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Determination of berberine, palmatine and jatrorrhizine in rabbit plasma by liquid chromatography–electrospray ionization-mass spectrometry

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ABSTRACT

Incurred rabbit plasmas samples were utilized for method quality assessment in this study, where an optimized protein precipitation method for the preparation of rabbit plasma samples and a rapid and sensitive liquid chromatography–electrospray ionization-mass spectrometry for the simultaneous determination of berberine, palmatine and jatrorrhizine was described. Plasma samples (100 μl) were pretreated by protein precipitation with the mixture of 3% formic acid and 50 ng/ml clozapine (internal standard) in acetonitrile followed by LC analysis using a C_{18} column and a mobile phase composed of 0.4% formic acid solution and 0.2% formic acid solution of methanol (60:40, v/v) operated at a flow rate of 0.4 ml/min. The analysis was performed in the multiple reaction monitoring mode via electrospray ionization source operating in the positive ionization mode. The method was linear over the concentration range of 0.1–400 ng/ml for all target components. The lower limits of quantification were 0.1 ng/ml for all analytes, all intra- and inter-day precision values were less than 7.10%, and accuracy (bias, %) was within ±7.11%. The mean absolute recovery was more than 72% for all analytes. The validated method has been successfully applied to the pharmacokinetic study of berberine, palmatine and jatrorrhizine in rabbit plasma after oral administration of San-Huang decoction to rabbits.

1. Introduction

San-Huang decoction, which consists of *Radix astragali*, *Coptis rhizome*, *Artemisia scoparira*, *Pollen typhae* and *Alisma orientalis*, is a classic traditional Chinese medicine preparation. It is widely used in traditional Chinese medicine to prevent and treat metabolic syndrome, and this natural preparation has played a sporadic but important role in addressing obesity in China [1].

In recent years, there has been a growing interest in the mechanism of metabolic syndrome and in therapeutic drugs used for its treatment. Metabolic syndrome is a combination of medical disorders that increase the risk of cardiovascular disease and diabetes, and is prevalent in up to 25% of the US population [2] and up to 16.5% of the Chinese population [3]. Since San-Huang decoction has exhibited potential for preventing and treating metabolic syndrome, there is an increasing enthusiasm among researchers for medical and related phytochemical studies of the compound.

Berberine, palmatine and jatrorrhizine are 3 important marker components in San-Huang decoction. The chemical structures of berberine, palmatine and jatrorrhizine are shown in Fig. 1. A sensitive method for determining berberine, palmatine and jatrorrhizine content in biological fluids is required for a pharmacokinetic study. Earlier publications have described methods for analysis of the target compounds in biological samples using HPLC–ultraviolet spectrometry (UV) [4–12], HPLC–fluorimetry [13] and HPLC–mass spectrometry (MS) [14–16]. Previously described methods suffered from several disadvantages, such as lack of sensitivity [4–16], use of a complex column-switching system [5], requirement for a large sample volume [4,8,11,15], long elution times [4,8,10,11,15–17], the lack of internal standards [4,5,8–11] and reproducible assessment of bioanalytical method using incurred samples [4–17].

In this study, an optimized protein precipitation method coupled with a rapid and sensitive liquid chromatography– electrospray ionization-mass spectrometry (LC–ESI-MS) was investigated for its ability to simultaneously determine berberine, palmatine and jatrorrhizine content in rabbit plasma, and the reproducibility of the presented bioanalytical method using incurred samples was proved, and this method was firstly applied to study the pharmacokinetics of the target compounds in rabbits after oral administration of San-Huang decoction.

2. Experimental

2.1. Herbal materials

Raw materials, including *R. astragali*, *C. rhizome*, *A. scoparira*, *P. typhae* and *A. orientalis*, were purchased from Chinese Herbal Pieces,

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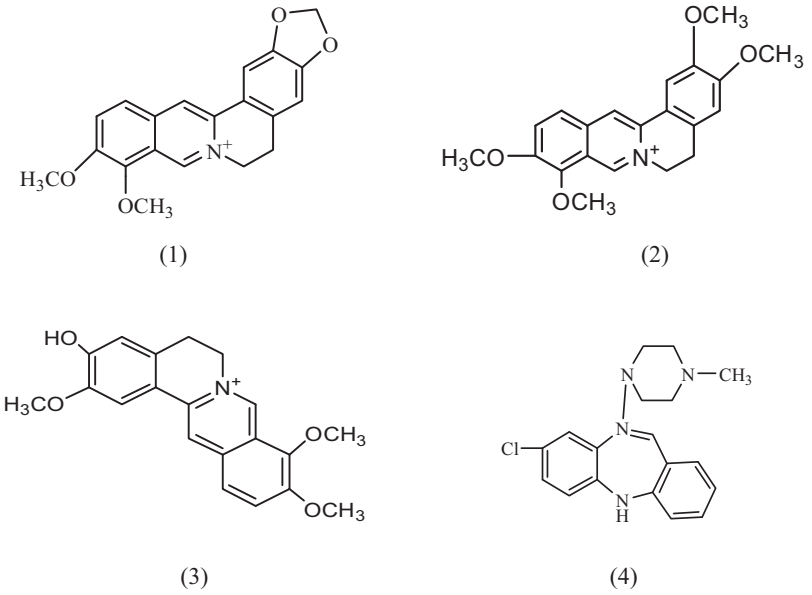

Fig. 1. Chemical structures of 3 compounds and IS: (1) berberine, (2) palmatine, (3) jatrorrhizine and (4) clozapine (internal standard).

Shanghai Hongqiao Co. Ltd., Shanghai, China, and were identified by Ms. Wenhuan Fu (Department of Pharmaceutical Analysis, Huashan Hospital, Fudan University, Shanghai, China).

2.2. Chemicals and reagents

The reference standards of berberine, palmatine, jatrorrhizine and clozapine (Internal standard, IS, Fig. 1) were obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). HPLC-grade methanol and acetonitrile were purchased from Merck (Darmstadt, Germany); and AR-grade formic acid, ammonium acetate, trichloroacetic acid and phosphoric acid were purchased from Sinopharm Group Chemical Reagent (Shanghai, China). Ultrapure water was prepared using a Milli-Q Academic water-purification system (Millipore, Milford, MA, USA).

2.3. Preparation of San-Huang decoction

Five crude herbs *R. astragali*, *C. rhizome*, *P. typhae*, *A. scoparira* and *A. orientalis* (30, 9, 15, 15 and 15 g) were decocted twice with 300 ml water, after thoroughly soaking for 25 min. The first and second decoctions were boiled using high heat for 60 min and 45 min, respectively. Finally, the decoction was condensed to 8.0 ml. It had been determined by LC–UV that the contents of berberine, palmatine and jatrorrhizine were 8.39, 1.74 and 0.96 mg/ml decoction, respectively. So 3.733 ml/kg dose of this decoction was equivalent to 31.320 mg/kg dose of berberine, 6.505 mg/kg dose of palmatine, and 3.565 mg/kg dose of jatrorrhizine.

2.4. LC–ESI-MS conditions

The HPLC system consisted of a Surveyor MS Pump and a Surveyor Autosampler (San Jose, CA, USA). Chromatographic separation was carried out on a CAPCELL PAKC_{18} MG (100 mm × 2.1 mm i.d., 5 μm; Shiseido, Japan) column with a Security Guard C_{18} column (4.0 mm × 2.0 mm i.d., 5 μm; Phenomenex, USA) maintained at 25 °C. The mobile phase consisted of 0.4% formic acid solution and 0.2% formic acid solution of methanol (60:40, v/v) at a flow rate of 0.4 ml/min. The total running time was 2.4 min for each injection. In addition, an automatic washing procedure of system was incorporated during each injection to remove dirt analytical and guard column and ion source and restore the chromatography system to the initial elution condition.

MS detection was performed on a Thermo Finnigan TSQ Quantum Triple Quadrupole Mass Spectrometer (San Jose, CA, USA) equipped with an ESI source in the positive ionization mode. The MS operating conditions were optimized as follows: the spray voltage was 3500 V, the source CID voltage was 10 eV, the heated capillary temperature was 500 °C, the sheath gas (nitrogen) was 20 psi, the auxiliary gas (nitrogen) was 50 psi, the collision gas (argon) pressure was 1.0 mtorr (1 torr = 133.3 Pa), and the collision energy was 37 eV. Data acquisition, peak integration and calibration were performed by Analyst 1.5 Software (San Jose, CA, USA). Quantification was obtained using the MRM mode of the transitions at m/z 336.2 → 320.1 for berberine, m/z 352.3 → 336.2 for palmatine, m/z 338.0 → 322.3 for jatrorrhizine, and m/z 327.1 → 270.1 for IS with a scan time of 0.3 s per transition.

2.5. Preparation of stock solutions, calibration standards and quality control samples

Standards and quality control samples (QCs) were made from 4 separate stock solutions (1 mg/ml in 50% acetonitrile for berberine, palmatine and jatrorrhizine). Plasma-based calibration standards were prepared at 0.1, 0.8, 2.0, 8.0, 20, 50 and 100, 400 ng/ml for berberine, palmatine and jatrorrhizine. Plasma QCs were prepared at 0.1 0.3, 16, and 320 ng/ml for berberine, palmatine and jatrorrhizine by appropriate dilution of the respective working solution with drug-free plasma. A 50 ng/ml working solution of IS was prepared by diluting the related stock solution with the mixture of 3% formic acid in acetonitrile.

All solutions, calibration standards and QCs were stored at −22 °C.

2.6. Sample preparation

Acetonitrile (200 μl, containing 3% formic acid and 50 ng/ml of the IS) was added to plasma samples (100 μl of blank, standard, control, or patient samples) in 1.5 ml polypropylene tubes. The mixture was vigorously vortexed for 30 s and centrifuged at 20,267 × g for

10 min at 4 °C. The supernatant was separated and dried under a nitrogen stream in a water bath at 40 °C. The residue was reconstituted in 100 μl of 20% methanol and centrifuged as described above, and then 10 μl of the supernatant was injected onto the column.

2.7. Data analysis

To obtain the pharmacokinetic parameters of berberine, palmatine and jatrorrhizine, the concentration–time data were analyzed by non-compartmental methods using the Win-Nonlin 5.0.1 (provided by Mountain View, CA, USA).

2.8. Assay validation

The method was validated for selectivity, linearity, LLOQ, accuracy, precision, recovery, stability, matrix effect (ME), cross-validation and reproducibility analysis according to the FDA Guidelines for Bioanalytical Methods Validation [18–20].

The calibration curves were obtained by linear least-squares regression analysis, plotting of peak to area ratios (berberine, palmatine and jatrorrhizine versus the IS) versus the ratio of concentrations of berberine, palmatine and jatrorrhizine using the 1/concentration^2 ($1/x^2$) as the weighting factor for each standard sample.

If the concentration was greater than the upper limit of the calibration range, duplication samples of analyte were analyzed after two-fold to four-fold dilution with drug-free plasma. The solutions obtained were assessed as described previously, and the adjusted calculated concentrations were compared with the nominal concentration.

The lower limit of detection (LLOD) was determined as the concentration of the drug giving a signal-to-noise ratio (S/N) of about 5:1. The lower limit of quantification (LLOQ) at which both precision and accuracy were $\leq$20% was determined as S/N $\geq$ 10 by analyzing samples in 6 replicates.

Three concentrations levels of 0.3, 16 and 320 ng/ml (5 samples for each concentration level) for berberine-, palmatine- and jatrorrhizine-spiked plasma standard samples were quantified to evaluate the precision (denoted as the relative standard deviation [R.S.D.] of replicate assay), accuracy (denoted as bias [%] from the nominal concentration and the mean value of the observed concentration) and extraction recovery (denoted as absolute recovery by comparing the analyte/IS peak area ratio of extracted analytes [R_1] with those of blank plasma extracts spiked with the standard solution [R_2]). Recovery of IS was also evaluated by comparing the mean peak areas of the 5 extracted medium samples to mean peak areas of the 5 reference solutions spiked in extracted plasma samples of the same concentration.

The stability of the analytes (low and high QCs) in rabbit plasma was evaluated after each storage period and analytical process, and the results were compared to the initial concentration (samples that were processed immediately after fresh preparation). The stability test included the following: (a) stability of samples at room temperature for 6 h; (b) stability of samples after 3 freeze–thaw cycles; (c) stability of the extracted samples in the autosampler for 24 h; and (d) stability of samples at −22 °C for 4 weeks. In addition, stock solution stability at 4 °C for 2 weeks was also assessed at 2 concentration levels (0.3 and 320 ng/ml). Samples were regarded as stable if the deviation (RE [%], bias from the spiked plasma concentration and the mean value of the calculated concentration) from the initial condition was within ±15%.

The ME was defined as the direct or indirect alteration or interference in response due to the presence of unintended analytes or other interfering substances in the sample [18]. First of all, ME on ion suppression or enhancement (post column infusion test) was assessed using six different samples of rabbit plasma fortified to contain 10 ng/ml of analytes (berberine, palmatine and jatrorrhizine). Peak areas of endogenous and exogenous compounds co-eluting with the analyte or internal standard should be less than 15% of the peak area of the standard and less than 5% of the response of the IS. What is more, a quantitative analysis of ME was performed as followed. In set 1, analytes were resolved in the blank plasma sample's reconstituted solution (the final solution of blank plasma after extraction and reconstitution). In set 2, analytes were resolved in mobile phase. The matrix effect of the method was determined by comparing the peak areas of set 1 (A) and set 2 (B). ME was calculated by using the formula: ME (%) = $A/B \times 100$. The matrix effect of the method was evaluated at three concentration levels of 0.3, 16 and 320 ng/ml, and five samples at each level were analyzed. The blank plasma samples used in this study were from five different batches of rabbit blank plasma. If the ME values exceed the range of 85–115%, an exogenous ME is implied.

Cross-validation was also operated to prove method performance with reference to drug–drug reactivity and stability of each target analytes in presence of each other. In set 1, one of the analytes (0.3 ng/ml, five replicates) was resolved in mobile phase (A_1) and the blank plasma samples (A_2), respectively. In set 2, the other analytes (1.0 ng/ml, five replicates) were resolved in mobile phase (B_1) and the blank plasma samples (B_2), respectively. The percentages of peak area ($B_1/A_1 \times 100$, $B_2/A_2 \times 100$) were used to determine the cross effect (CE, %) of the analysts. The blank plasma samples used in this study were from five different batches of rabbit blank plasma.

A formulation development study, comprising of 60 incurred plasma samples (concentration range was from 0.1 ng/ml to 400 ng/ml) which were analyzed over 1 month, was used to explore the method reproducibility including precision, accuracy and stability of incurred samples after implementation. Incurred rabbit plasma samples were processed in duplicates on a single day (n = 22), or a single plasma sample was reassayed on a second occasion (n = 22) to estimate precision of the developed method in authentic rabbit plasma samples. Moreover, accuracy of berberine, palmatine and jatrorrhizine in incurred samples was evaluated by a standard adding procedure. A standard solution (20 μl, corresponding to 0.3 ng/ml or 16 ng/ml of berberine, palmatine and jatrorrhizine in plasma) was added to randomly selected incurred samples (100 μl) with unknown concentrations before processing by protein precipitation. Sample analysis was performed in 10 analytical batches. The recovery of spiked amount of berberine, palmatine and jatrorrhizine was calculated from the concentration difference between the spiked study sample and the original study sample: (result for spiked sample − result for sample)/nominal spiking concentration. The goal of this experiment was to estimate the average recovery (method accuracy) which would be feasible with a sufficient number of observations. In addition, the stability of berberine, palmatine and jatrorrhizine in authentic rabbit plasma samples was investigated in a pilot study (n = 4) over four analytical occasions. Samples were regarded as stable if the deviation (average [%] for incurred samples is given as the ratio: second/initial result × 100) from the initial condition was within ±30%.

2.9. Sample collection (including incurred samples)

Sixteen male New Zealand white rabbits (2.00 ± 0.20 kg) were purchased from the Center of Experimental Animals, Fudan University, China, and were fasted overnight (16 h) prior to oral administration of 3.733 ml/kg San-Huang decoction (according to human dosage in clinical practice and human-rabbit coefficient of skin surface area). Blood samples (1.0 ml) were collected into heparinized tubes from the ear vein at 0, 0.25, 0.5, 1.0, 1.5, 2,

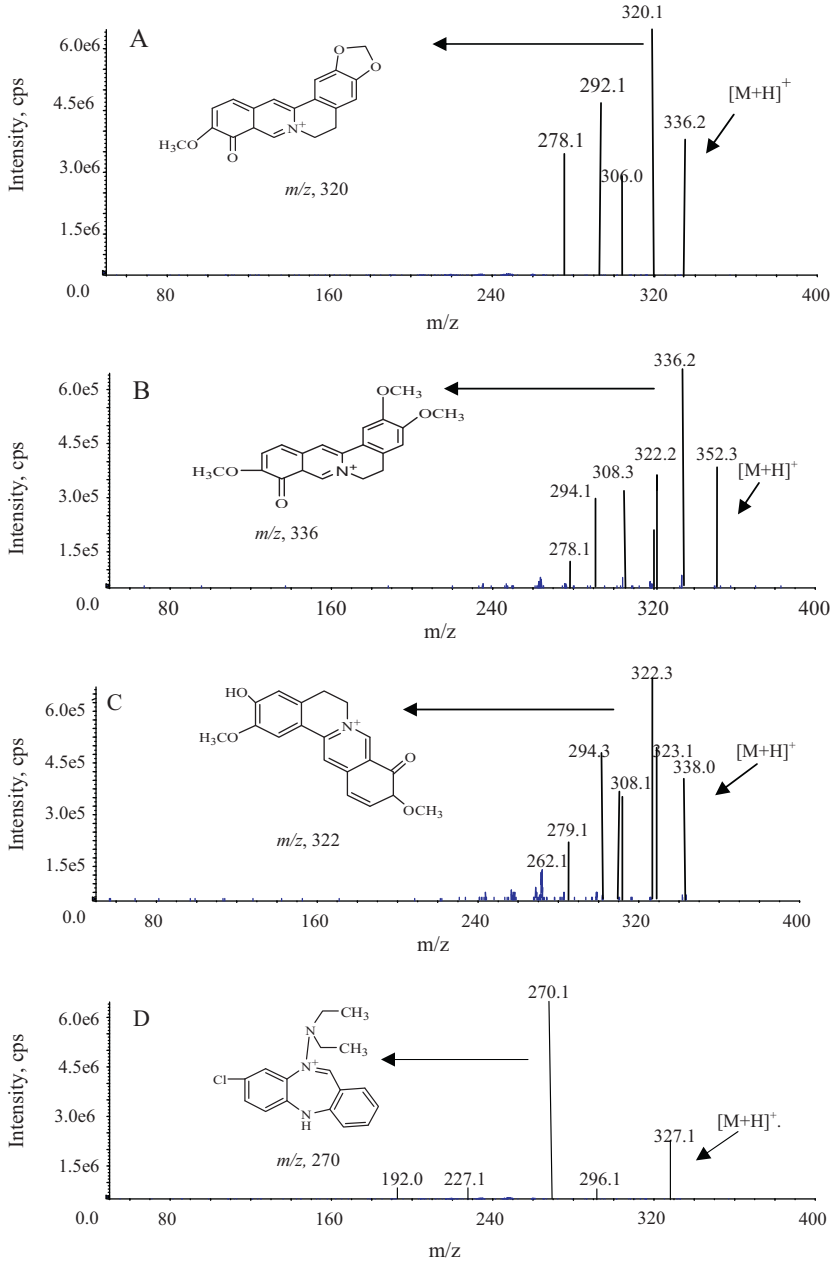

Fig. 2. Product ion mass spectra of $[M+H]^+$ ion of (A) berberine, (B) palmatine, (C) jatrorrhizine and (D) clozapine.

3, 5, 7, 11, 17 and 24 h after the oral dose. Following centrifugation (1441 × *g* for 10 min), the plasma samples were harvested and stored at −22 °C until analysis.

3. Results and discussion

3.1. Method development and optimization

3.1.1. Mass spectrometry

Considering that berberine, palmatine and jatrorrhizine are basic compounds, a positive ion-monitoring mode was adopted in the LC–ESI-MS. In order to select the target ion for monitoring the 3 target substances, the ESI mass spectra obtained by full-scan monitoring at different fragmentor voltages were investigated. Test results showed that all 3 substances formed predominantly quaternary ammonium ion at *m*/*z* 336.2 for berberine, *m*/*z* 352.3 for palmatine, *m*/*z* 338.0 for jatrorrhizine; IS formed predominantly ion at *m*/*z* 327.1. The ion spectra of the product substances can be seen in Fig. 2. The most abundant fragment ion was found at *m*/*z* 320.1 for berberine, *m*/*z* 336.2 for palmatine, *m*/*z* 322.3 for jatrorrhizine, and *m*/*z* 270.1 for IS; therefore, the MRM transitions at *m*/*z* 336.2 → 320.1, *m*/*z* 352.3 → 336.2, *m*/*z* 338.0 → 322.3, and *m*/*z* 327.1→270.1 were selected to analyze berberine, palmatine, jatrorrhizine and IS, respectively. Moreover, the spray voltage, source CID voltage, heated capillary temperature, sheath gas (nitrogen), auxiliary gas (nitrogen), collision gas (argon) pressure, and collision energy were carefully optimized in order to achieve the highest level of sensitivity of the MRM.

3.1.2. Chromatography and internal standard

The selection of an appropriate mobile phase is critical to achieve appropriate ionization and good chromatographic peak shape. We systemically investigated analytical columns with different packing materials and several modifiers in the mobile phase,

Table 1
Precision and accuracy data for berberine, palmatine and jatrorrhizine in QC samples (3 days, five replicates per day, $n = 5$) and incurred samples ($n = 22$).

Sample	Quality control sample				Incurred sample		
	Spiked concentration (ng/ml)	Measured concentration (ng/ml)	R.S.D. (%)	Bias (%)	R.S.D. (%)	Average (%)	Recovery (%)
Berberine							
[a]Intra-day ([b]same run)	0.1	0.09 ± 0.00	6.51	−7.01	2.81	99.7	[c]101.9; [d]98.8
	0.3	0.28 ± 0.02	7.10	−6.65			
	16	15.49 ± 0.45	2.90	−3.19			
	320	312.76 ± 1.54	0.49	−2.26			
[a]Inter-day ([b]between runs)	0.1	0.09 ± 0.00	6.06	−6.32	8.68	100.4	
	0.3	0.29 ± 0.01	3.45	−3.32			
	16	15.55 ± 0.35	2.25	−2.81			
	320	316.13 ± 1.79	0.56	−1.21			
Palmatine							
Intra-day (same run)	0.1	0.11 ± 0.00	5.09	6.09	1.93	99.4	[c]95.6; [d]97.8
	0.3	0.31 ± 0.01	3.22	3.34			
	16	16.07 ± 0.33	2.05	0.44			
	320	313.71 ± 1.18	0.38	−1.96			
Inter-day (between runs)	0.1	0.09 ± 0.00	4.02	−7.11	7.14	101.2	
	0.3	0.28 ± 0.01	3.57	−6.65			
	16	15.82 ± 0.23	1.45	−1.12			
	320	321.45 ± 1.56	0.48	−0.45			
Jatrorrhizine							
Intra-day (same run)	0.1	0.09 ± 0.00	5.01	−7.01	2.43	100.6	[c]98.3; [d]96.3
	0.3	0.29 ± 0.02	6.90	−3.33			
	16	15.73 ± 0.23	1.46	−1.69			
	320	315.22 ± 5.25	1.66	1.49			
Inter-day (between runs)	0.1	0.09 ± 0.00	4.91	−6.02	9.72	99.3	
	0.3	0.28 ± 0.01	3.56	−6.67			
	16	15.89 ± 0.17	1.07	0.69			
	320	312.18 ± 4.33	1.39	−2.44			

[a] For QC sample.
[b] For incurred sample.
[c] The spiked concentration was 0.3 ng/ml.
[d] The spiked concentration was 16 ng/ml.

such as formic acid and ammonium acetate alone or in combination in different concentrations, and found that a weakly acidic condition of the mobile phase was beneficial to the separation of berberine, palmatine and jatrorrhizine from interference by endogenous compounds of the plasma. Starting with 0.1% formic acid solution and 0.1% formic acid solution of methanol, it was found increasing acid concentration led to less ME and more separation effect. A high-purity reversed phase silica column was selected with the intent of facilitating removal of late eluting endogenous components. Finally, the best peak shape and ionization were achieved with a CAPCELL PAKC18 MG column and a mobile phase comprising of 0.4% formic acid solution and 0.2% formic acid solution of methanol (60:40, v/v).

The autosampler carry-over was minimized by utilizing an active external needle rinse with a low-pH solution, in which the solubility of berberine, palmatine and jatrorrhizine was higher than at neutral pH. A mixture of methanol–water–formic acid (90:10:1, v/v/v) was found effective for wash of the injector needle.

Clozapine was chosen as the IS because of its similarity with the analytes in structure, chromatographic behavior and mass spectrographic behavior.

3.1.3. Extraction method

Owing to the very low concentrations (ng/ml level) of berberine, palmatine and jatrorrhizine in rabbit plasma, and the presence of various structural types of the alkaloids with different polarities and a large amount of acidic endogenous compounds, such as chlorogenic acid, in San-Huang decoction, a selective sample-extracting method is needed. Protein precipitation [14] and liquid–liquid extraction (LLE) [15,16] have been reportedly used in the extraction of berberine, palmatine and jatrorrhizine in the past, and we found that protein precipitation was more effective than LLE. Therefore, we selected the protein precipitation method for preparing samples and carefully optimized deproteinized agents, such as the mixture of trichloroacetic acid, formic acid or phosphoric acid in acetonitrile. Owing to the reason that formic acid is easier to volatilize than the other acids the mixture of formic acid in acetonitrile was selected as the optimized deproteinized agents. In addition, the recovery of target compounds was influenced by the concentration of formic acid. Starting with 0.1% formic acid, it was found that increasing acid concentration led to more recovery of target compounds thus the optimal level was 3%. Based on these results, the best extraction was achieved using the mixture of 3% formic acid in acetonitrile as deproteinized agent.

All in all, analytical column, mobile phase, and extraction method were explored and optimized to achieve the better sensitivity in the presented method.

3.2. Method validation

3.2.1. Selectivity

No endogenous interference to berberine, palmatine, jatrorrhizine, or IS was observed in 6 different sources of blank plasma. Good separation was achieved with retention times of 1.77, 1.80, 1.42 and 1.83 min for berberine, palmatine, jatrorrhizine and IS, respectively. This method yielded good peaks without producing any significant drift of the baseline in the entire runtime of 2.4 min. The typical chromatograms of blank plasma; LLOQ in plasma sample (0.1 ng/ml); and a plasma sample from a rabbit (2.1 ng/ml for berberine, 20.3 ng/ml for palmatine, 12.5 ng/ml for jatrorrhizine, and 0.6 μg/ml for IS) are presented in Fig. 3.

3.2.2. Calibration curves and dilution test

Excellent linearity of calibration curves was obtained for each compound over a range of 0.1–400 ng/ml in rabbit plasma,

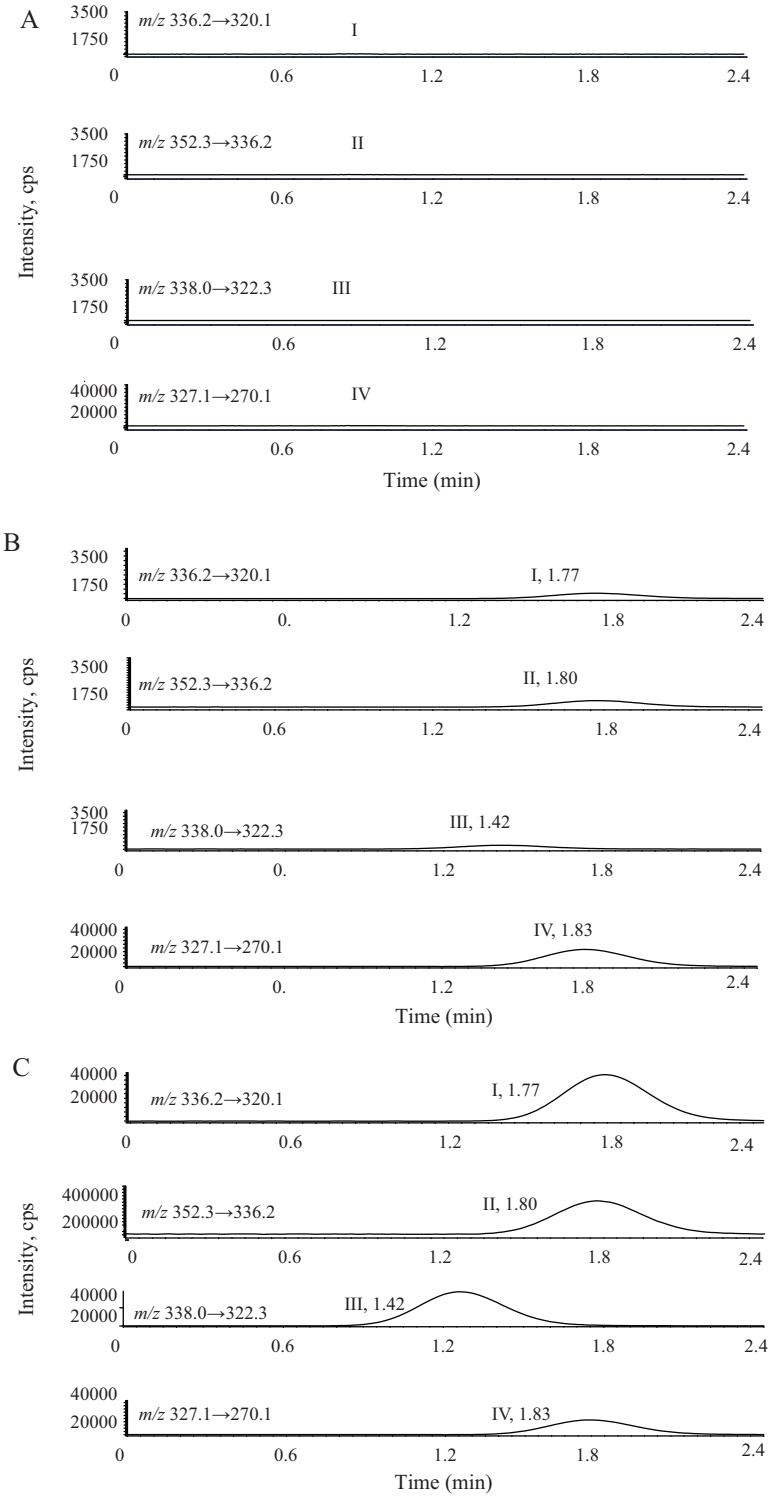

Fig. 3. Representative MRM chromatograms for berberine, palmatine, jatrorrhizine and IS in rabbit plasma: (A) a blank plasma sample; (B) a blank plasma sample spiked with berberine, palmatine and jatrorrhizine at the LLOQ of 0.1 ng/ml (spiked with IS); (C) an incurred plasma sample (2.1 ng/ml for berberine, 20.3 ng/ml for jatrorrhizine, and 12.5 ng/ml for palmatine; spiked with IS). I, berberine; II, jatrorrhizine; III, palmatine; IV, clozapine (IS).

and the correlation coefficients (r) for each run were >0.9990. Representative regression equations for berberine, palmatine and jatrorrhizine were as follows: $y = 4.72 \times 10^{-2}x + 2.81 \times 10^{-2}$ ($r = 0.9991$); $y = 4.58 \times 10^{-2}x + 4.81 \times 10^{-3}$ ($r = 0.9993$); and $y = 4.51 \times 10^{-2}x - 3.51 \times 10^{-3}$ ($r = 0.9992$), respectively.

A weighting factor of (x^{-2}) was chosen to compare the plots' linearity and precision by examining different weighting factors to fit the standard curves.

The dilution test was reproducible (recovery 100.21 ± 0.52% for berberine; 99.15 ± 0.34% for palmatine; 100.37 ± 0.41% for jatrorrhizine), which will be useful in cases where drug concentrations in study samples exceed the linear range of this method.

3.2.3. Precision, accuracy, and LLOQ

The intra- and inter-day precision and accuracy of data for berberine, palmatine, and jatrorrhizine are shown in Table 1. All

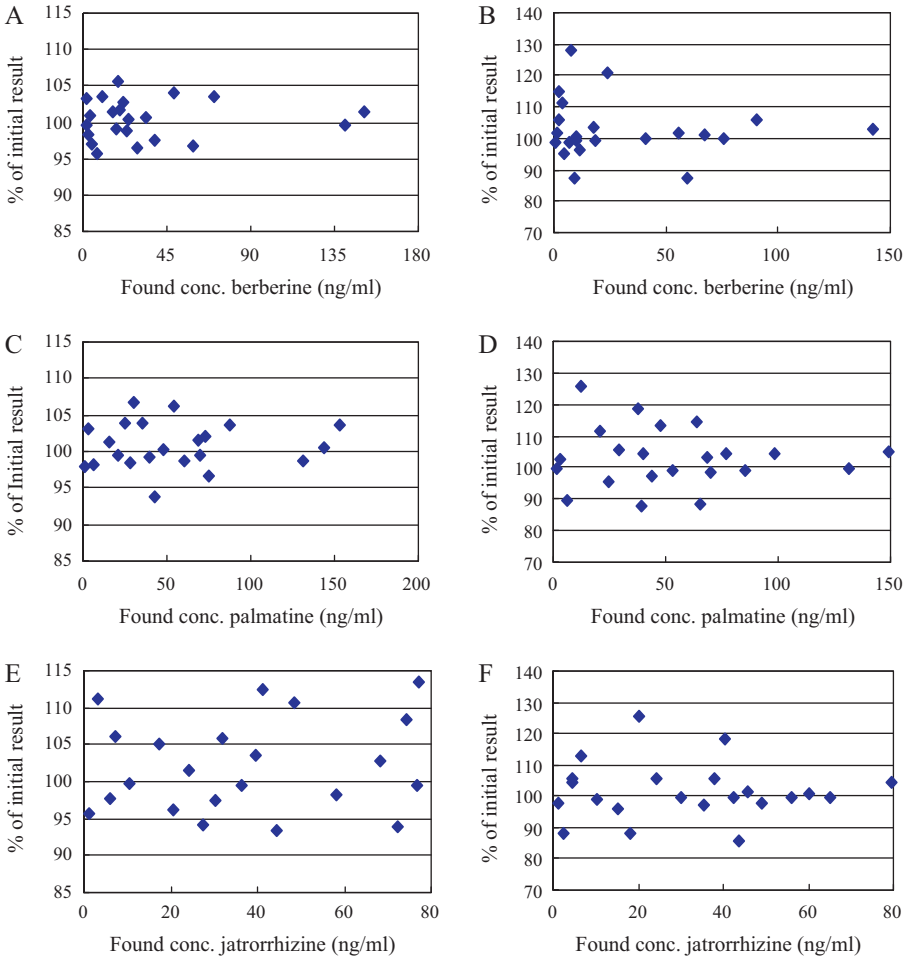

Fig. 4. Incurred rabbit plasma sample analysis. Data are presented as second replicate result (% of the initial, $n = 22$). See Table 1 for summary statistics. (1) A, C and E: each sample was processed in duplicates in the same analytical run. (2) B, D and F: each sample was processed in two different analytical runs.

values of accuracy and precision were within the recommended limits. The intra-day and inter-day precisions were no more than 7.10% for each analyte. The bias, determined from QC samples (0.3, 16 and 320 ng/ml), was within ±7.11%. All values were within the ±15% limit of the confidence.

The LLOD was 0.05 ng/ml and the LLOQ was 0.10 ng/ml for berberine, palmatine and jatrorrhizine. The LLOQ was obtained with enough precision (R.S.D. of no more than 7.10%, $n = 5$) and accuracy (bias within ±7.11%, $n = 5$).

The LLOQ of 0.02 ng/ml has been reported using LC–ESI-MS [15], which required 1 ml of plasma. Notably, the LLOQ is better than the 1.0 ng/ml [14] and 0.31 ng/ml [16] LLOQs in previous reports. Our method enabled the LLOQ for berberine, palmatine and jatrorrhizine to be 0.1 ng/ml and used only 100 μl plasma, which was more sensitive than the results from the studies described above. Considering that the very low concentrations of berberine, palmatine and jatrorrhizine in rabbit plasma and limitedly accessible volume of rabbit plasma, our method is obviously more suitable for

Table 2
Stability results for berberine, palmatine and jatrorrhizine in QC samples ($n = 5$) and incurred samples ($n = 4$) under various storage conditions. Statistics are based on data from the entire study.

Storage condition	Quality control sample				Incurred sample		
	Spiked concentration (ng/ml)	RE (%)			Average (%)		
		Berberine	Palmatine	Jatrorrhizine	Berberine	Palmatine	Jatrorrhizine
Room temperature (6 h)	0.3	−3.31	3.32	−6.61	4.32	−3.51	5.24
	320	−2.26	−0.78	−2.63			
Freeze–thaw cycles (3 cycles, −22 °C to room temperature)	0.3	−3.29	−6.59	−3.32	−11.2	5.93	6.27
	320	2.72	−1.16	−2.71			
Autosampler (24 h extracted samples)	0.3	−3.32	3.29	6.62	3.96	−4.25	17.9
	320	−0.28	−1.41	−0.67			
Long-term stability (4 weeks, −22 °C)	0.3	3.28	−3.41	−6.58	8.23	−5.61	−7.76
	320	−2.39	−2.64	1.58			

Table 3
Matrix effects data for berberine, palmatine and jatrorrhizine at 0.3, 16, 320 ng/ml and IS 50.0 ng/ml in five different sources of rabbit plasma ($n = 5$).

Sample	Spiked concentration (ng/ml)	Peak area				ME (%)
		Set 1		Set 2		
		Mean ± S.D.	R.S.D. (%)	Mean ± S.D.	R.S.D. (%)	
Berberine	0.3	22,050 ± 617	2.83	22,950 ± 597	2.61	96.1
	16	190,853 ± 5153	2.71	198,689 ± 3576	1.87	96.0
	320	4,161,359 ± 58,259	1.45	4,275,637 ± 55,583	1.35	97.3
Palmatine	0.3	8310 ± 241	2.92	8415 ± 219	2.64	98.8
	16	149,662 ± 4041	2.79	151,662 ± 4246	2.85	98.7
	320	3,204,324 ± 67,291	2.15	3,293,782 ± 62,582	1.94	97.3
Jatrorrhizine	0.3	6645 ± 193	2.95	6782 ± 190	2.83	98.0
	16	156,851 ± 3294	2.13	157,963 ± 3001	1.95	99.3
	320	3,116,528 ± 46,748	1.58	3,098,758 ± 24,790	0.87	100.6
IS	50	280,227 ± 6445	2.31	281,853 ± 3664	1.38	99.4

pharmacokinetic study of berberine, palmatine and jatrorrhizine in rabbit.

3.2.4. Recovery

The mean absolute recovery of berberine, palmatine and jatrorrhizine determined from the QC samples (0.3, 16 and 320 ng/ml) and IS (0.6 μg/ml) following the extraction procedure was 74.32 ± 1.37%, 72.53 ± 2.16%, 75.34 ± 1.08% and 76.59 ± 1.43%, respectively.

3.2.5. Stability

The stability data of the QC samples (0.3 and 320 ng/ml) is summarized in Table 2. The bias, determined from standard solutions (1 ng/ml) kept for 2 weeks at 4 °C, was within ±2.0%.

The results showed that all samples were stable during each storage period and analytical process.

3.2.6. Matrix effects

The presence of ME can decrease or increase the response of the analyte and thus affects the sensitivity of the method. Therefore studies of ME should be an integral part of the validation of any LC–MS/MS method, especially when ESI is utilized.

Two approaches have been reported to study ME [14–16,18]. The first approach is a qualitative method, which a solution of the analyte is constantly infused into the eluent from the column via post-column tee connection using a syringe pump. The continuous post-column infusion produces constant signals in the detector, unless compounds that elute from the column suppress or enhance ionization, which would lead to a decreased or increased detector response, respectively. The second approach is a quantitative method with two kind of comprehensive strategy having been recently published [14–16]. The above methods were introduced to assess ME in this study.

No significant matrix ionization effects were observed for berberine, palmatine or jatrorrhizine, which were demonstrated in two ways. Firstly, constant-infusion experiments revealed that the chromatograms were free of matrix ionization effects in the region where berberine, palmatine and jatrorrhizine elute (Fig. 3). Secondly, the data in Table 3 show peak areas of berberine, palmatine and jatrorrhizine in serum samples were not significantly different from the peak areas of these compounds in methanol standards. RSD value of absolute ME in five different sources of rabbit plasma was below 3.0%, indicating that the relative MEs for the analytes were minimal in this study. In addition, no ionization suppression on IS was observed.

3.2.7. Cross-validation

The three analysts have similar structures and the same fragmentation mechanism, and they were analyzed in the selected MRM transitions, which may jointly lead to a cross reactivity and even may affect their stability and the accuracy of quantification. As shown in Table 4, the possible interference did not significantly affect the peak area of the analysts. Therefore, the developed analytical method was reliable and suitable for this study. Moreover, the cross-validation of the analysts in stock solutions has been validated in published study [14].

3.2.8. Reproducibility of incurred samples analysis

Bioanalytical methods are usually developed and validated by using spiked biological matrix samples. While this approach is generally effective, more bioanalytical issues have arised upon applying a validated method to the analysis of incurred samples, which are usually quite different from the spiked samples. Analysis

Table 4
Cross effect data for berberine, palmatine and jatrorrhizine at 0.3 ng/ml in mobile phase and rabbit plasma ($n = 5$).

Solution condition	Sample	Spiked concentration (ng/ml)	Concentration of the other analytes (ng/ml)	Peak area				CE (%)
				Set 1		Set 2		
				Mean ± S.D.	R.S.D. (%)	Mean ± S.D.	R.S.D. (%)	
Mobile phase	Berberine	0.3	1.0, 1.0	23,065 ± 577	2.51	22,950 ± 427	1.86	99.5
	Palmatine	0.3	1.0, 1.0	8431 ± 152	1.82	8415 ± 176	2.09	99.8
	Jatrorrhizine	0.3	1.0, 1.0	6762 ± 142	2.17	6782 ± 191	2.82	100.3
Blank plasma	Berberine	0.3	1.0, 1.0	22,340 ± 424	1.95	22,050 ± 337	1.53	98.7
	Palmatine	0.3	1.0, 1.0	8352 ± 226	2.73	8310 ± 241	2.90	99.5
	Jatrorrhizine	0.3	1.0, 1.0	6586 ± 132	2.01	6645 ± 129	1.94	100.9

Table 5
Pharmocokinetic parameters of berberine, palmatine and jatrorrhizine after an oral administration of San-Huang decoction 7.67 ml/kg body weight ($n = 6$).

Component	t_{max} (h)	C_{max} (ng/ml)	$AUC_{0\rightarrow\infty}$ (h ng/ml)	$T_{1/2}$ (h)
Berberine	0.25 ± 0	327.73 ± 29.64	299.04 ± 22.07	5.89 ± 0.46
Palmatine	0.75 ± 0	147.33 ± 9.01	1440.81 ± 284.91	20.41 ± 5.67
Jatrorrhizine	0.50 ± 0	71.30 ± 7.72	1099.54 ± 292.67	18.12 ± 4.74

of real-life samples may be influenced by many variables, such as sampling procedures, feeding conditions, individual variations of endogenous substance levels. Early observations of deviations are important, as method modifications might be warranted to improve robustness.

Within-day repeatability for incurred samples analyzed in duplicates is presented in Fig. 4A, C and E as the second duplicate result (% of the initial/first duplicate). The precision was (%R.S.D.) 2.81, 1.93, 2.43 for berberine, palmatine and jatrorrhizine, respectively (Table 1). Method reproducibility over the study is shown in Fig. 4B, D and F, the precision for incurred samples between runs was 8.68, 7.14 and 9.72 for berberine, palmatine and jatrorrhizine, respectively (Table 1). The precision for incurred samples indicates that the method provided consistent data over the study course.

The accuracy of berberine, palmatine and jatrorrhizine in incurred samples was shown in Table 1; the recovery ranged from 95.6% to 101.9%.

The stability of berberine, palmatine and jatrorrhizine in authentic rabbit plasma samples was shown in Table 2; the bias ranged from −11.2% to 17.9%.

Overall, all repeats agreed within ±20% for incurred samples analysis, which is in accord with the recommendatory criteria [19,20]. Therefore, QC samples performance seemed to reflect the variability of authentic samples in the concentration range studied.

3.3. Results of pharmacokinetic study

After the oral administration of San-Huang decoction (3.733 ml/kg body weight) to 6 rabbits, the plasma concentrations of berberine, palmatine and jatrorrhizine were simultaneously determined by the described LC–ESI-MS method. Fig. 5 shows the mean plasma concentration–time profiles ($n = 6$). There was only 1 typical peak observed in both the individual and mean plasma-concentration curves of berberine, palmatine and jatrorrhizine. Moreover, the concentration of berberine was very low (ng/ml level) in rabbit plasma, which is consistent with the results from other rabbit, Beagle dog and human [6,8,11,12] oral administration studies, and may be explained by poor absorption and extensive metabolism [5,7,10,21].

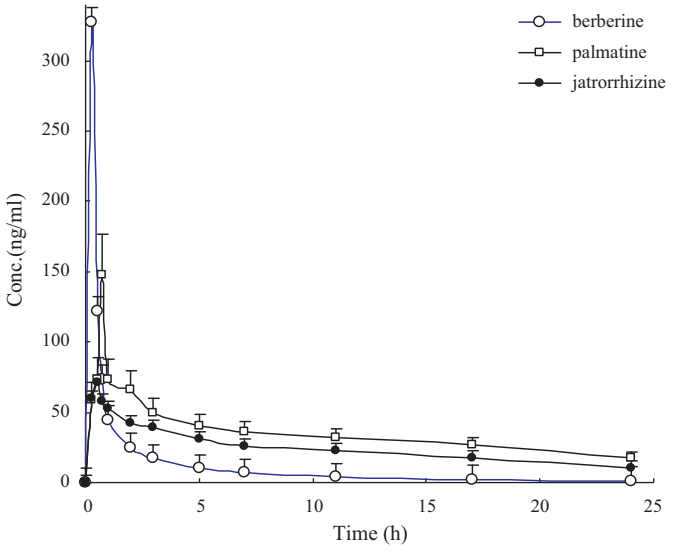

Fig. 5. Mean plasma concentration–time curves of berberine, palmatine and jatrorrhizine after oral administration of San-Huang decoction 7.467 ml/kg body weight, equivalent to 62.64 mg/kg berberine, 13.01 mg/kg palmatine and 7.13 mg/kg jatrorrhizine, respectively. Data are shown as mean ± SD ($n = 5$).

Li et al. [5] reported that berberine was absorbed quickly and eliminated very slowly in the rabbit after the oral administration of Xiexin-tang decoction. The mean t_{max} was also 0.5 h, and the higher concentration lasted for 4.5 h and resulted in a concentration platform. Table 5 lists some important pharmacokinetic parameters. The terminal elimination half-life for berberine, palmatine and jatrorrhizine was 5.89 ± 0.46, 20.41 ± 5.67 and 18.12 ± 4.74, respectively. The time to reach the maximum plasma drug concentration was 0.25, 0.5, 0.75 h for berberine, palmatine and jatrorrhizine, respectively, which showed the target compounds were also absorbed quickly and eliminated slowly in rabbits.

4. Conclusion

In this paper, we first developed a selective protein precipitation method coupled with LC–ESI-MS for the simultaneous determination of berberine, palmatine and jatrorrhizine levels in rabbits after oral administration of San-Huang decoction. The advantages of this optimized method include smaller plasma volume, simpler sample preparation method, better sensitivity, a relatively short analysis time of 2.4 min for each sample and reproducibility of the method. The LC–ESI-MS assay was firstly successfully used in the pharmacokinetic studies of berberine, palmatine and jatrorrhizine in rabbit plasma.

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