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Simultaneous determination of active xanthone glycosides, timosaponins and alkaloids in rat plasma after oral administration of Zi-Shen Pill extract for the pharmacokinetic study by liquid chromatography–tandem mass spectrometry

Fei Cai[a,b], Wen Xu[a], Hua Wei[a], Lianna Sun[c], Shouhong Gao[a], Qi Yang[a], Jin Feng[a], Feng Zhang[a], Wansheng Chen[a,b,*]

[a] Department of Pharmacy, Changzheng Hospital, Second Military Medical University, No. 415 Fengyang Road, Shanghai 200003, PR China
[b] Modern Research Center for Traditional Chinese Medicine, Second Military Medical University, No. 325 Guohe Road, Shanghai 200433, PR China
[c] Department of Pharmacognosy, School of Pharmacy, Second Military Medical University, No. 325 Guohe Road, Shanghai 200433, PR China

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ABSTRACT

A sensitive and reliable liquid chromatography–electrospray ionization-tandem mass spectrometry (LC–ESI-MS/MS) has been developed and validated for simultaneous determination of active components, i.e., xanthone glycosides (neomangiferin and mangiferin), timosaponins (timosaponin E1, timosaponin B-II and timosaponin B) and alkaloids (palmatine and berberine) in rat plasma after oral administration of Zi-Shen Pill extract. Plasma samples were pretreated by protein precipitation with acetonitrile containing the internal standards ginsenoside Re (for xanthone glycosides and timosaponins) and tetrahydroberberine (for alkaloids). LC separation was achieved on a Zorbax SB-C_{18} column (150 mm × 2.1 mm I.D., 3.5 μm) with gradient elution using a mobile phase consisting of acetonitrile-0.1% formic acid in water at a flow rate of 0.25 mL/min. The detection was carried out by a triple–quadrupole tandem mass spectrometer in multiple reaction monitoring (MRM) mode via polarity switching between negative (for xanthone glycosides and timosaponins) and positive (for alkaloids) ionization mode. Linear calibration curves were obtained over the concentration range of 5–1000 ng/mL for mangiferin, 0.5–100 ng/mL for neomangiferin, timosaponin E1, timosaponin B-II and timosaponin B, and 0.05–10 ng/mL for palmatine and berberine. The mean recovery of all the analytes ranged from 64.7 to 93.8%. The intra- and inter-day precision (% R.S.D.) was within 11.7% and accuracy (% bias) ranged from −9.0 to 10.9%. This fully validated method was successfully applied to pharmacokinetic study of the above seven compounds in rats.

1. Introduction

Traditional Chinese Medicine (TCM) originating from oriental philosophy and culture has long been practiced in China, Japan, Korea and other eastern countries. During the past decades, TCM has been spread globally and increasingly accepted by more countries and regions, partly due to alterations in disease types, prevalence of some chronic diseases and limitations of modern medicines in terms of adverse reactions [1].

Zi-Shen Pill (ZSP), a traditional Chinese medicinal formula, has been used in the treatment of benign prostatic hyperplasia (BPH), prostatitis and frequent urination for a long period of time. Our previous study demonstrated that ZSP extract had an inhibitory effect on rat BPH induced by testosterone after castration [2]. In view of adverse reactions induced by conventional chemical drugs, ZSP might be an excellent alternative and complementary medicine for the treatment of human BPH. ZSP is prepared from Rhizoma Anemarrhenae (*Anemarrhena asphodeloides* Bge.), Cortex Phellodendri Amurensis (*Phellodendron amurense* Rupr.) and Cortex Cinnamomi (*Cinnamomum cassia* Presl) at a weight ratio of 10:10:1. In general, multiple constituents involved in a TCM formula are believed to be responsible for their pharmacological and biological effects. Many studies suggested that inflammation appeared to be directly associated with the pathogenesis and progression of BPH and prostatitis [3–5]. At the same time, pharmacological studies showed that xanthone glycosides such as neomangiferin and mangiferin [6,7], timosaponins (e.g., timosaponin E1, timosaponin B-II and timosaponin B) [8–10], as well as alkaloids like palmatine and berberine [11,12] had various anti-inflammatory or anti-superoxidative potential. Consequently, the above compounds could be main bioactive constituents of ZSP in the treatment of BPH

* Corresponding author at: Department of Pharmacy, Changzheng Hospital, Second Military Medical University, No. 415 Fengyang Road, Shanghai 200003, PR China. Tel.: +86 21 81871350; fax: +86 21 33100038.
E-mail addresses: chenwanshengsmmu@yahoo.com.cn, chenws@vnet.citiz.net (W. Chen).

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and prostatitis. A full-scale investigation into the pharmacokinetics of bioactive compounds in ZSP could not only link data from pharmacological assays to clinical effects but also help design rational dosage regimens, minimize unacceptable side effects and avoid undesirable drug–drug interactions.

Due to the distinguished physicochemical difference of xanthone glycosides, timosaponins and alkaloids, it is rather difficult to develop a robust assay for simultaneous quantification of these compounds in biological samples. Although identification of these constituents in rat serum and urine by liquid chromatography–mass spectrometry can be retrieved in the literature [13,14], to our knowledge, several analytical methods have been previously described to determine only one or two of these relevant compounds in plasma separately [15–18] and none of these methods is able to simultaneously determine all compounds of interest in biological samples. The present study was designed to develop a rapid and sensitive liquid chromatography–tandem mass spectrometry (LC–MS/MS) with polarity switching and utilize it for full pharmacokinetic investigation of the main active constituents in ZSP following oral administration of its extract.

2. Experimental

2.1. Herbal materials and chemicals

Rhizoma Anemarrhenae, Cortex Phellodendri Amurensis and Cortex Cinnamomi were all collected from Bozhou of Anhui Province of China, and authenticated by one of us, Prof. Wansheng Chen. Mangiferin, palmatine hydrochloride, berberine hydrochloride and ginsenoside Re (internal standard, IS) were purchased from the Chinese National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China); tetrahydroberberine (IS) was kindly offered by Dr. Lianna Sun from School of Pharmacy, the Second Military Medical University; other chemical standards (neomangiferin, timosaponin E1, timosaponin B-II and timosaponin B) were isolated from Rhizoma Anemarrhenae in our laboratory. Their structures were elucidated based on their spectral data (IR, MS, ^{1}H NMR and ^{13}C NMR). Purity of these standards was determined as higher than 98% by normalization of the peak areas detected by HPLC equipped with an evaporative light scattering detector. Their chemical structures are shown in Fig. 1.

Acetonitrile was of HPLC grade from Merck (Darmstadt, Germany); formic acid was purchased from Tedia (Fairfield, USA); and ultrapure water was prepared from a Milli-Q water purification system (Millipore, Milford, MA, USA). All other chemicals were of analytical grade.

2.2. Preparation of ZSP extract and determination of seven main compounds in the extract

The powder (1050 g) of crude herbs consisting of Rhizoma Anemarrhenae, Cortex Phellodendri Amurensis and Cortex Cinnamomi (10:10:1, w/w/w) was used for preparing ZSP extract. The mixed powder was extracted by percolating with 95% aqueous ethanol (16 L) and water (5 L) in sequence at room temperature. Then the percolates were pooled and evaporated to an extract (378 g).

To calculate the administration dosage, the contents of the seven main constituents in ZSP extract were quantitatively determined. One hundred milligrams of the extract was suspended in 100 mL acetonitrile–water (50:50, v/v), ultrasonically dissolved for 30 min, and then cooled at room temperature; acetonitrile–water (50:50) was added to compensate for the lost volume. Finally, the solution filtered through a 0.45 μm membrane was quantified by HPLC coupled with diode array and evaporative light scattering detectors. The contents of neomangiferin, mangiferin, timosaponin E1, timosaponin B-II, timosaponin B, palmatine and berberine were 0.818, 1.18, 0.680, 4.55, 1.39, 0.833 and 1.31 g/100 g extract, respectively.

2.3. LC–MS/MS analysis

LC–MS/MS analysis was performed on an Agilent 1200 Series liquid chromatograph (Agilent Technologies, Palo Alto, CA, USA) coupled with an Agilent 6410 triple quadrupole mass spectrometer (USA) with an electrospray ionization (ESI) source. Data were acquired and analysed with MassHunter Workstation Software (Agilent Technologies, USA).

Chromatographic separation was achieved using an Agilent Zorbax SB-C_{18} column (150 mm × 2.1 mm I.D., 3.5 μm) with an Agilent C_{18} guard column (12.5 mm × 2.1 mm I.D., 5 μm). The mobile phase was composed of acetonitrile (A) and 0.1% formic acid in water (B) using the following gradient program: 20% A → 46% A at 0–1.0 min; 46% A at 1.0–2.5 min; 46% A → 20% A at 2.5–4.0 min; 20% A at 4.0–9.0 min. Chromatography was performed at 18 °C with a flow rate of 0.25 mL/min, and the injection volume was 20 μL. The overall run time for each injection was 9.0 min (6.5 min for negative mode followed by 2.5 min for positive mode). The effluent from the LC column was directed from the waste to the mass spectrometer source after the first 1.0 min of each run. An injector rinse solvent consisting of acetonitrile–water (50:50, v/v) was used.

The mass spectrometer was operated in either negative or positive ion mode using multiple reaction monitoring (MRM) to detect the mass transitions. High purity nitrogen served as both nebulizing and drying gas. Compound-dependent parameters are listed in Table 1. Other parameters of the mass spectrometer were set as follows: drying gas flow 10 L/min; drying gas temperature 350 °C; nebulizer pressure 40 psi; capillary voltage 4000 V.

2.4. Preparation of standards and quality control samples

The stock solutions of neomangiferin, palmatine, berberine and the IS (ginsenoside Re and tetrahydroberberine) were individually prepared in methanol, while the stock solutions of timosaponin E1, timosaponin B-II as well as timosaponin B were individually prepared in acetonitrile–water (90:10, v/v), and the stock solution of mangiferin was prepared in methanol–dioxane (80:20, v/v), to obtain a final concentration of 200 μg/mL. All the individual stocks were stored at −20 °C and were stable for 3 months. The stock solutions of the standards were further diluted in acetonitrile–water (50:50, v/v) to produce combined standard working solutions at concentrations of 100, 200, 400, 1000, 2000, 4000, 10000 and 20000 ng/mL for mangiferin, 10, 20, 40, 100, 200, 400, 1000 and 2000 ng/mL for neomangiferin, timosaponin E1, timosaponin B-II as well as timosaponin B, and 1, 2, 4, 10, 20, 40, 100 and 200 ng/mL for palmatine and berberine. Calibration samples were prepared by a 1:20 dilution of the corresponding combined standard working solutions with pooled blank rat plasma to obtain final concentrations in the range of 5–1000 ng/mL for mangiferin, 0.5–100 ng/mL for neomangiferin, timosaponin E1, timosaponin B-II and timosaponin B, and 0.05–10 ng/mL for palmatine and berberine. Quality control (QC) samples were also prepared in the same way (8, 80, 640 ng/mL for mangiferin; 1, 10, 80 ng/mL for neomangiferin, timosaponin E1, timosaponin B-II and timosaponin B; 0.1, 1, 8 ng/mL for palmatine and berberine).

2.5. Sample preparation

A 100 μL aliquot of the plasma sample was treated with 120 μL acetonitrile containing the IS ginsenoside Re (100 ng/mL) and

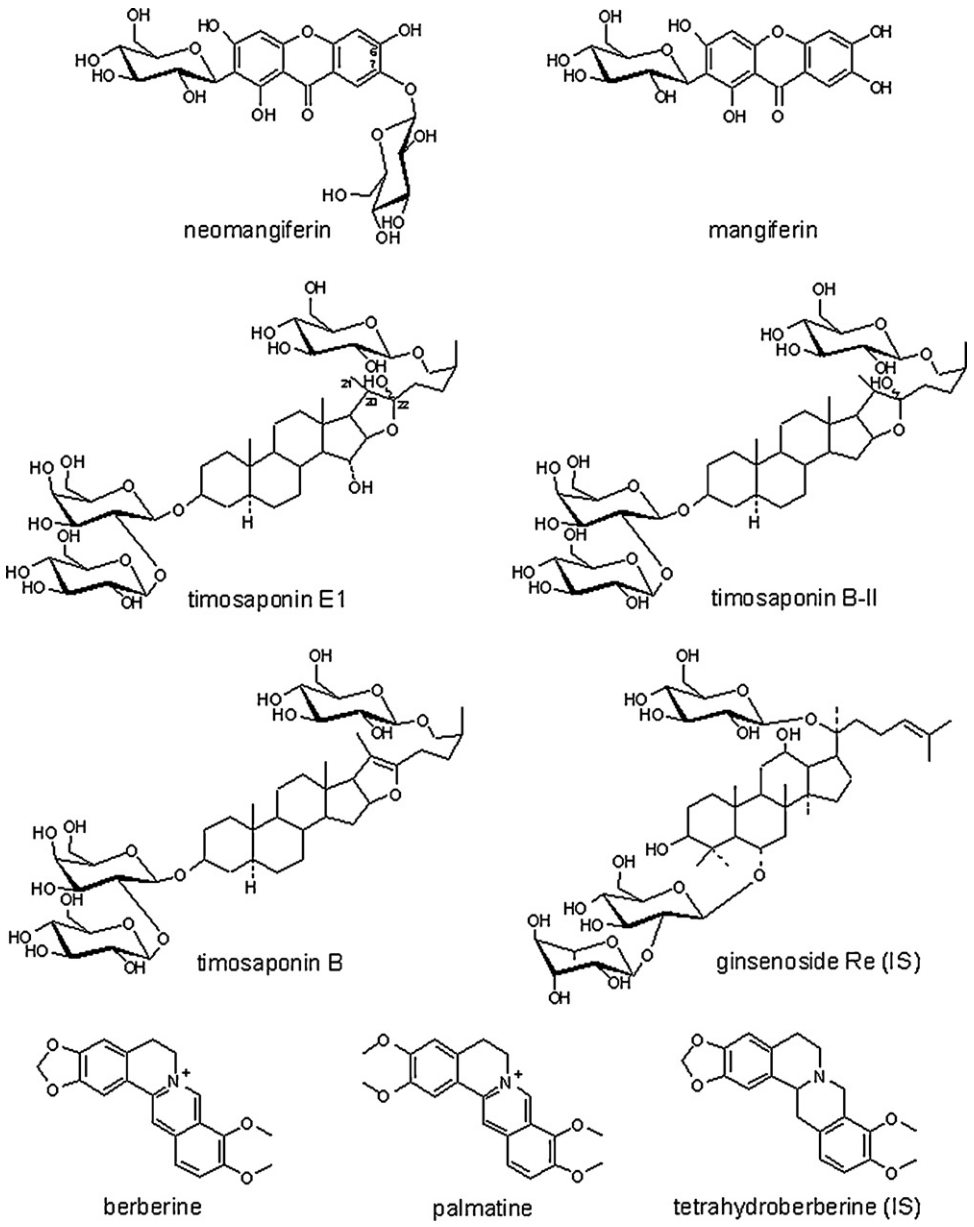

Fig. 1. Chemical structures of neomangiferin, mangiferin, timosaponin E1, timosaponin B-II, timosaponin B, palmatine, berberine, ginsenoside Re (IS), and tetrahydroberberine (IS).

tetrahydroberberine (1 ng/mL). The mixture was vortex mixed for 2 min and centrifuged at 13,000 × *g* for 10 min. Then 150 μL of the supernatant was added to 300 μL water. After mixing, the solution was transferred to a 1.5 mL autosampler vial and 20 μL of the solution was injected into the LC–MS/MS system for analysis.

2.6. Matrix effects and extraction recovery

To evaluate the matrix effects [19], six different lots of blank plasma from untreated rats ($n = 6$) were processed according to the protein precipitation described above and then spiked with the analytes at the final concentrations after extraction and dilution

Table 1
MS/MS transitions and parameters for the detection of the analytes and internal standards.

Target compound	Molecular mass	Ionization mode	Precursor ion (*m*/*z*)	Product ion (*m*/*z*)	Fragmentor (V)	Collision energy (V)
Neomangiferin	584.1	ESI^-	583.2	331.1	200	35
Mangiferin	422.1	ESI^-	421.1	301.1	125	23
Timosaponin E1	936.5	ESI^-	935.6	773.5	300	53
Timosaponin B-II	920.5	ESI^-	919.5	757.4	300	53
Ginsenoside Re (IS)	946.6	ESI^-	945.6	475.4	300	51
Timosaponin B	902.5	ESI^-	901.5	739.5	300	48
Tetrahydroberberine (IS)	339.2	ESI^+	340.2	176.1	125	24
Palmatine	352.2	ESI^+	352.3	336.2	100	26
Berberine	336.1	ESI^+	336.2	320.2	125	30

of water. The absolute matrix effects were expressed as the ratios of the mean peak areas of analytes spiked post-extraction to that of the neat standards at corresponding concentrations. The value of 100% indicates no absolute matrix effect was observed, and the value of <100% illustrates ionization suppression while the value of >100% indicates ionization enhancement. The same evaluation was performed for the IS. The recovery of the analytes and IS was determined by calculating the ratios of the mean peak areas of six regularly prepared samples to that of post-extraction spiked samples.

2.7. Precision and accuracy

The intra-day precision and accuracy were determined at three different levels (low, mid and high) of concentrations from five replicate QC samples on the same day. The inter-day precision and accuracy were determined at three different levels of concentrations from five replicate QC samples on three independent days. The precision was expressed as relative standard deviation (R.S.D.), and the accuracy was calculated as the percentage bias from the nominal concentration (% bias).

2.8. Stability

The stability of seven analytes in plasma was assessed by analyzing five replicate QC samples at three different concentrations after three cycles of freezing at −20 °C and thawing, stored for 4 h at ambient temperatures, and stored for 2 weeks at −20 °C. The post-preparative stability where the pretreated samples were re-injected after being left in the HPLC autosampler at room temperature for 12 h was also assessed. The measured concentrations were then compared to those of freshly prepared QC samples and the percentage concentration deviation was calculated to evaluate stability.

2.9. Animals and pharmacokinetic study

Male Sprague–Dawley rats (240–260 g) were supplied by the Animal Center of the Chinese Academy of Sciences (Shanghai, China). They were housed in controlled conditions (temperature 22–24 °C; humidity 55–60%; 12 h light/dark cycle) and received a standard rat chow and tap water *ad libitum* for a week prior to experiments. Animal experiments were carried out according to the Guidelines for the Care and Use of Laboratory Animals, and were approved by the Animal Ethics Committee of the Second Military Medical University. Six rats were intragastric administered with 1.94 g/kg ZSP extract suspended in 0.5% CMC-Na (w/v). About 250 μL blood samples were collected in heparinized tubes from each rat at 5, 10, 15, 30 min, 1, 2, 3, 4, 6, 8, 12, 24, 36 and 48 h. Within 30 min after blood withdrawal, the samples were centrifuged and then the separated plasma samples were stored at −20 °C prior to analysis. The plasma collected from six vehicle-administered rats served as the blank.

All pharmacokinetic parameters including maximum plasma concentration (C_{max}), time to maximum concentration (T_{max}), apparent elimination half-life time ($t_{1/2}$), area under the curve (AUC), mean residence time (MRT), and total body clearance (CL/F) were estimated using Drug And Statistics 2.0 (DAS 2.0, Mathematical Pharmacology Professional Committee of China, Shanghai, China) in which non-compartmental analysis was chosen.

3. Results and discussion

3.1. LC–MS/MS optimization

The analytes and IS were at first characterized by MS^2 scan and MS/MS product ion to ascertain their precursor ions and to select product ions for use in MRM mode, respectively. The full-scan mass spectra showed that the ionization of the glycosides (xanthone glycosides and timosaponins) was more efficient in negative than positive ion mode, whereas the alkaloids responded much better in positive ion mode. The MS/MS product ion spectra of the analytes and IS are shown in Fig. 2. To get the richest relative abundance of precursor and product ions, the parameters for fragmentor energy and collision energy were optimized. Table 1 shows the MS/MS transitions and energy parameters of all the compounds.

It is well known that ionization in ESI mode occurs in the solution state. The additives may have a significant influence on the response of the analyte. After optimization, a proper concentration of formic acid (0.1%, v/v) was chosen. Because it so dramatically enhanced the abundance of $[M+H]^+$ ions of the alkaloids that an adequate lower limit of quantification (0.05 ng/mL for palmatine and berberine) was obtained in this way, while the addition of formic acid only slightly suppressed the intensity of $[M–H]^+$ ions of xanthone glycosides and timosaponins.

With respect to the mobile phase, it was recommended [20] that the analysis of furostanol saponins (e.g., timosaponins) by HPLC–MS was performed using acidified aqueous acetonitrile mobile phase and avoiding methanol due to the interconversion of the C-22 hydroxy and C-22 methoxy forms. Besides, acetonitrile displayed higher resolving power than methanol in the test. So aqueous acetonitrile rather than aqueous methanol was chosen as the mobile phase. It was reported [18] that cross-talk may exist and affect the accuracy of MS quantification while the analytes have very similar structures and same fragmentation mechanisms. To eliminate the undesirable cross-talk effects, it seems advisable to achieve a complete chromatographic resolution for this method. Firstly, early various efforts had been done to adjust the mobile phase composition and gradient time, and thereafter the optimized gradient elution (see Section 2.3) was employed to obtain a basically acceptable resolution. Secondly, the column temperature, which had a notable impact on the separation of the three alkaloids in the test, was selected at 18 °C to further enhance the resolution. So an improved separation of the seven analytes was achieved under the specified chromatographic conditions and no cross-talk was observed.

It is necessary to use an IS to get high accuracy when performing MS quantitation. An appropriate IS will control variability in extraction, HPLC injection and ionization. The selection of ginsenoside Re and tetrahydroberberine as IS was based on their structural similarity with most of the corresponding analytes. Hence, ginsenoside Re and tetrahydroberberine were chosen as IS for the glycosides (xanthone glycosides and timosaponins) in negative ion mode and for the alkaloids in positive ion mode, respectively, giving better results for linearity and quantitation.

3.2. Sample preparation

Sample preparation is a critical step for an accurate and reliable LC–MS/MS method. Initially, several conventional liquid–liquid extraction (LLE) procedures were investigated using different extraction solvents (ethyl acetate, diethyl ether, cyclohexane, *tert*-butyl methyl ether, *n*-butanol or their different combinations), but no satisfactory recovery was obtained for all the timosaponins. Finally, protein precipitation (PPT) using acetonitrile was tried and found to give good and consistent recovery for all the analytes. At the earlier stage of the method development, the supernatant was separated out and evaporated to dryness under vacuum at 40 °C after vortex-mixing and centrifugation. It was found that neomangiferin and mangiferin in the residue could not be completely reconstituted with the mobile phase, even with addition of a solubilizer (e.g., dioxane and dimethyl formamide). For the sake of symmetric peak shape, the supernatant was diluted with water before being injected into the LC–MS/MS system. Although PPT gen-

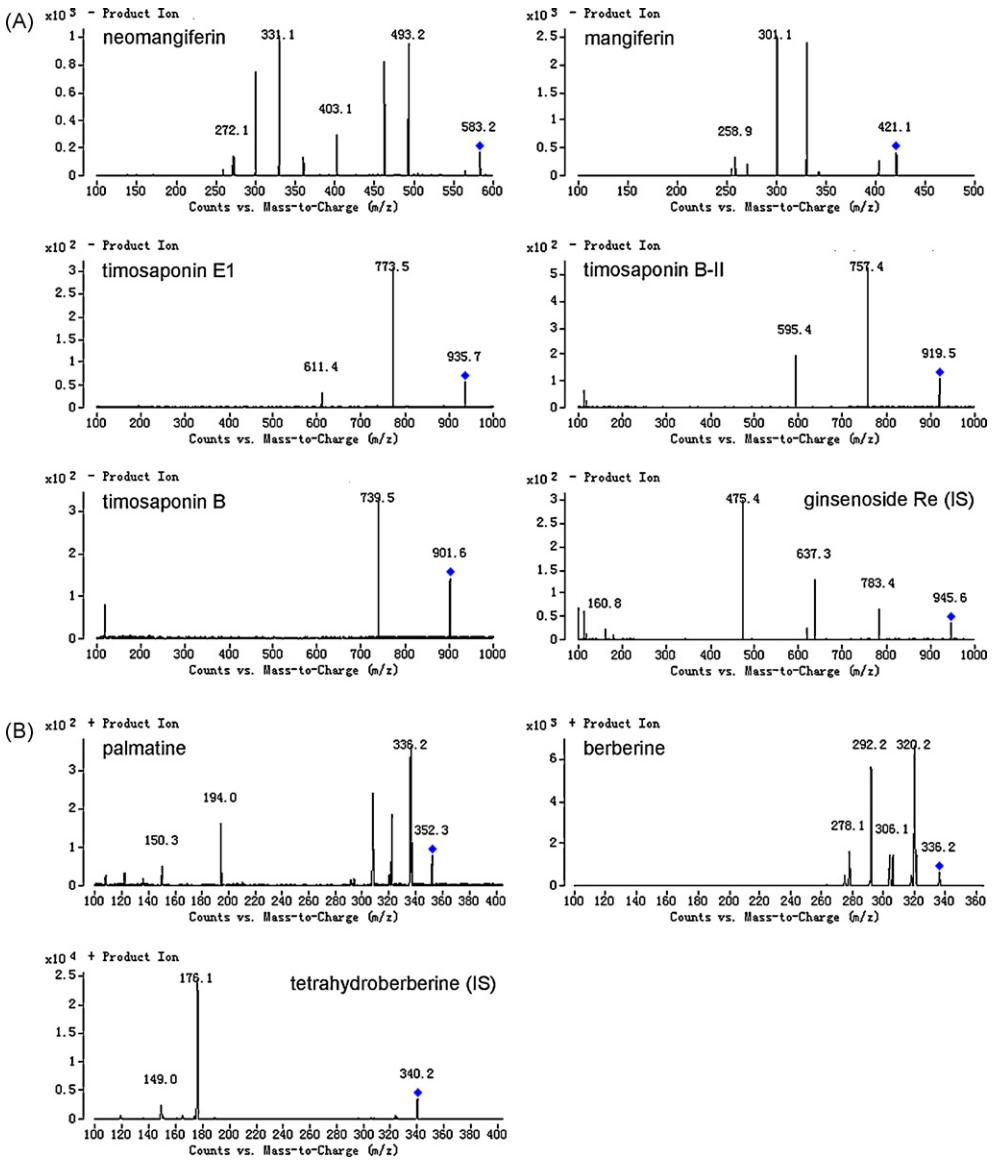

Fig. 2. Product ion mass spectra of the analytes and internal standards: (A) negative ion mode and (B) positive ion mode.

erally yields stronger matrix effects than other sample preparation methods like LLE and solid phase extraction, it is a simple and fast technique. Hence, PPT was chosen as isolation procedure.

3.3. Selectivity

Blank plasma samples from six sources were screened and found to be free of interference from endogenous components or other sources at the same mass transitions and retention times as the analytes and IS. Under the conditions set forth, the retention time was 1.57 min for neomangiferin, 2.00 min for mangiferin, 5.88 min for timosaponin E1, 6.04 min for timosaponin B-II, 6.04 min for ginsenoside Re (IS), 6.37 min for timosaponin B, 6.85 min for tetrahydroberberine (IS), 7.08 min for palmatine, and 7.38 min for berberine. Fig. 3 shows the representative chromatograms of the blank plasma, blank plasma spiked with seven analytes and two IS, and plasma obtained 4 h after oral administration of ZSP extract.

3.4. Linearity and lower limit of quantification

Calibration curves were calculated using linear regression with a $1/x$ weighting, where y represents the peak area ratios (analyte/IS) and x represents the relative concentrations (analyte/IS). The method was linear over the concentration range of 5–1000 ng/mL for mangiferin, 0.5–100 ng/mL for neomangiferin, timosaponin E1, timosaponin B-II and timosaponin B, and 0.05–10 ng/mL for palmatine and berberine. The mean values of regression equations of the analytes are listed as follows:

Neomangiferin	$y = 2.8898x + 0.0594$	$r^2 = 0.9981$
Mangiferin	$y = 13.780x + 0.4843$	$r^2 = 0.9970$
Timosaponin E1	$y = 5.7814x + 0.0048$	$r^2 = 0.9974$
Timosaponin B-II	$y = 8.3044x + 0.1281$	$r^2 = 0.9977$
Timosaponin B	$y = 13.308x + 0.0074$	$r^2 = 0.9969$
Palmatine	$y = 0.3376x + 0.0606$	$r^2 = 0.9975$
Berberine	$y = 0.4368x + 0.1274$	$r^2 = 0.9961$

The lower limit of quantification (LLOQ) is defined as the lowest concentration of the analyte that can be measured with an acceptable accuracy within ±20% (% bias) and precision ≤20% (% R.S.D). The LLOQ samples of six different rat plasma independent of the calibration curves were analyzed. A signal-to-noise (S/N) > 10 at the LLOQ was observed for all the analytes. It was found that the LLOQ was 0.2 ng/mL for mangiferin, 0.5 ng/mL for neomangiferin, timosaponin E1, timosaponin B-II and timosaponin B, and 0.05 ng/mL

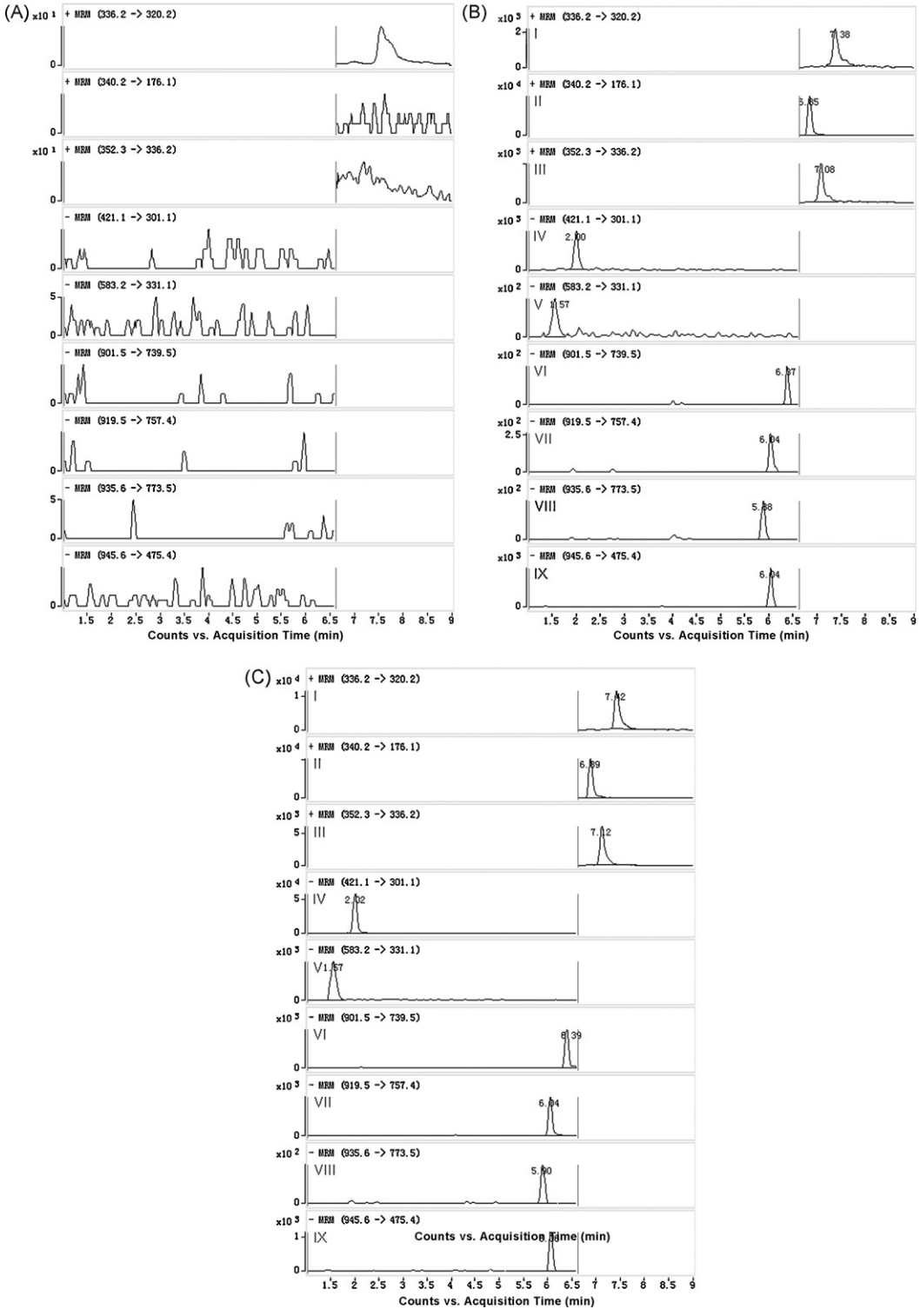

Fig. 3. Representative MRM chromatograms of (I) berberine, (II, IS) tetrahydroberberine, (III) palmatine, (IV) mangiferin, (V) neomangiferin, (VI) timosaponin B, (VII) timosaponin B-II, (VIII) timosaponin E1, and (IX, IS) ginsenoside Re: (A) blank plasma sample, (B) blank plasma sample spiked with seven analytes at LLOQ and IS, and (C) plasma sample collected from a rat 4 h after oral administration of ZSP extract at a dose of 1.94 g/kg.

for palmatine and berberine. These limits are sufficient for pharmacokinetic study of the seven active constituents following an oral administration of ZSP extract to rats. Carry-over of berberine from the injector became problematic at concentrations higher than 10 ng/mL, even with an injector wash step. At the concentration of 10 ng/mL, carry-over of berberine was equivalent to about 900 area units, approximately 6% of the peak area at the LLOQ (0.05 ng/mL), while the carry-over effect was inferior to 1% for other compounds at the upper end of the curve.

3.5. Matrix effects and extraction recovery

The evaluation of matrix effects on the quantitative analysis of drugs in biological fluids by mass spectrometry is an important

Table 2
Matrix effects and extraction recovery for the analytes in rat plasma ($n = 6$).

Analyte	Spiked concentration (ng/mL)	Absolute matrix effect		Extraction recovery	
		Mean (%)	R.S.D. (%)	Mean (%)	R.S.D. (%)
Neomangiferin	1	60.6	10.9	83.1	9.2
	10	59.6	11.6	80.2	10.6
	80	58.1	1.7	87.4	5.3
Mangiferin	8	172.3	10.4	87.0	8.3
	80	177.7	4.9	90.7	4.4
	640	172.5	4.5	92.9	8.1
Timosaponin E1	1	86.5	7.8	93.3	11.6
	10	83.7	10.5	93.8	4.4
	80	84.3	5.4	88.6	7.8
Timosaponin B-II	1	92.8	14.5	83.7	6.7
	10	85.8	4.8	86.3	8.6
	80	93.9	4.8	88.4	4.9
Timosaponin B	1	79.5	11.1	83.9	7.9
	10	80.1	7.8	86.4	5.2
	80	84.3	6.0	90.2	7.1
Palmatine	.0.1	69.5	8.3	73.9	6.0
	1	67.9	1.7	75.9	3.8
	8	67.8	4.1	77.5	5.1
Berberine	.0.1	73.9	11.6	64.7	8.0
	1	73.7	6.1	69.1	2.9
	8	75.3	3.9	68.1	4.2
Ginsenoside Re (IS)	100	103.3	4.7	82.6	3.1
Tetrahydroberberine (IS)	1	78.0	4.9	75.6	5.6

aspect of assay validation [21]. When analyzing the supernatant from a plasma sample using protein precipitation, salts and endogenous materials are present and can cause ion suppression or enhancement, which may be greater than that of solid-phase and liquid–liquid extracts. The assessment of matrix effects in this study is therefore of practical importance during validation of the assay. Table 2 shows the results of matrix effects for the analytes and IS. Although the matrix effects caused intense ion suppression for neomangiferin, which were 60.6, 59.6 and 58.1% at 1, 10 and 80 ng/mL, respectively, the results showed that there was no severe variation across all QC concentration levels. In contrast, the mean value of the absolute matrix effect for mangiferin ranged from 172.3 to 177.7% over the entire QC concentration range, suggesting significant ion enhancement under the experimental conditions, but the matrix effects were consistent and concentration-independent. Thus, despite the absolute matrix effects were indeed observed, the present analytical method is stable and reliable. As shown in Table 2, the PPT procedure used in this study yielded excellent

Table 3
Precision and accuracy for the analytes in rat plasma ($n = 15$, 5 replicates per day for 3 days).

Analyte	Nominal concentration (ng/mL)	Intra-day			Inter-day		
		Measured concentration (ng/mL)	Precision (% R.S.D.)	Accuracy (% bias)	Measured concentration (ng/mL)	Precision (% R.S.D.)	Accuracy (% bias)
Neomangiferin	1	0.963	4.6	−3.7	0.979	4.2	−2.1
	10	10.39	8.0	3.9	10.25	6.0	2.5
	80	79.30	4.8	−0.9	79.89	3.8	−0.1
Mangiferin	8	8.454	6.7	5.7	8.318	5.8	4.0
	80	74.23	4.7	−7.2	74.51	6.0	−6.9
	640	645.01	1.9	0.8	653.36	3.9	2.1
Timosaponin E1	1	1.035	6.3	3.5	1.019	7.3	1.9
	10	9.86	4.1	−1.4	9.72	5.7	−2.8
	80	78.76	1.3	−1.5	81.32	4.8	1.6
Timosaponin B-II	1	1.023	11.7	2.3	1.000	9.2	0.0
	10	10.41	4.9	4.1	10.11	6.1	1.1
	80	79.98	3.6	0.0	79.82	4.4	−0.2
Timosaponin B	1	0.964	7.5	−3.6	0.966	5.7	−3.4
	10	9.69	6.2	−3.1	9.70	7.6	−3.0
	80	78.51	2.2	−1.9	81.42	4.9	1.8
Palmatine	.0.1	0.108	4.2	7.7	0.106	4.8	5.8
	1	0.96	5.5	−3.5	0.96	5.1	−3.6
	8	7.80	4.1	−2.6	8.01	4.8	0.2
Berberine	.0.1	0.091	10.1	−9.0	0.095	8.6	−4.5
	1	1.11	1.3	10.9	1.07	6.8	7.0
	8	7.89	7.2	−1.4	7.98	6.3	−0.3

Table 4
Stability of the analytes in rat plasma ($n = 5$).

Analyte	Spiked concentration (ng/mL)	Stability (% RE[a])			
		Three freeze-thaw	Short-term (4 h at room temperature)	Long-term (2 weeks at −20 °C)	Post-preparative (12 h at room temperature)
Neomangiferin	1	−2.6	8.0	4.5	−10.9
	10	−6.2	−1.5	7.5	8.0
	80	−1.3	6.1	6.3	−4.1
Mangiferin	8	−5.8	4.1	6.8	1.5
	80	−10.9	−5.1	6.2	2.9
	640	−3.9	−7.4	5.9	−1.6
Timosaponin E1	1	11.6	3.4	5.3	12.9
	10	−6.8	−1.1	−11.3	−0.6
	80	4.3	2.1	5.3	−1.3
Timosaponin B-II	1	2.1	4.9	9.3	−10.1
	10	11.3	8.2	10.6	2.7
	80	2.0	9.6	7.3	−2.0
Timosaponin B	1	8.1	7.4	11.6	11.8
	10	−1.8	4.2	1.5	7.1
	80	−0.9	6.1	9.3	−3.3
Palmatine	.0.1	−5.7	−7.8	−10.1	−2.7
	1	−6.8	−0.9	−0.4	−3.4
	8	−8.9	−0.2	−6.8	−5.8
Berberine	.0.1	−8.2	−8.1	4.9	5.1
	1	−3.2	−7.8	10.5	−1.2
	8	−8.7	0.1	−0.7	−4.1

[a] RE is expressed as (measured concentration/freshly prepared concentration − 1) × 100%.

extraction recovery of greater than 80% for all analytes, except for palmatine (range 73.9–77.5%) and berberine (range 64.7–69.1%), indicating that the overall extraction recovery was efficient and consistent.

3.6. Precision and accuracy

Intra- and inter-day precision was assessed from the results of QC samples by using a one-way analysis of variance (ANOVA). Table 3 summarizes the intra- and inter-day precision and accuracy for the seven analytes from the QC samples. The precision, represented as R.S.D., ranged from 1.3 to 11.7% and 3.8 to 9.2% for intra- and inter-day determination, respectively. The accuracy, represented as percentage bias against the nominal concentration, ranged from −9.0 to 10.9% and −6.9 to 7.0% for intra- and inter-day determination, respectively. All intra- and inter-day precision and accuracy were acceptable.

3.7. Stability

The stability of all the analytes was assessed under various conditions. The results presented in Table 4 indicate that these analytes were all stable in plasma after three freeze–thaw cycles, at room temperature for 4 h, and at −20 °C for 2 weeks. Post-preparative stability of the analytes also showed that no significant degradation occurred when the extracted samples were kept in the autosampler at room temperature for 12 h.

3.8. Pharmacokinetic study

The LC–MS/MS method developed was applied to investigate the pharmacokinetics of the seven active constituents in ZSP after an oral administration of 1.94 g extract/kg (neomangiferin 15.9 mg/kg, mangiferin 23.0 mg/kg, timosaponin E1 13.2 mg/kg, timosaponin B-II 88.5 mg/kg, timosaponin B 27.0 mg/kg, palmatine 16.2 mg/kg and berberine 25.5 mg/kg) to six rats. Fig. 4 shows the plasma concentration–time profiles of the analytes in rats given single oral administration. The main pharmacokinetic parameters were calculated and summarized in Table 5.

As showed in Fig. 1, neomangiferin is the derivative of mangiferin, in which the hydroxyl group attached to C-7 is substituted by glucose, but their pharmacokinetic parameters are remarkably different. With T_{max} at 0.58 h, neomangiferin was absorbed more rapidly than mangiferin (T_{max} at 4.22 h), where T_{max} of the latter was in conformity with the previous reference [15]. The contents of neomangiferin and mangiferin were 0.818 and 1.18 g/100 g extract, respectively, while the value of C_{max} and AUC of neomangiferin was much lower than that of mangiferin. This phenomenon might be attributed to the biotransformation of neomangiferin to mangiferin by intestinal bacteria and enzymes *in vivo*, resulting in great increase of mangiferin in plasma. In addition, it is likely that constituents in herbal preparations may be substrates, inhibitors, or inducers of cytochrome P450, and thus have an impact on the pharmacokinetics of each other [22].

As shown in Table 5 and Fig. 4B, owing to the very similar structures, timosaponin E1, timosaponin B-II and timosaponin B have parallel pharmacokinetic parameters and concentration–time curves *in vivo*, being absorbed and eliminated with the similar rate. Notably, distinct double-peaks were observed in both individual and mean plasma concentration–time curves of all these timosaponins. Previous pharmacokinetic studies [23,24] also revealed the double-peak phenomenon in several other saponins. It is well known that drug absorption is a very complex process that manifests itself through potential interaction with a host of physicochemical and physiological variables. Some factors that may affect the absorption process include presystemic metabolism/efflux, “absorption window” along the gastrointestinal tract, enterohepatic recirculation, variable gastric emptying and drug–drug interactions [23,25]. Therefore, atypical drug absorption profiles such as double-peak and absorption window-type absorption profiles are often encountered [25]. Further detailed absorption studies are needed to

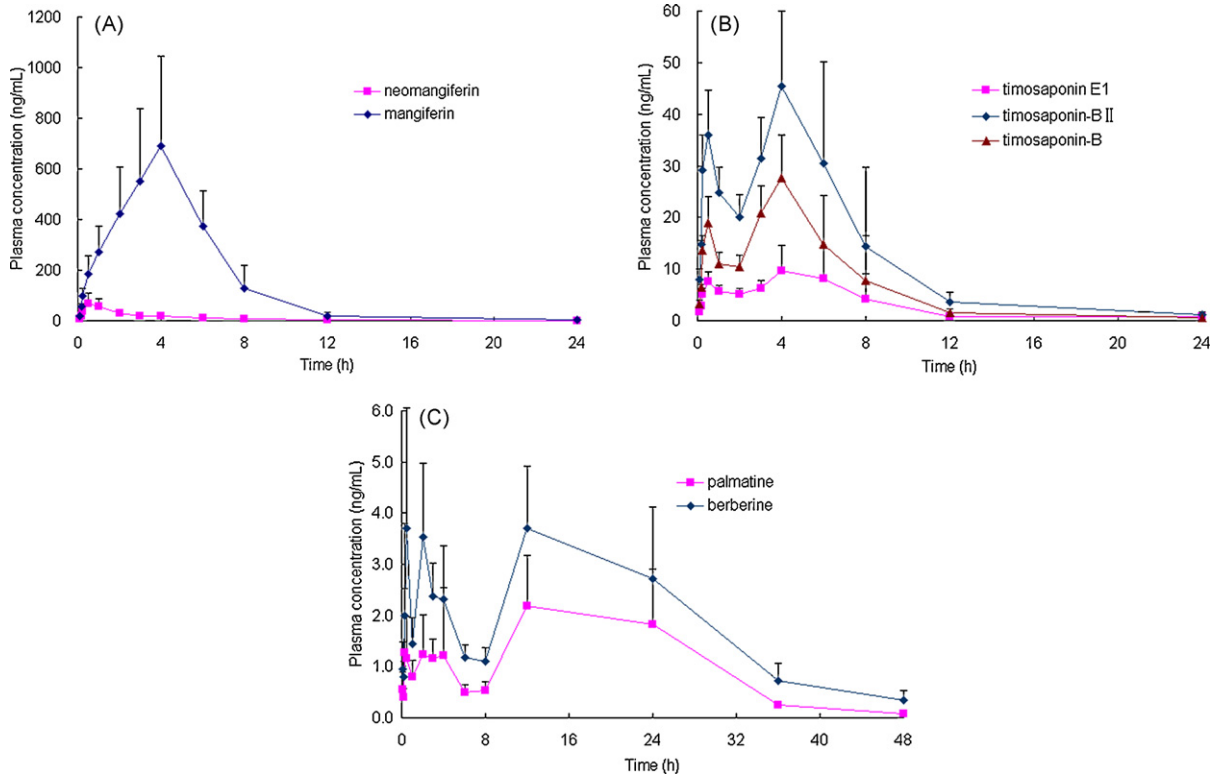

Fig. 4. Mean plasma concentration–time profiles of (A) xanthone glycosides, (B) timosaponins, and (C) alkaloids in rats after oral administration of ZSP extract at a dose of 1.94 g/kg (each point represents mean ± S.D., $n = 6$).

Table 5
Pharmacokinetic parameters of the seven constituents in rats after oral administration of ZSP extract (mean ± S.D., $n = 6$).

Compound	C_{max} (ng/mL)	T_{max} (h)	$t_{1/2}$ (h)	AUC_{0-t} (ng h/mL)	$AUC_{0-\infty}$ (ng h/mL)	MRT (h)	CL (L/kg/h)
Neomangiferin	71.4 ± 38.6	0.58 ± 0.25	3.48 ± 1.92	222.3 ± 93.1	224.7 ± 92.9	4.56 ± 1.28	86.6 ± 47.2
Mangiferin	874.9 ± 302.3	4.22 ± 1.79	2.80 ± 0.97	3593.0 ± 1279.6	3607.9 ± 1278.4	4.87 ± 1.25	10.1 ± 3.7
Timosaponin E1	15.2 ± 5.3	3.00 ± 2.05	4.70 ± 2.01	77.1 ± 30.4	81.8 ± 30.0	8.45 ± 4.55	228.0 ± 58.1
Timosaponin B-II	63.5 ± 17.0	3.44 ± 2.05	4.91 ± 2.92	296.0 ± 101.7	304.0 ± 99.9	6.80 ± 2.26	373.5 ± 85.5
Timosaponin B	35.0 ± 8.5	4.00 ± 0.82	3.06 ± 1.08	158.8 ± 50.4	162.5 ± 51.0	5.38 ± 1.96	214.2 ± 52.2
Palmatine	3.8 ± 1.1	13.33 ± 4.55	6.78 ± 3.35	59.9 ± 16.7	67.9 ± 27.1	19.54 ± 8.78	411.8 ± 145.6
Berberine	6.8 ± 2.1	11.83 ± 5.02	7.88 ± 3.06	99.1 ± 23.3	114.1 ± 35.6	21.00 ± 9.29	314.9 ± 96.6

elucidate the mechanism of the double-peak phenomenon in pharmacokinetics.

It is interesting to note that multiple plasma concentration peaks of palmatine and berberine were observed in Fig. 4C, as was reported by Yu et al. [17] and Deng et al. [18]. The factors mentioned above may also contribute to this result; besides another probable factor is distribution re-absorption. If the concentration of one drug in tissue is much higher than that in plasma, it is possible for the drug to transfer from tissue to plasma, causing another peak in plasma. It was reported [26] that berberine distributed rapidly after absorption, with a higher concentration in tissue. The poor absorption and extensive metabolism may take responsibility for the extremely low plasma concentration of berberine after oral administration [17]. Reasonably, the plasma concentration of palmatine is also quite low, for its structure is similar to that of berberine.

4. Conclusions

A rapid, sensitive and selective LC–MS/MS method involving switching of the ionization polarity has been developed and validated for the simultaneous determination of the main active constituents of ZSP in rat plasma. It was successfully applied to the preliminary pharmacokinetic study of these constituents after oral administration of ZSP extract. The excellent selectivity, sensitivity, precision, accuracy and dynamic range proved that the method is suitable for pharmacokinetic study. Furthermore, the relative short chromatographic run time and straightforward sample pretreatment procedure allow for fast and easy analysis of plasma samples. In addition, the method can be further adapted for the analysis of other biological samples such as urine, bile and various tissues. This new method will be used in our ongoing pharmacokinetic interaction study of ZSP in rats.

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