4	95.2	394.65 ± 24.77	6.3	93.4

Table 2
Room temperature stability and freeze–thaw stability of brucine and strychnine.

Analytes	Concentration added (ng/ml)	Room temperature stability ($n = 4$)		Freeze–thaw stability ($n = 4$)	
		Concentration found (mean ± SD (ng/ml))	RSD (%)	Concentration found (mean ± SD (ng/ml))	RSD (%)
Str	4.96	4.73 ± 0.34	7.2	4.85 ± 0.18	3.7
	37.23	36.90 ± 1.04	2.8	37.68 ± 2.00	5.3
	397.1	371.40 ± 31.79	8.6	380.18 ± 16.47	4.3
Bru	5.28	5.53 ± 0.26	4.8	5.45 ± 0.21	3.8
	39.6	38.05 ± 2.43	6.4	38.30 ± 1.40	3.6
	422.4	393.24 ± 20.77	5.3	402.55 ± 23.99	6.0

3.4. Accuracy and precision

The results of intra-day and inter-day precision and accuracy for Str and Bru in plasma QC samples are summarized in Table 1. The intra-day precisions were ≤7.8 for Str and ≤8.8 for Bru. The inter-day precisions were ≤11.7 for Str and ≤13.8 for Bru.

3.5. Recovery and matrix effect

The recoveries of three concentrations QC samples for Str and Bru were evaluated as front described formula. The ER was ranged from 89.2% to 99.3% for Str and 81.9% to 87.9% for Bru. The ME for Str and Bru was varied between 90.5–99.4% and 88.7–94.1%, respectively. The IS also had a high extraction recovery of 91.0%. The ionization suppression or enhancement effects were between ±15%, so it would not affect the determination of analytes.

3.6. Stability

The stability of the analytes in mice plasma under room temperature conditions and freeze–thaw conditions was evaluated, and the results are given in Table 2. All the stability studies were conducted at three concentration levels as QC samples. For room temperature stability, the results showed that all analytes were stable at least 12 h in mice plasma samples. Freeze–thaw stability results indicated that the repeated freeze and thawing (three cycles) did not affect the stability of Str and Bru.

3.7. Applications to the analysis of mice plasma

This method was successfully applied to determine Str and Bru in mice plasma. Mean (n = 3) plasma concentration–time profiles after oral administration of Semen Strychni are shown in Fig. 4. After 12 h of administration, the mean concentrations of Str and Bru were 11.43 and 2.52 ng/ml, respectively. While in toxicokinetics researches, the concentration of strychnine and brucine will be higher in biofluids than in the mice plasma. In previous reports, concentrations for Str of 2100 ng/ml at 3 h [20] and 3800 ng/ml at 0.5 h [21] in patient's serum caused death. The concentration was still 380 ng/ml post ingestion of Str for 74 h [4]. So the UPLC–MS/MS method was sensitive, fast and effective enough for the research of pharmacokinetics and metabolic kinetics under toxical dose for Str and Bru *in vivo*. Further researches will be carried out on toxicokinetics of Str and Bru in different tissues.

4. Conclusion

An efficient, reliable, sensitive and simple UPLC–MS/MS method has been developed and fully validated for the determination of Str and Bru in mice plasma. The simple liquid–liquid extraction method was used to extract the analytes and IS which provided excellent specificity and reproducibility. The run-time was only 3 min. This validated method is suitable for the quantitative determination of Str and Bru in plasma as well as pharmacokinetic and toxicological analysis.

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