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ScienceDirect
Journal of Pharmaceutical and Biomedical Analysis 44 (2007) 532–539
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Simultaneously determination of five ginsenosides in rabbit plasma using solid-phase extraction and HPLC/MS technique after intravenous administration of 'SHENMAI' injection

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Received 1 November 2006; received in revised form 18 January 2007; accepted 18 January 2007
Available online 24 January 2007

Abstract

In this study, a sensitive and reliable analytical method for the simultaneous determination of five ginsenosides (R_{g1}, R_f, R_e, R_d and R_{b1}) in rabbit plasma was developed by high performance liquid chromatography-electrospray ionization mass spectrometry (HPLC-ESI-MS). Chromatographic separation was carried out on a Zorbax SB-C_{18} Column (150 mm × 2.1 mm i.d., 5.0 μm particle size) with a simple linear gradient elution. The detection was conducted on a single quadrupole mass spectrometer by selected ion monitoring mode via electrospray ionization source. Good linearity over the investigated concentration ranges was observed with the values of R^2 higher than 0.991 for all the analytes. Limits of detection of the analytes varied from 0.25 ng/ml to 1.45 ng/ml, and the average recoveries, examined at three concentration levels, ranged from 90.6% to 106.9%. The validated method was successfully applied to the determination of the ginsenosides in the rabbit plasma after intravenous administration of 'SHENMAI' injection.

Keywords: 'SHENMAI' injection; Ginsenosides; HPLC-ESI-MS; Rabbit plasma; Solid-phase extraction; Quantitative analysis

1. Introduction

Traditional Chinese medicine (TCM), considered as the alternative medicine in the West, has a long history for the medicinal practice in China and some Oriental countries [1,2]. An increasing effort over recent decades to prepare TCM as botanical drugs improves the therapeutic effect and ease of use of these tradition medicines. Due to the fact that the TCM-derived botanical drugs are commonly prepared from multiple medicinal plants, a huge quantity of work has been performed in order to develop various analytical methods for the identification, quantification and quality control of the active components in raw plant materials, extracts and the final products. However, the studies on the absorption, distribution, metabolism and excretion of these botanical drugs are seldom reported. One of the main reasons lies on the lack of the sensitive, specific and reliable analytical methods for the pharmacokinetic studies of the TCM-derived botanical drugs [3–5].

Ginsenosides are the major active components contained in the important Oriental herb "ginseng" derived from the roots and rhizomes of different *Panax* species, which is mainly used to increase resistance to physical, chemical and biological stress [6]. Immune system modulation, antistress activities and antihyperglycemic activities are among the most attractive features of ginseng in laboratory and clinical trials [7]. Moreover, some studies also indicate that some ginsenosides have antitumor properties and other bioactivities related to cancer. Owing to their potent pharmacological activities, ginseng is widely used to prepare the TCM-derived botanical drugs. For example, 'SHENMAI' injection, which is derived from a TCM formula named "SHEN MAI SAN" and mainly composed of red ginseng and ophiopogon, is now commonly used in China [8,9]. Therefore, the pharmacokinetic studies of ginsenosides are of great importance for the further development and the rational use of the related botanical drugs. Up to now, a number of analytical methods have been developed for the determination of some major

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doi:10.1016/j.jpba.2007.01.032

ginsenosides in the raw materials or the final ginseng products based on thin layer chromatography [10], gas chromatography [11], micellar electrokinetic chromatography [12], HPLC [13,14] and some other techniques [15]. Among these analytical techniques, HPLC has become the routine choice. Although ultraviolet (UV) detector was widely employed for HPLC analysis, it is not sufficient for the sensitive analysis of ginsenosides within the biological matrices. The main problem is attributed to the high level of baseline noise and the poor sensitivity caused by the weak UV absorption of ginsenosides. In recent years, HPLC/MS technique has been successfully applied to the sensitive analysis of ginsenosides [16–18], and some advantages of mass spectrometry such as the higher specificity, selectivity, sensitivity, and the lower limit of detection enhance its application to the development of the analytical methods for the pharmacokinetic studies of ginsenosides [19–21]. However, most of these methods were concentrating on one or two constituents in ginseng, thus the effort to develop the method for the simultaneous quantifying the multiple active components of the raw materials or final products of ginseng in the biological matrices is still necessary for the pharmacokinetic studies.

'SHENMAI' injection is used for the treatment of coronary atherosclerotic cardiopathy and viral myocarditis, and it is also capable of raising tumor patient's immunity. Ginsenosides such as R_{g1}, R_f, R_e, R_d and R_{b1} (their chemical structures have been shown in Fig. 1) are the main effective components contained in this botanical drug. Development of a sensitive method for the simultaneous determination of these components in plasma or other biological matrices is very helpful for the pharmacokinetic study of 'SHENMAI' injection. In our early studies [22,23], HPLC/MS technique was not only used to characterize the constituents of 'SHENMAI' injection, but also to develop a HPLC/MS fingerprinting for its quality evaluation. In this work, a sensitive HPLC-ESI-MS method coupled with solid-phase extraction was developed for the simultaneous determination of ginsenosides R_{g1}, R_f, R_e, R_d and R_{b1} in rabbit plasma. This method was applied to the development of the concentration–time profiles of the five components in rabbit plasma after intravenous administration of 'SHENMAI' injection.

2. Experimental

2.1. Chemicals and reagents

Standard compounds ginsenosides R_{g1}, R_f, R_e, R_d and R_{b1} were all purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China).

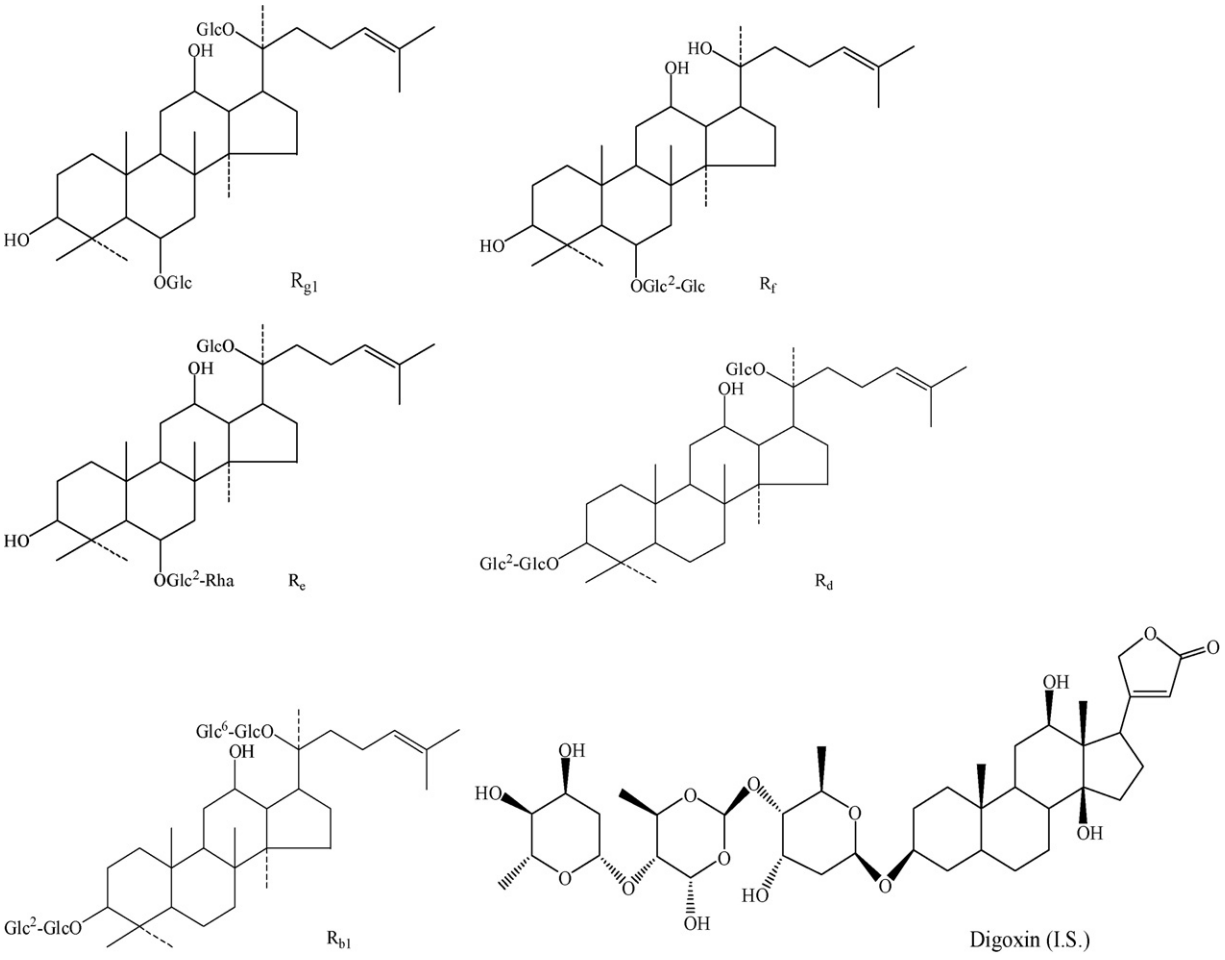

Fig. 1. Chemical structures of five ginsenosides and the I.S. compound.

'SHENMAI' injection was supplied by a Chinese pharmaceutical company (Qingchunbao, Hangzhou, China). Digoxin was kindly provided by the Laboratory for the Control of Drugs of Henan Province (Zhengzhou, China). It was adopted as the internal standard (I.S.) because of its similarity of retention, ionization with the analytes [19,20].

Acetonitrile and methanol were of HPLC grade and both were purchased from Merck (Darmstadt, Germany). Acetic acid and heparin sodium (A.R. grade) were purchased from Hangzhou Reagent Company (Hangzhou, China). Water used throughout the experiments was generated by a Milli-Q academic water purification system (Milford, MA, USA). The experiment animals were supplied by the Animal Center of Zhejiang Province (Hangzhou, China).

2.2. Instrumentation and analytical conditions

The Agilent 1100 series LC-MSD system (single quadruple) equipped with an 1100 well plate autosampler, a diode array detector, a column heater–cooler and ChemStation software (Agilent Technologies, Palo Alto, CA, USA) was used in this study. An 1100 binary pump connected to eluent A (0.01% acetic acid in water, v/v) and B (0.01% acetic acid in acetonitrile, v/v) was employed for the separation. All chromatographic separations were performed on a Zorbax SB-C_{18} Column (150 mm × 2.1 mm i.d., 5.0 μm) using a linear gradient elution. The mobile phases were consecutively programmed as follows: a linear gradient of 23–45% B for the first 10 min, an isocratic elution of 45% B for the next 6 min, and then followed a linear gradient of 45–90% B within 1 min. After holding the solvent composition of 90% B for a further minute the column was returned to its starting conditions. The flow rate was set at 0.2 ml/min, the column temperature was maintained at 30 °C, and the injection volume was set at 10 μl. Prior to the analytical column, a C_{18} guard column (Agilent Technologies) was placed to prevent column degradation.

Electrospray ionization (ESI) in the mass spectrometer was performed with the following parameters: ionization, negative ion mode; V-cap voltage, 3.5 kV; fragmentor voltage, 250 V; flow of heated dry nitrogen gas, 10 l/min; heater temperature of gas, 350 °C; nebulizer gas pressure, 45 psi. MS data was acquired by the selective ion monitoring (SIM) with $m/z = 779$ (for I.S.), 799 (for R_f), 859 (for R_{g1}), 945 (for R_e and R_d) and 1107 (for R_{b1}). When the factors associated with an effect on the performance of ESI were tested, intensities of the monitoring ions for the investigated components were most strongly affected by the fragmentor voltage among all factors examined. The test of the fragmentor voltage with the range of 50–350 V was performed, and 250 V providing the higher intensities for these analytes was chosen.

The plasma sample clean-up procedure consisted of a solid-phase extraction (SPE), which was performed using C_{18} cartridges (300 mg, Supelco, Bellefonte, PA, USA). These cartridges were placed in a vacuum manifold proceeding station (Agilent Technologies) coupled with a vacuum pump from Taikang (Henan Province, China).

2.3. Preparation of calibration standards and quality control samples

The standard stock solutions of ginsenosides R_{g1} (1.98 mg/ml), R_f (1.20 mg/ml), R_e (3.60 mg/ml), R_d (3.17 mg/ml) and R_{b1} (1.85 mg/ml) were prepared in 80% (v/v) methanol aqueous solution. The stock solutions were appropriately diluted to prepare a series of standard working solutions. The I.S. (digoxin) stock solution (1.15 mg/ml) was also prepared in the methanol aqueous solution. I.S. working solution (0.46 μg/ml) was prepared by diluting the stock solution. All solutions were stored at 4 °C and protected away from light.

Calibration curves were prepared from 1.0 ml aliquot of plasma by spiking drug-free control rabbit plasma using the working solutions. Ten calibration standards were prepared, nominally ranging from 1.24 to 994.0 ng/ml for ginsenoside R_{g1}, from 0.50 to 400.0 ng/ml for R_f, from 0.75 to 600.0 ng/ml for R_e, from 1.50 to 1 200 ng/ml for R_d and from 2.90 to 2 320 ng/ml for R_{b1}, respectively.

For the validation of the measurement, three pools of quality control (QC) plasma samples were prepared containing ginsenoside R_{g1} (9.9 ng/ml, 248.5 ng/ml and 745.5 ng/ml, respectively), R_f (4.0 ng/ml, 100.0 ng/ml and 300.0 ng/ml, respectively), R_e (6.0 ng/ml, 150.0 ng/ml and 450.0 ng/ml, respectively), R_d (12.0 ng/ml, 300.0 ng/ml and 900.0 ng/ml, respectively), and R_{b1} (23.2 ng/ml, 580.0 ng/ml and 1 740.0 ng/ml, respectively).

2.4. Extraction procedure

The SPE cartridges were conditioned with 3 ml methanol, and followed by 3 ml water. The samples (0.3 ml plasma and 0.1 ml I.S. working solution) were introduced to the cartridges manually and washed with 2 ml water. After the samples had been absorbed completely at high vacuum, the SPE cartridges were then eluted with 2 ml methanol. The obtained eluent was evaporated to dryness with nitrogen. The residue was reconstituted with 0.1 ml 23% (v/v) acetonitrile aqueous solution, ultrasonically mixed and centrifuged at 6640 × g for 10 min under room temperature. The supernatant was transferred into autosampler vials after filtering through a 0.45 μm syringe filter.

2.5. Validation of the assay

Calibration curves for all the analytes were obtained in six independent days using six batches of prepared calibration standards. QC samples at three concentration levels were assayed with the interval of two hours on the same day to examine the intra-day precision and accuracy, while on each of six separate days to determine the inter-day precision and accuracy. For sensitivity determination, a series of different diluted plasma standard samples were prepared. The limit of quantification (LOQ) and detection (LOD) were estimated by considering a signal to noise ratio (s/n) of 10:1 and 3:1, respectively. The reproducibility and precision examinations with the sample concentration to be accepted as LOQ

were also estimated (R.S.D. less than 20%). The recoveries were determined by comparing the QC samples with drug free plasma samples which were extracted using the same SPE procedure but spiked before nitrogen evaporation. I.S. solution (0.46 μg/ml) was added before SPE procedure in all cases.

The QC samples were also used to investigate the stability of the five ginsenosides during the sample storing and processing procedures. The QC samples were stored at ambient temperature for 48 h, and the long-term storage stability was examined by the assay of the QC samples stored at −20 °C for a month. The freeze–thaw stability was examined after three freeze and thaw cycles. In the freeze and thaw cycles, the QC samples were first frozen at −20 °C for 24 h and thawed at room temperature. After that, the samples were refrozen with the same condition when they were completely thawed. The freeze and thaw cycle was repeated twice before the examination.

3. Results and discussion

3.1. Separation, specificity and matrix effect

To obtain a better separation, three kinds of Agilent columns including Zorbax-SB C_{18} (150 mm × 2.1 mm i.d., 5.0 μm), Zorbax-SB C_{18} (150 mm × 4.6 mm i.d., 5.0 μm), Zorbax-XDB C_8 (150 mm × 4.6 mm i.d., 5.0 μm) were attempted to separate the analytes. We found that the Zorbax-SB C_{18} column with 2.1 mm i.d. showed the good separation of ginsenosides, and the flow rate of 0.2 ml/min was suitable for MS detection with splitless mode. It was also found that the addition of acetic acid to the mobile phase would improve separation and enhance response with little ion suppress. Finally, gradient elution with two mobile phases as described in Section 2.2 was adopted to achieve the separation.

A number of studies based on mass spectrometry have been carried out on ginsenosides to investigate the influence of ionization mode, mobile phase and metal ions adducts on the sensitivity of the analysis [24–26]. In general, ion sensitivities for the ginsenosides were greater in the negative ion mode, which afforded the $[M-H]^-$ ion together with several adduct ions depending on the additive in the mobile phase. In the present study, negative ion mode was used, and the mass spectrometric parameters were optimized to obtain the higher signal of the select ions (*m/z* 779 for I.S., 799 for R_f, 859 for R_{g1}, 945 for R_e and R_d and 1107 for R_{b1}). The representative SIM chromatograms of the blank plasma, plasma samples spiked with the standard solution, and plasma sample obtained after intravenous administration of the 'SHENMAI' injection were shown in Fig. 2. The chromatographic peaks were symmetrical with good resolution, and no endogenous components interfered with the analytes at the corresponding retention times of these ginseno-

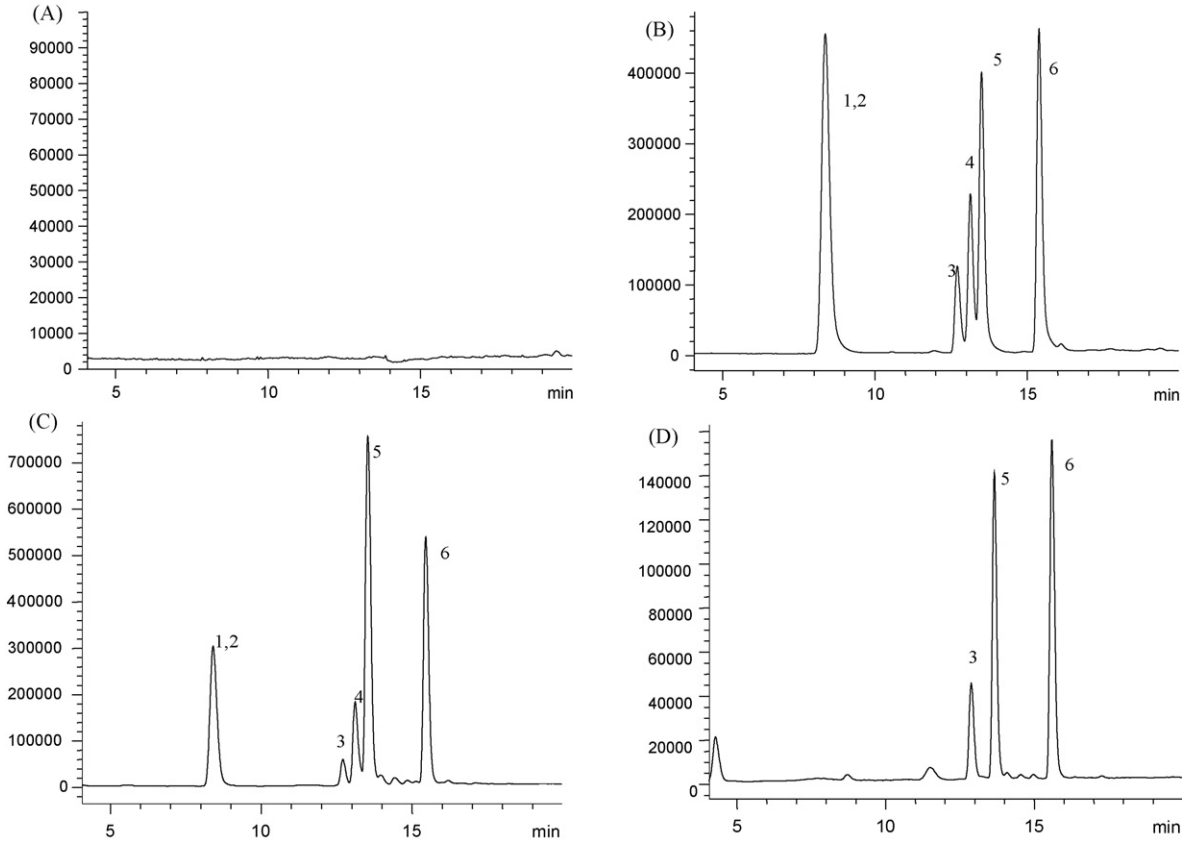

Fig. 2. Representative LC/MS SIM chromatograms of the ginsenosides: (A) blank plasma; (B) plasma spiked with (1) ginsenoside R_{g1} (248.5 ng/ml), (2) ginsenoside R_e (150 ng/ml), (3) I.S. (460 ng/ml), (4) ginsenoside R_f (100 ng/ml), (5) ginsenoside R_{b1} (580 ng/ml), and (6) ginsenoside R_d (300 ng/ml); (C) plasma 15 min after intravenous administration of 'SHENMAI' injection; (D) plasma 60 h after intravenous administration.

sides. Because of the high specificity of SIM detection, the investigated ginsenosides were well separated except R_{g1} and R_e. Comparing the retention time, relative molecular mass and MS spectra of the peaks shown in the real sample solution with those of the reference compounds in the standard solution, the chromatographic peaks of the investigated ginsenosides were identified. As shown in Fig. 3, the extracted ions were highly sensitive and specific for the quantitative analysis. All the components were identified by their dominant $[M-H]^-$ mass peaks, except R_{g1}, which was monitored as its dominant $[M+AcO]^-$ mass peak at $m/z = 858.7$. Ginsenoside R_{g1} and R_e were not separated with this chromatographic condition; however, their different m/z ratio ensured the quantitative analysis. Meanwhile, although R_e and R_d exhibited the same m/z ratio, their different retention time guaranteed the resolution of these two compounds.

3.2. Linearity, work range and limits of detection

The calibration standards were assayed using the method described above. The peak area ratios of ginsenosides R_{g1}, R_f, R_e, R_d and R_{b1} to I.S. compound in rabbit plasma varied linearly with concentrations over the ranges described in Section 2.3. Best fits ($R^2 > 0.991$) for the calibration curves produced by 10 equidistant concentrations of calibration standards were achieved by the linear regression function of $y = ax + b$, where y was the peak area ratio, x was the corresponding concentration, a was the slope and b was the intercept of the regression line, respectively. The limits of detection (LOQ) of the analytes were in the range of 0.25–1.45 ng/ml, while the limits of quantification (LOQ) were from 0.50 ng/ml to 2.90 ng/ml. The detailed descriptions of the calibration curves and linear ranges, together with the values of LOD and LOQ, were all listed in Table 1.

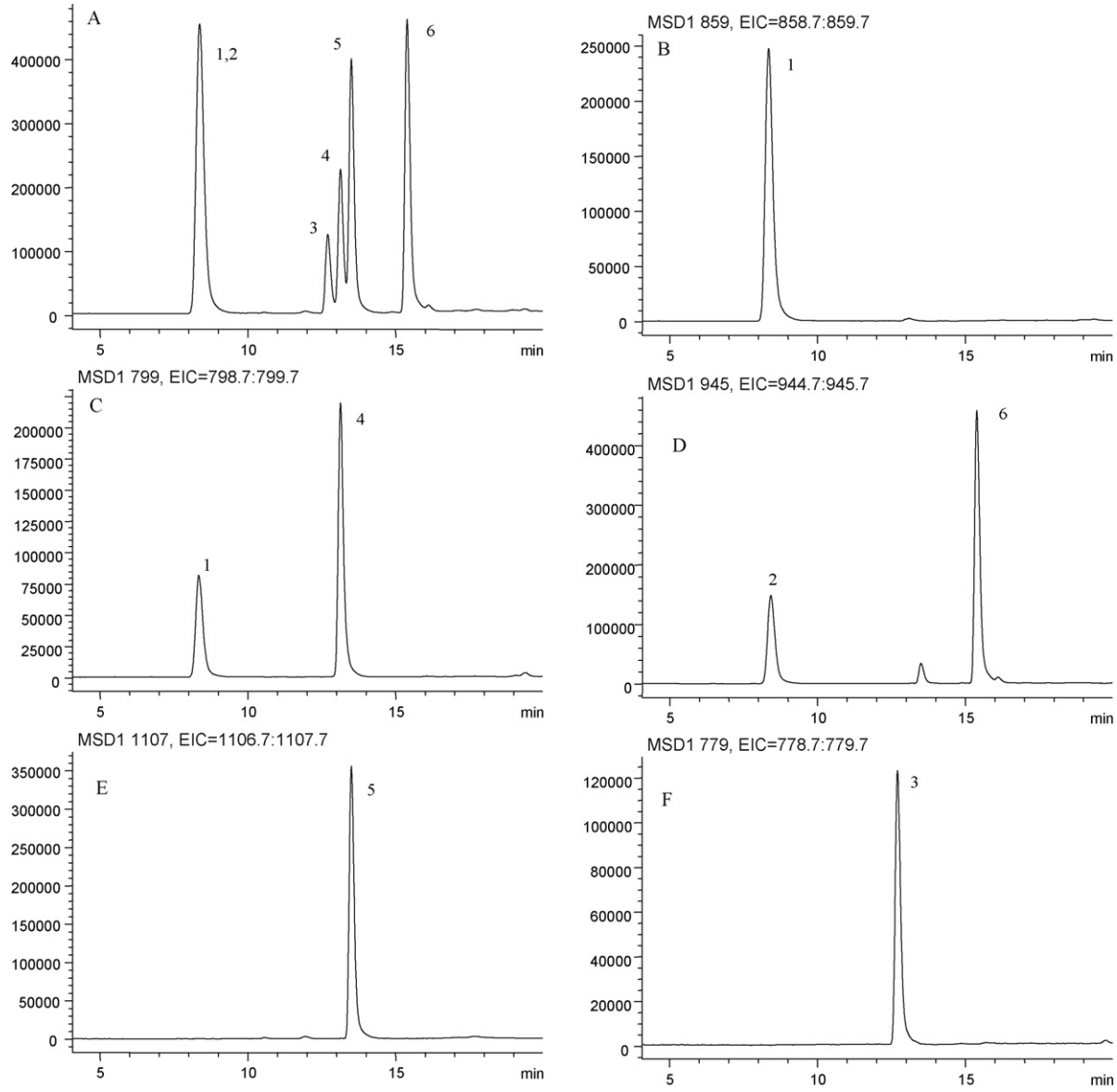

Fig. 3. The extracted ions for quantitative analysis: (A) spiked plasma; (B) adduct ion $[M+OAc]^-$ of ginsenoside R_{g1}; (C)–(E) Quasi-molecular ions $[M-H]^-$ of the remained four ginsenosides; (F) Quasi-molecular ion $[M-H]^-$ of I.S. compound. The peaks marked with 1–6 were the same with those presented in Fig. 2.

Table 1
Calibration curves for five ginsenosides of 'SHENMAI' injection in rabbit plasma

Components	Calibration curves ($y = ax + b$)	Linear range (ng/ml)	R^2	LOD (ng/ml)	LOQ (ng/ml)
Ginsenoside R_{g1}	$y = (0.0092 \pm 0.0010)x + (0.2348 \pm 0.0297)$	1.24–994	0.9942	0.62	1.24
Ginsenoside R_f	$y = (0.0165 \pm 0.0006)x + (0.0706 \pm 0.0499)$	0.50–400	0.9992	0.25	0.50
Ginsenoside R_e	$y = (0.0104 \pm 0.0008)x + (0.0803 \pm 0.0110)$	0.75–600	0.9978	0.38	0.75
Ginsenoside R_d	$y = (0.0106 \pm 0.0008)x - (0.2456 \pm 0.1184)$	1.50–1 200	0.9920	0.75	1.50
Ginsenoside R_{b1}	$y = (0.0039 \pm 0.0006)x - (0.2116 \pm 0.1113)$	2.90–2 320	0.9910	1.45	2.90

LOD is defined as the concentration where the signal-to-noise ratio is 3, and LOQ is defined as the concentration where the signal-to-noise ratio is 10. The slope and intercept were given as the form of mean ± S.D. of six experiments.

Table 2
Absolute recovery of the developed method and the repeatability of the extraction procedure ($n = 6$)

Components	Nominal concentration (ng/ml)	Recovery (%)	R.S.D. (%)
Ginsenoside R_{g1}	745.5	106.1	3.5
	248.5	107.8	2.1
	9.94	95.2	6.2
Ginsenoside R_f	300.0	97.4	3.0
	100.0	100.3	0.7
	4.0	99.8	2.5
Ginsenoside R_e	450.0	106.9	1.5
	150.0	105.1	1.3
	6.0	90.9	4.8
Ginsenoside R_d	900.0	90.6	4.8
	300.0	101.6	0.9
	12.0	93.9	2.9
Ginsenoside R_{b1}	1740.0	97.9	1.2
	580.0	95.9	0.6
	23.2	98.3	1.9

3.3. Absolute recovery and the repeatability of extraction procedure

Six replicates of QC samples at three concentration levels were used to examine the absolute recovery of the SPE procedure. As presented in Table 2, the absolute recovery was from 90.6% to 106.9%. Repeatability of the extraction procedure was also determined by using six replicates of the QC samples. The result showed that the R.S.D. values of the extraction repeatability were all lower than 6.5%.

3.4. Precision and accuracy

The QC samples at three concentration levels were analyzed with the method mentioned above (Section 2.5). The results of precision and accuracy of the assay were both summarized in Table 3. The precision was presented as percentage of R.S.D. and accuracy was presented as percentage of the relative error (RE) against the nominal concentration. R.S.D. values of intra-day precision were less than 10%, and the corresponding RE values ranged from −7.1% to 9.6%, except for ginsenoside R_f and R_d

Table 3
Intra-day and inter-day precision for the assay of five ginsenosides in rabbit plasma

Nominal concentration (ng/ml)	Intra-day precision ($n = 6$)			Inter-day precision ($n = 6$)		
	Measured concentration (ng/ml)	R.S.D. (%)	RE (%)	Measured concentration (ng/ml)	R.S.D. (%)	RE (%)
Ginsenoside R_{g1}						
745.5	754.68 ± 24.19	3.2	1.2	784.39 ± 34.35	4.4	5.2
248.5	272.41 ± 10.54	3.9	9.6	270.13 ± 22.00	8.1	8.7
9.9	10.28 ± 0.61	7.9	3.4	8.95 ± 1.11	12.4	−10.0
Ginsenoside R_f						
300.0	279.94 ± 15.28	5.5	−6.7	295.48 ± 11.61	3.9	−1.5
100.0	92.86 ± 2.66	2.9	−7.1	98.23 ± 3.29	3.3	−1.8
4.0	4.72 ± 0.28	6.0	18.0	3.32 ± 0.15	4.8	−17.0
Ginsenoside R_e						
450.0	442.47 ± 8.79	2.0	−1.7	477.05 ± 19.46	4.1	6.0
150.0	150.79 ± 6.12	4.1	0.5	150.14 ± 6.69	4.5	0.1
6.0	6.32 ± 0.63	9.9	5.3	6.10 ± 0.75	12.3	1.7
Ginsenoside R_d						
900.0	868.0 ± 25.2	2.9	−3.6	936.93 ± 43.01	4.6	4.1
300.0	298.2 ± 6.9	2.3	−0.6	317.27 ± 15.51	4.9	5.8
12.0	10.39 ± 0.66	6.3	−13.4	9.06 ± 1.11	12.2	−24.5
Ginsenoside R_{b1}						
1740.0	1639.0 ± 97.2	5.9	−5.8	1758.96 ± 114.59	6.5	1.1
580.0	571.2 ± 38.9	6.8	−1.5	583.19 ± 46.22	7.9	0.6
23.2	22.79 ± 1.79	7.9	−1.8	19.28 ± 1.92	10.0	−16.9

Table 4
Stability of five ginsenosides in rabbit plasma ($n = 6$)

Components	Concentration (ng/ml)	Three freeze–thaw stability/R.S.D.%	Stored at −20 °C/R.S.D.%	Stored at ambient temperature/R.S.D.%
Ginsenoside R_{g1}	745.5	5.4	1.4	6.0
	248.5	6.2	3.4	4.5
	9.94	11.8	4.0	6.3
Ginsenoside R_f	300.0	2.7	1.1	1.3
	100.0	1.8	4.7	2.6
	4.0	5.4	1.0	2.7
Ginsenoside R_e	450.0	2.6	3.0	4.7
	150.0	4.5	2.1	5.9
	6.0	11.5	1.0	6.8
Ginsenoside R_d	900.0	6.3	0.06	1.8
	300.0	3.6	4.5	2.5
	12.0	10.7	12.9	2.1
Ginsenoside R_{b1}	1740.0	8.4	4.7	2.6
	580.0	4.5	5.0	8.7
	23.2	8.6	8.5	7.1

at the low concentration levels. R.S.D. values of the inter-day precision were in the range of 3.3–12.4%, and the corresponding RE values were less than 10%, except for ginsenoside R_f, R_d and R_{b1} at the low concentration levels. The results showed that the developed method has a satisfactory precision and accuracy. Although the accuracy of this measurement was deteriorated at the lower concentration levels, this method is still satisfactory for the quantification of the investigated components due to the relatively higher concentration levels of the target components in the rabbit plasma.

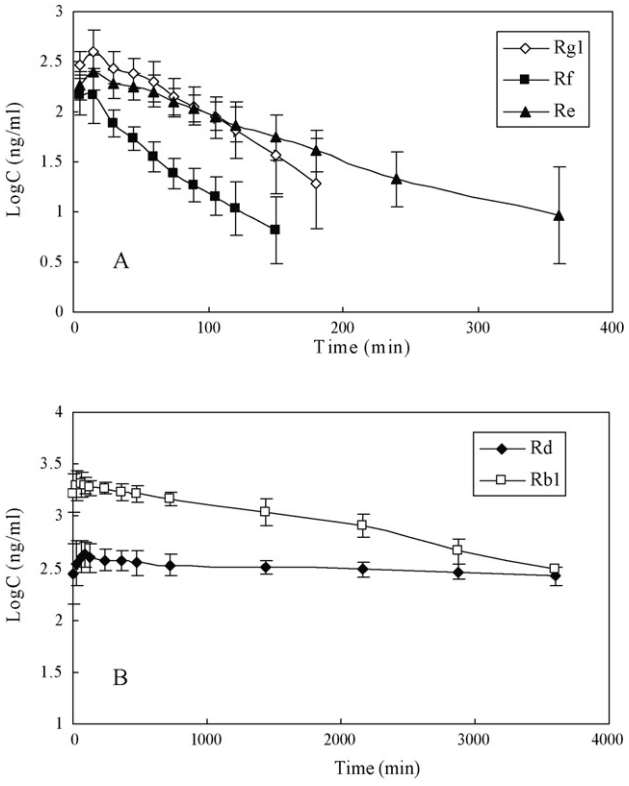

Fig. 4. The blood concentration–time profiles of ginsenosides R_{g1}, R_f, R_e, R_d and R_{b1} after the intravenous injection of a dose of 1.0 mg/kg 'SHENMAI' injection. Each data point was given as the form of mean ± S.D. of six experiments. (A) For ginsenosides R_{g1}, R_f and R_e and (B) for ginsenosides R_d and R_{b1}.

3.5. Stability

Stability of ginsenosides R_{g1}, R_f, R_e, R_d and R_{b1} in the biological matrices during the sampling storing and processing procedures were also evaluated. The results of the stability tests shown in Table 4 were satisfactory for the pharmacokinetics studies.

3.6. Application

The developed method was applied to monitoring the concentrations of the five ginsenosides in rabbit plasma after a single intravenous administration of 'SHENMAI' injection with a dose of 1.0 ml/kg. The plasma concentration–time profiles of the investigated components were shown in Fig. 4. It was observed that the speed of elimination of ginsenosides R_{g1}, R_f and R_e was much faster than that of ginsenosides R_d and R_{b1}, and this effect should be attributed to the longer half-life of these two components. As shown in Fig. 4, the validated method successfully determined these ginsenosides in rabbit plasma, and this was helpful for the further pharmacokinetic study of 'SHENMAI' injection.

4. Conclusion

In the present study, a sensitive, specific and reliable method for the simultaneous determination of ginsenosides R_{g1}, R_f, R_e, R_d and R_{b1} in the rabbit plasma after intravenous administration of 'SHENMAI' injection was developed using HPLC-ESI-MS technique. Solid-phase extraction was employed for the extraction of these components from the plasma samples. The developed method provided a simple sample preparation and a

stable analytical result for the determination of these ginsenosides in the plasma samples. This method was applicable for the pharmacokinetic study of 'SHENMAI' injection.

Acknowledgements

This work was financially supported by the Key Program in Major Research Plan of National Natural Science Foundation of China (No. 90209005) and National Basic Research Program of China (No. 2005CB523402).

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