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Simultaneous determination of ginsenoside Rg1, Re, Rd, Rb1 and ophiopogonin D in rat plasma by liquid chromatography/electrospray ionization mass spectrometric method and its application to pharmacokinetic study of 'SHENMAI' injection

Chunhua Xia[a,b], Guangji Wang[a,*], Jianguo Sun[a], Haiping Hao[a], Yuqing Xiong[b], Shenghua Gu[a], Lili Shang[a], Chaonan Zheng[a]

[a] *Key Lab of Drug Metabolism and Pharmacokinetics, China Pharmaceutical University, Nanjing 210009, China*
[b] *Clinical Pharmacology Institute, Medical College of Nanchang University, Nanchang 330006, China*

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Abstract

A sensitive and rapid liquid chromatography–mass spectrometric method for the simultaneous determination of ginsenoside Rg1, Re, Rd, Rb1 and ophiopogonin D in rat plasma was developed and validated. Chromatographic separation was performed on a C_{18} column using a step gradient program with the mobile phase of 0.5 mmol/L ammonium chloride solution and acetonitrile. The analytes and I.S. were detected using an electrospray negative ionization mass spectrometry in the selected ion monitoring (SIM) mode. The method was linear over the investigated concentration range with a good correlation coefficient higher than 0.997. The lower limits of detection (LLOD) of these analytes were all lower than 2.0 ng/mL. The intra- and inter-day precisions were all no more than 7.5% and accuracies were within the range of 97.5–107.0%. The validated method was successfully applied to investigate the pharmacokinetics of ginsenoside Rg1, Re, Rd, Rb1 and ophiopogonin D in rat after intravenous administration of 'SHENMAI' injection.
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Keywords: Ginsenoside Rg1; Re; Rd; Rb1; Ophiopogonin D; LC–ESI-MS; Pharmacokinetics; 'SHENMAI' injection

1. Introduction

'SHENMAI' injection, composed of red ginseng and ophiopogon, is now widely used in China for the treatment of coronary atherosclerotic cardiopathy, viral myocarditis and shock. Ginsenosides and ophiopogonins, isolated from ginseng and ophiopogon, were regarded as the principal constituents responsible for the pharmacological activities. Up to now, more than 30 different ginsenisides and ophiopogonins were identified and isolated. According to their sapogenins with a dammarane skeleton, the former can be divided into two groups, i.e. 20(S)-protopanaxatriol (e.g. ginsenoside Rg1, Re, etc.) and 20(S)-protopanaxadiol (e.g. ginsenoside Rb1, Rd, etc.) and these two types of saponins showed different pharmacological effects. Chemical and pharmacological studies demonstrated that ophiopogonins mainly included phiopogonin A, B, C, D, J and cinnamonvine aglycone, etc. and phiopogonin D was one of the major active components in ophiopogon.

To date, there are many literatures described about the quantification of ginsenosides based on thinlayer chromatography (TLC) [1], enzyme immunoassay (EIA) [2], high performance liquid chromatography with ultraviolet detection (HPLC-UV) [3], high performance liquid chromatography with fluorescence detection (HPLC-FLD) [4], liquid chromatography-evaporative light-scattering detection (LC-ELSD) [5], liquid chromatography mass spectrometry (LC–MS) [6–9] and liquid chromatography-tadem mass spectrometry (LC–MS/MS) [10,11]. However, these methods were mainly focused on the quantification of some ginsenosides in the raw materials or final ginseng products or one of ginsenosides in plasma, and few of them were reported for the simultaneous determination of multiple ginsenosides in biological fluids.

* Corresponding author. Tel.: +86 25 8327 1128.
E-mail address: guangjiwang@hotmail.com (G. Wang).

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Recently, Yu et al. developed a method to simultaneously detect five ginsenosides in plasma for a pharmacokinetic study of 'SHENMAI' injection [12]. But the analytes were not involved in any representative active component from ophiopogon. The studies on the absorption, distribution, metabolism and excretion of ophiopogonin in body are not reported until today, though there are several methods such as HPLC-UV [13], HPLC-ELSD [14–16] and TLC [17] established for the identification and quantification of ophiopogonin (B, D, D', J) in medical materials or extracts.

It was confirmed that ginsenoside Rg1, Re, Rd, Rb1 and ophiopogonin D were all the major active constituents in 'SHENMAI' injection. For a comprehensive understanding of the pharmacokinetics of 'SHENMAI' injection, it is crucial to develop a reliable method for the simultaneous determination of ginsenosides and ophiopogonins in plasma after intravenous administration of 'SHENMAI' injection. In the paper, therefore, we developed a sensitive and rapid LC–ESI-MS method for the simultaneous determination of ginsenoside Rg1, Re, Rd, Rb1 and ophiopogonin D in rat plasma.

2. Experimental

2.1. Chemicals and reagents

Ginsenoside Rg1, Re, Rd, Rb1 and digoxin (purity > 98.0%) were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Ophiopogonin D (purity > 98.0%) was provided by Shanghai kaimi Co. Ltd. (Shanghai, China). 'SHENMAI' injection was supplied by Yaan pharmaceutical Co. Ltd. (Yaan, China). Acetonitrile (Fisher Company, USA) and methanol (Merck, Germany) were of HPLC grade, ammonium chloride was of analytical grade. Distilledwater, doubly distilled in the laboratory, was used throughout the study.

2.2. Equipments and chromatographic conditions

The HPLC system consisted of a LC-10AD pump, a DGU-14 AM degasser, a Shimadzu 10ATvp autoinjector and a CTO-10 Avp column oven (Shimadzu, Japan). A Shimadzu 2010 liquid chromatograph–mass spectrometer (Shimadzu, Japan) equipped with an ESI probe and a QoQ system was used in this study.

The chromatographic separation was carried on a LUNA 5 μ C18 column (150 mm × 2.00 mm, Phenomenex®, USA) with the mobile phase of 0.5 mmol/L ammonium chloride solution (A) and acetonitrile (B) at a flow rate of 0.2 mL/min. A gradient elution program was used as follows: 0.03 → 6.0 min, B% 30 → 90; 6.0 → 7.0 min, B% 90 → 90; 7.0 → 7.5 min, B% 90 → 30. The column and autosampler tray temperature were kept constant at 40 °C and 4 °C, respectively.

Samples were ionized by negative-ion electrospray ionization (ESI) probe in the negative ion mode under the following tuning conditions: curve dissolution line (CDL) voltage was fixed as that in tuning and probe high voltage was set at 4.5 kV, Qarray voltage −70 V and RF 150 V. Mass spectra were obtained at a dwell time of 0.2 s and 1 s for SIM and scan mode accordingly. Analysis was carried out using selected ion monitoring (SIM) at *m/z* 835.80 for ginsenoside Rg1, 981.80 for ginsenoside Re and Rd, 1143.65 for Rb1, 899.50 for ophiopogonin D and 815.40 for digoxin $[M + Cl]^-$. Peak areas for all components were automatically integrated using LC/MS solution Version 2.04.

2.3. Preparation of standard solution and quality control samples

Ginsenoside Rg1, Re, Rd, Rb1, ophiopogonin D and digoxin (I.S.) were all dissolved in methanol for the final concentrations of 1.040 mg/mL, 1.224 mg/mL, 1.316 mg/mL, 1.112 mg/mL, 1.244 mg/mL and 400 μg/mL, respectively. A series of working solutions of these analytes were obtained by further diluting the stock solutions with methanol. The effective concentrations in plasma for calibration curve ranged from 4.5 ng/mL to 1728.0 ng/mL for ginsenoside Rg1, from 3.0 ng/mL to 1152.0 ng/mL for Re and Rd, from 6.0 ng/mL to 2304.0 ng/mL for Rb1 and from 3.5 ng/mL to 672.0 ng/mL for ophiopogonin D, respectively. For the validation of the method, three concentration levels of QC plasma samples were prepared containing ginsenoside Rg1 (10.0 ng/mL, 100.0 ng/mL, 1000.0 ng/mL), Re and Rd (8.0, 80.0, 800.0 ng/mL), Rb1 (15.0 ng/mL, 150.0 ng/mL, 1500.0 ng/mL) and ophiopogonin D (7.5 ng/mL, 50.0 ng/mL, 500.0 ng/mL).

2.4. Sample preparation

In our study, a conventional liquid–liquid extraction method was used to prepared plasma samples. After 100 μL of plasma sample was transferred into a 1.5 mL plastic test tube together with 10 μL of IS solution, 1.2 mL of *n*-butanol was added. The analytes and IS were extracted from plasma by vortex-mixing for 3 min. Then the sample was centrifuged at 20,000 × *g* for 5 min and 1000 μL of the organic layer was transferred into another test tube and evaporated to dryness using evaporator at 50 °C. Finally, the residue was reconstituted in 100 μL methanol and centrifuged at 30,000 × *g* for 10 min, 5 μL aliquot was injected into chromatographic system for analysis.

2.5. Method validation

The lower limit of quantification (LLOQ) was determined as the lowest concentration point of the standard curve. The lower limit of detection (LLOD) was defined as the amount that could be detected with a signal-to-noise ratio of 3.

Plasma samples were quantified by using the ratio of the peak area of analyte to that of I.S. as the assay parameter. Standard curves representing peak area ratios versus analyte concentrations were described in the form of $y = a + bx$. To evaluate linearity, plasma calibration curves were prepared and assayed in duplicate on separate 5 days.

The accuracy and precision were calculated by determining QC samples at high, middle, low and LLOQ concentration levels on three different validation days. The accuracy was expressed by (mean measured concentration)/(spiked concentration) × 100% and the precision by relative standard deviation (RSD%).

The extraction recovery of analyte at three QC levels were determined by comparing the peak areas obtained from QC samples with the un-extracted standard working solutions at the same concentration in the same solvent.

The stability of analyte in rat plasma was assessed by analyzing QC samples at three concentrations exposed to different time and temperature conditions. The long-term stability was assessed after the QC samples had been stored at −20 °C for 30 days. The freeze-thaw stability was determined after three freeze–thaw cycles (−20 °C to 20 °C) on 3 consecutive days.

2.6. Application

Six Sprague–Dawley rats (200–230 g) were obtained from Sino-British Sippr/BK Lab Animal Ltd. (Shanghai, China). After an intravenous administration of 'SHENMAI' injection at a dosage of 5 mL/kg though the tail vein, blood samples (about 250 μL) were drawn in heparinized polythene tubes at times 0 h, 0.03 h, 0.17 h, 0.5 h, 1 h, 1.5 h, 2 h, 3 h, 5 h, 8 h, 12 h, 24 h, 48 h, 72 h and immediately centrifuged to separate 100 μL plasma. The obtained plasma samples were stored at −20 °C until analysis.

3. Results and discussion

3.1. Method development

The mass spectra of ginsenoside Rg1, Re, Rd, Rb1, ophiopogonin D and internal standard (I.S.) were presented in Fig. 1. In the full scan spectra, it can be observed that predominant chloridized adduct ions $[M+Cl]^-$ of ginsenoside Rg1, Re, Rd, Rb1, ophiopogonin D and IS were formed with *m/z* 835.80, 981.80, 981.80, 1143.65, 889.50 and 815.40, respectively. A higher signal of detection of these analytes was obtained when monitoring the adduct ions $[M+Cl]^-$ instead of $[M-H]^-$, and the addition of ammonium chloride in mobile phase could enhance the formation of chloride adduct of the analyte molecule $[M+Cl]^-$. For the analysis of multiple components, a gradient program was often used to improve the resolution and peak shapes. In our research, a gradient elution program was employed and 30% organic phase was adopted as the initiated concentration of gradient program which was crucial to the peak shape and retention time of these analytes. With the gradient procedure, the assay of every sample could be completed within 13 min.

It is necessary to use an I.S. for accuracy when a mass spectrometer is equipped with HPLC as the detector. In present study,

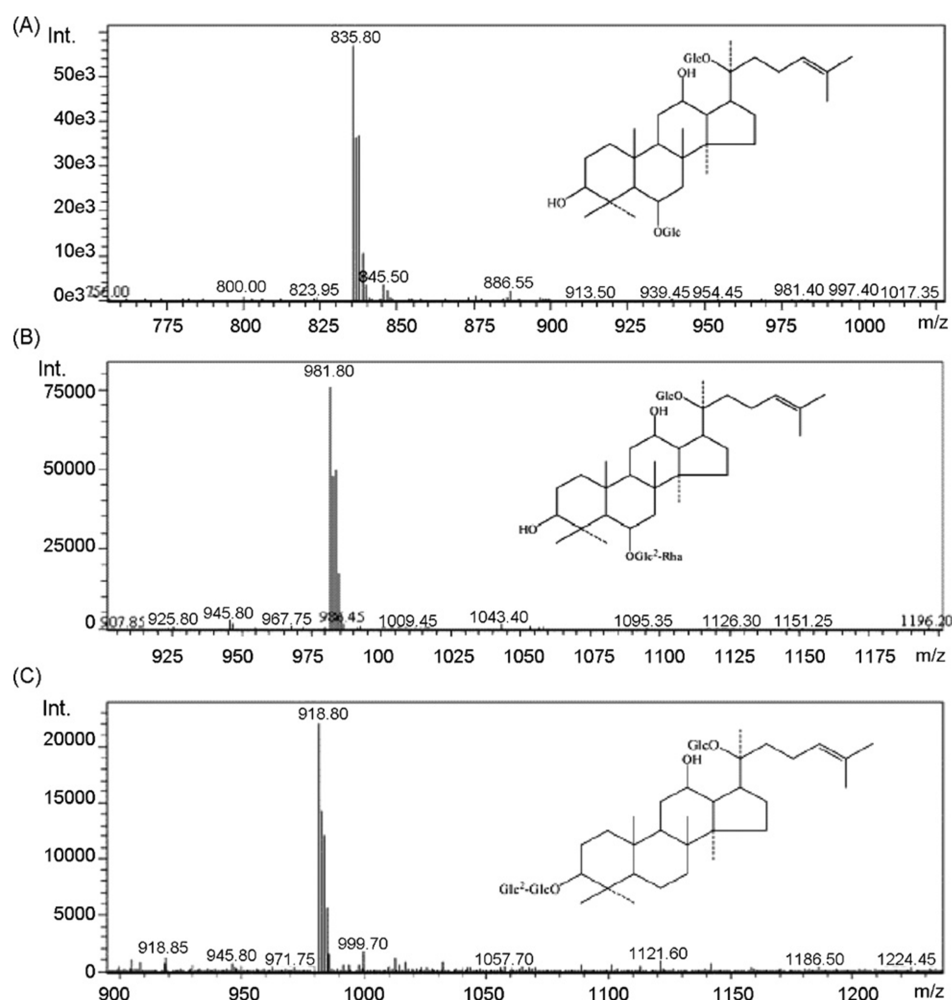

Fig. 1. Mass spectra of ginsenoside Rg1(A), Re(B), Rd(C), Rb1(D), ophiopogonin D (E) and I.S. (F) in the negative ionization mode of full scan.

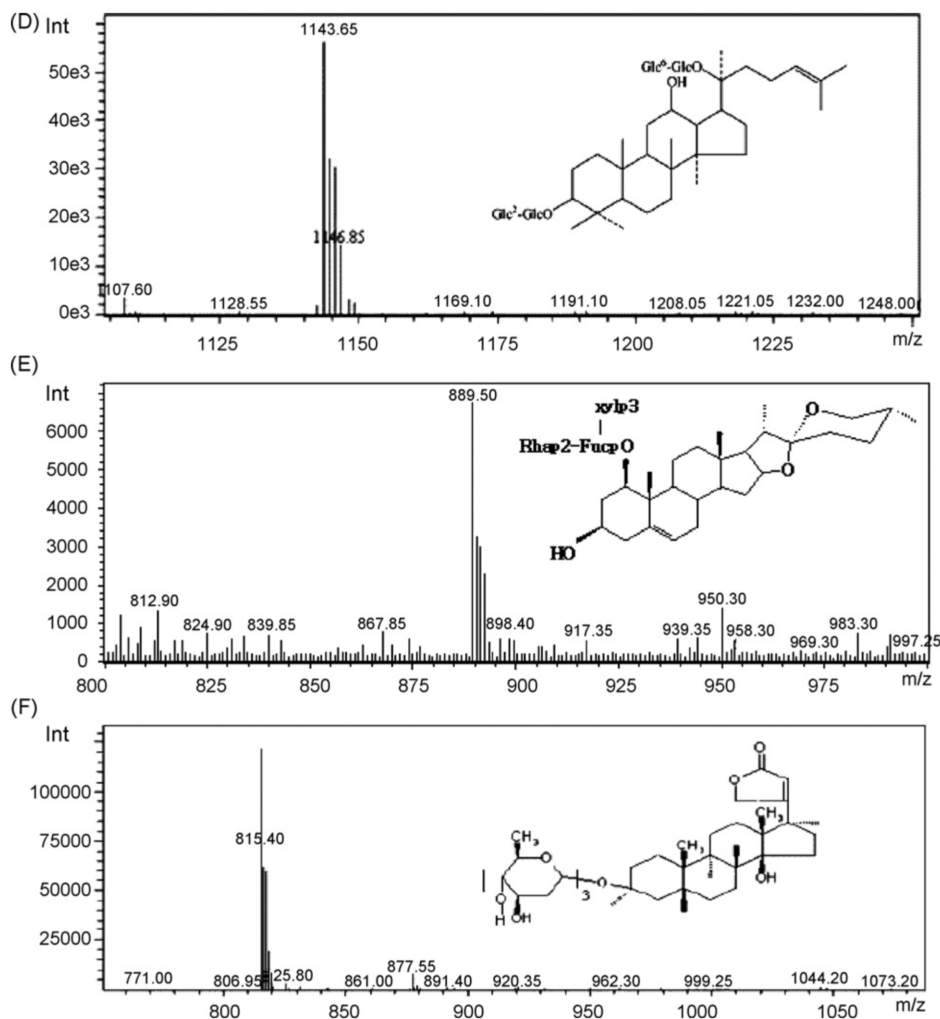

Fig. 1. (*Continued*).

therefore, digoxin was chosen as the internal standard because of its similarity of chemical structure, retention action, ionization and extraction efficiency as well as its less endogenous interference at chlorinated adducts of molecular ions *m/z* 815.40. On account of a certain difference in the physiochemical properties, it is also essential to select a proper method to prepare the sample with a high recovery for all these analytes. A solid extraction was often used as an effective technique for the purpose, but the expensive cost and insufficient reproducibility limited its extensive application. Thus, in our study, a conventional liquid–liquid extraction program was used and ethyl acetate, *n*-butanol, trichloromethane and diethyl ether were all tested as extraction solvent, only *n*-butanol was finally adopted because of its higher extraction efficiency (>70%) for all these saponins.

3.2. Method validation

3.2.1. Specificity

The typical chromatograms of ginsenoside Rg1, Re, Rd, Rb1 and ophiopogonin D were presented in Fig. 2. Under the described chromatographic conditions, a good separation was achieved and no obvious interferences from endogenous plasma substances were observed. The retention time of ginsenoside Rg1, Re, Rd, Rb1, ophiopogonin D and I.S. was 4.7 min, 4.6 min, 6.5 min, 5.8 min, 9.3 min and 6.1 min, respectively.

3.2.2. Linearity and lower limit of detection

The linear range and regression equations for quantification of ginsenoside Rg1, Re, Rd, Rb1 and ophiopogonin D were presented in Table 1. The correlation coefficients of these calibration curves were all higher than 0.997. The lower limits of detection (LOD) of these analytes using 100 μL plasma were 1.2–2.0 ng/mL. Compared with some methods reported using MS detector [6,12], a higher sensitivity for Rg1, Rb1 and Rd was obtained in our study and made this method more advantageous to measure the trace concentration of these analytes in plasma.

3.2.3. Extraction recovery, precision and accuracy

The extraction recoveries of ginsenoside Rg1, Re, Rd, Rb1 and ophiopogonin D in rat plasma were shown in Table 2. At three concentration levels of these analytes, the extraction recov-

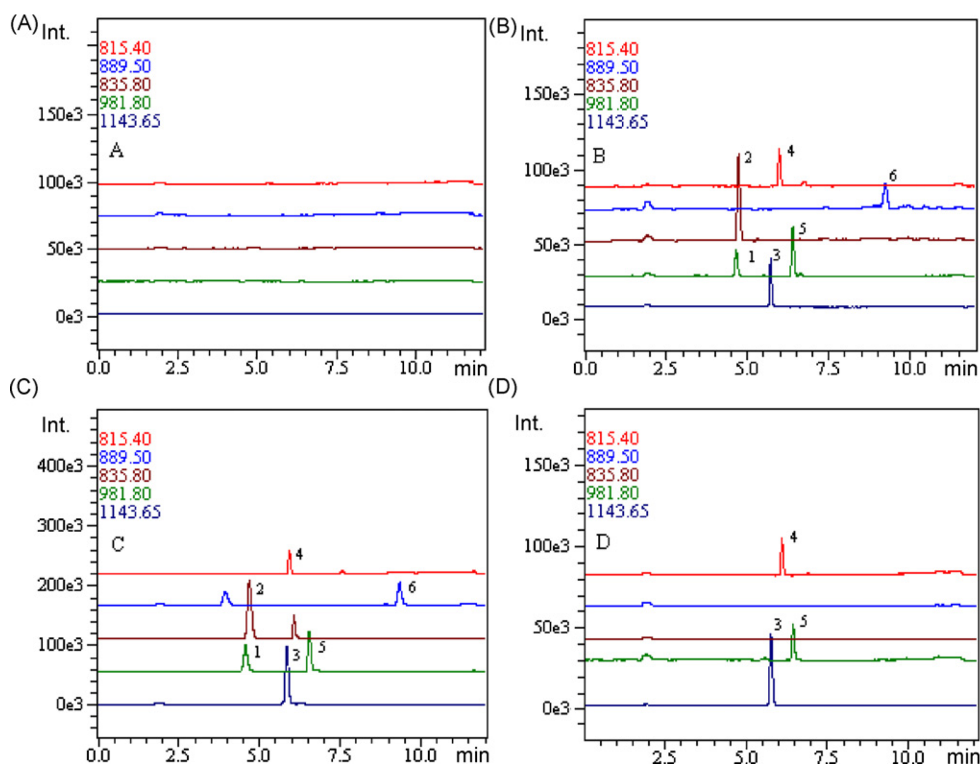

Fig. 2. SIM chromatograms of ginsenoside Rg1(2#), Re(1#), Rd(5#), Rb1(3#), ophiopogonin D (6#) and IS (4#) (A) blank plasma; (B) blank plasma spiked with ginsenoside Rg1, Re, Rd, Rb1 and ophiopogonin D; (C) plasma sample at 10 min after administration of 'SHENMAI' injection; (D) plasma sample at 12 h after administration of 'SHENMAI' injection.

Table 1
The linearity, LLOD and LLOQ of the assay for ginsenoside Rg1, Re, Rd, Rb1 and ophiopogonin D

Analytes	Calibration curves ($y = ax + b$)	Linear range (ng/mL)	R^2	LLOQ (ng/mL)	LLOD (ng/mL)
Ginsenoside Rg1	$y = (358.50 \pm 21.32)x - (3.9765 \pm 1.1422)$	4.5–1728.0	0.998	4.5	1.5
Ginsenoside Re	$y = (869.06 \pm 36.33)x - (9.1839 \pm 3.6355)$	3.0–1152.0	0.998	3.0	1.5
Ginsenoside Rd	$y = (632.32 \pm 41.56)x - (11.969 \pm 4.0124)$	3.0–1152.0	0.997	3.0	1.5
Ginsenoside Rb1	$y = (440.23 \pm 19.18)x - (2.7614 \pm 1.5238)$	6.0–2304.0	0.998	6.0	2.0
Ophiopogonin D	$y = (243.14 \pm 15.39)x - (4.8515 \pm 1.7794)$	3.5–672.0	0.999	3.5	1.2

Table 2
The extraction recoveries of ginsenoside Rg1, Re, Rd, Rb1 and ophiopogonin D in rat plasma ($n = 5$)

Analytes	Spiked conc. (ng/mL)	Recovery (%)	RSD (%)
Ginsenoside Rg1	10.0	86.4 ± 4.3	5.0
	100.0	83.3 ± 5.2	6.3
	1000.0	85.4 ± 3.7	4.4
Ginsenoside Re	8.0	76.0 ± 3.7	4.9
	80.0	79.2 ± 3.5	4.4
	800	77.7 ± 5.6	7.1
Ginsenoside Rd	8.0	71.3 ± 3.9	5.5
	80.0	72.0 ± 4.5	6.3
	800.0	70.0 ± 2.9	4.1
Ginsenoside Rb1	15.0	83.1 ± 3.9	4.7
	150.0	85.1 ± 3.7	4.4
	1500.0	84.4 ± 3.5	4.1
Ophiopogonin D	7.5	80.5 ± 5.2	6.5
	50.0	82.6 ± 4.2	5.0
	500.0	81.3 ± 2.7	3.3

eries were all more than 70%. Table 3 summarized the intra- and inter-day precisions and accuracies of ginsenoside Rg1, Re, Rd, Rb1 and ophiopogonin D at different concentration levels (included LLOQ concentration). As shown in Table 3, the intra- and inter-day accuracies of these analytes were within the range of 97.5–107.0% and 98.1–106.3%, respectively. The intra- and inter-day precisions (RSD) of these analytes were all no more than 7.5%. The results demonstrated that the values were all within the acceptable range and the method was accurate and precise.

3.2.4. *Stability*

The stability of ginsenoside Rg1, Re, Rd, Rb1 and ophiopogonin D was shown in Table 4. The results indicated that these analytes in rat plasma were all stable for three cycles of freeze-thaw, 24 h at room temperature, 30 days at −20 °C with accuracy in the range of 102.7–108.0%. Stock solutions of these analytes were all stable for 24 h at room temperature and at least 3 weeks at 4 °C with the accuracy of 101.7–106.5%.

Table 3
The intra- and inter-day accuracies and precisions of ginsenoside Rg1, Re, Rd, Rb1 and ophiopogonin D in rat plasma at high, middle, low and LLOQ concentration levels ($n = 5$)

Spiked conc. (ng/mL)	Intra-day			Inter-day		
	Measured conc. (ng/mL)	Accuracy (%)	Precision (%)	Measured conc. (ng/mL)	Accuracy (%)	Precision (%)
Ginsenoside Rg1						
4.5	4.8 ± 0.3	106.9	7.1	4.8 ± 0.3	106.0	6.9
10.0	10.5 ± 0.5	104.6	5.1	10.3 ± 0.5	103.2	5.2
100.0	107.0 ± 7.6	107.0	7.1	105.3 ± 6.5	105.3	6.2
1000.0	1040.4 ± 39.8	104.0	3.8	1047.5 ± 34.9	104.7	3.3
Ginsenoside Re						
3.0	2.9 ± 0.2	97.7	7.2	2.9 ± 0.2	98.1	7.1
8.0	7.8 ± 0.6	97.5	7.5	7.9 ± 0.5	98.8	6.6
80.0	83.3 ± 3.9	104.2	4.7	83.6 ± 3.7	104.5	4.4
800.0	845.1 ± 36.7	105.6	4.3	840.2 ± 36.4	105.0	4.3
Ginsenoside Rd						
3.0	3.2 ± 0.2	106.3	7.2	3.1 ± 0.2	104.3	7.0
8.0	8.5 ± 0.5	106.0	5.6	8.3 ± 0.5	103.5	6.0
80.0	84.3 ± 3.3	105.4	3.9	83.3 ± 3.5	104.1	4.2
800.0	835.8 ± 35.0	104.5	4.2	835.7 ± 32.0	104.5	3.8
Ginsenoside Rb1						
6.0	6.4 ± 0.4	107.0	6.7	6.4 ± 0.4	106.3	6.6
15.0	15.9 ± 0.8	106.3	4.7	15.7 ± 1.0	104.4	6.4
150.0	157.6 ± 7.8	105.1	4.9	157.1 ± 7.3	104.7	4.6
1500.0	1552.3 ± 56.5	103.5	3.6	1573.6 ± 68.7	104.9	4.4
Ophiopogonin D						
3.5	3.4 ± 0.2	98.0	7.0	3.5 ± 0.3	99.4	7.5
7.5	7.4 ± 0.3	98.4	4.2	7.4 ± 0.5	98.1	6.1
50.0	52.1 ± 3.1	104.2	5.9	52.0 ± 3.5	104.0	6.7
500.0	527.0 ± 35.8	105.4	6.8	527.2 ± 32.8	105.4	6.2

Table 4
The stability of ginsenoside Rg1, Re, Rd, Rb1 and ophiopogonin D in rat plasma and stock solution ($n = 5$)

Spiked C (ng/mL)	At −20 °C for 1 month in plasma		At room temperature for 24 h in plasma		After three freeze-thaw cycles in plasma		At room temperature for 24 h in stock solution		At 4 °C for 3 weeks in stock solution	
	Measured C (ng/mL)	Accuracy (%)	Measured C (ng/mL)	Accuracy (%)	Measured C (ng/mL)	Accuracy (%)	Measured C (ng/mL)	Accuracy (%)	Measured C (ng/mL)	Accuracy (%)
Ginsenoside Rg1										
10.0	10.5	105.2	10.3	102.6	10.4	104.4	10.2	102.3	10.3	103.3
100.0	104.3	104.3	103.9	103.9	105.3	105.3	105.2	105.2	101.7	101.7
1000.0	1041.7	104.2	1052.7	105.3	1037.8	103.8	1025.6	102.6	1045.1	104.5
Ginsenoside Re										
8.0	8.3	103.8	8.5	106.3	8.4	105.0	8.5	106.3	8.2	102.5
80.0	84.2	105.3	82.9	103.6	84.7	105.9	83.6	104.5	84.4	105.5
800.0	845.8	105.7	833.7	104.2	827.6	103.5	828.3	103.5	839.9	105.0
Ginsenoside Rd										
8.0	8.4	105.0	8.2	102.5	8.6	107.5	8.2	102.5	8.3	103.8
80.0	83.9	104.9	85.2	106.5	83.6	104.5	83.8	104.8	83.1	103.9
800.0	844.7	105.6	832.5	104.1	844.3	105.5	851.9	106.5	828.5	103.6
Ginsenoside Rb1										
15.0	15.4	102.7	15.8	105.3	16.2	108.0	15.5	103.3	15.6	104.0
150.0	159.8	106.5	153.6	102.4	157.3	104.9	158.3	105.5	157.6	105.1
1500.0	1588.5	105.9	1573.4	104.9	1582.2	105.5	1592.4	106.2	1581.5	105.4
Ophiopogonin D										
7.5	7.9	105.3	8.0	106.7	7.7	102.7	7.9	105.3	7.7	102.7
50.0	53.3	106.6	52.7	105.4	53.3	106.6	52.1	104.2	50.9	101.8
500.0	524.5	104.9	531.2	106.2	521.4	104.3	518.7	103.7	521.4	104.3

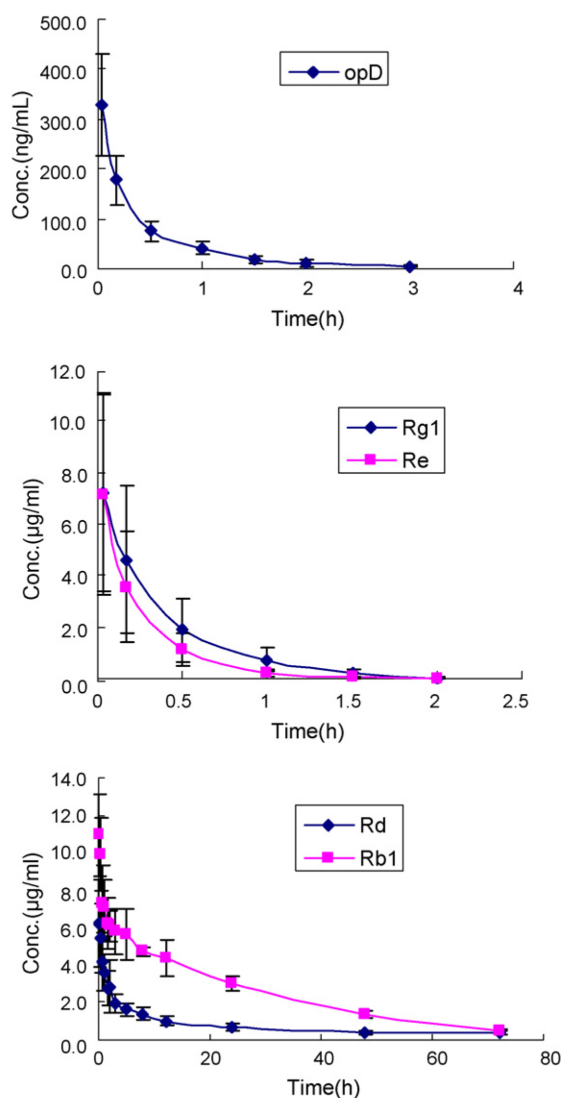

Fig. 3. The mean plasma concentration-time curves of ginsenoside Rg1, Re, Rd, Rb1 and ophiopogonin D in rat after administration of 'SHENMAI' injection ($n = 6$).

3.3. Pharmacokinetic study

The method was successfully applied to the pharmacokinetic study of ginsenoside Rg1, Re, Rd, Rb1 and ophiopogonin D in rats following intravenous administration of 'SHENMAI' injection. The mean plasma concentration–time profiles were illustrated in Fig. 3. From the figure, it can be seen that ginsenoside Rg1 and Re (20(S)-protopanaxatriol) were eliminated quickly in body, similar with that of ophiopogonin D. However, the pharmacokinetic behaviors of ginsenoside Rd and Rb1 (20(S)-protopanaxadiol) were markedly different from those of ginsenoside Rg1 and Re in rat, their half-time in plasma were about 22 h.

4. Conclusion

In the present study, a sensitive and rapid LC-ESI-MS method for the simultaneous determination of ginsenoside Rg1, Re, Rd, Rb1 and ophiopogonin D in rat plasma was developed and validated. A simple liquid–liquid extraction procedure was used to prepare the samples with a high recovery. The established assay was proved to be precise, accurate, specific, reproducible and has been successfully applied to the pharmacokinetic study of 'SHENMAI' injection. The pharmacokinetic study indicated there was a significant difference in the elimination speed of ginsenoside Rg1, Re, Rd, Rb1 and ophiopogonin D in 'SHENMAI' injection.

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