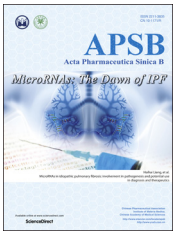
Acta Pharmaceutica Sinica B 2016;6(6):584–592

Chinese Pharmaceutical Association
Institute of Materia Medica, Chinese Academy of Medical Sciences

Acta Pharmaceutica Sinica B

www.elsevier.com/locate/apsb
www.sciencedirect.com



ORIGINAL ARTICLE

Pharmacokinetic evaluation of Shenfu Injection in beagle dogs after intravenous drip administration

Yuqiao Zhang[a], Dali Tian[b], Yuyou Huang[b], Ling Li[a], Juan Mao[a], Juan Tian[a], Jinsong Ding[a,*]

[a]*School of Pharmaceutical Sciences, Central South University, Changsha 410013, China*
[b]*China Resources San-Jiu (Ya'an) Pharmaceutical Co., Ltd., Ya'an 625000, China*

Received 1 March 2016; revised 4 April 2016; accepted 15 April 2016

KEY WORDS

HPLC–MS/MS;
Ginsenoside;
Aconitum alkaloids;
Pharmacokinetics;
Shenfu Injection;
Beagle dogs

Abstract Shenfu Injection (SFI) is a well-defined Chinese herbal formulation that is obtained from red ginseng and processed aconite root. The main active constituents in SFI are ginsenosides and aconitum alkaloids. In this work, ginsenosides (ginsenoside Rg1, ginsenoside Rb1 and ginsenoside Rc) and aconitum alkaloids (benzoylmesaconine and fuziline) were used as the index components to explore the pharmacokinetic behavior of SFI. A selective and sensitive HPLC–MS/MS method was developed for the quantification of ginsenosides and aconitum alkaloids in dog plasma and was used to characterize the pharmacokinetics of the five index components after intravenous drip of three different dosages of SFI in beagle dogs. The pharmacokinetic properties of the index components were linear over the dose range of 2–8 mL/kg.

*Corresponding author. Tel.: +86 731 82650250.
E-mail address: dingjs0221@163.com (Jinsong Ding).

Peer review under responsibility of Institute of Materia Medica, Chinese Academy of Medical Sciences and Chinese Pharmaceutical Association.

http://dx.doi.org/10.1016/j.apsb.2016.05.006

1. Introduction

Shenfu Injection (SFI) is a commonly used traditional Chinese medicine (TCM) composed of the extracts of red ginseng (steamed roots of *Panax ginseng*) and aconite (processed lateral roots of *Aconitum carmichaeli*). SFI has been widely accepted as an effective therapeutic approach in clinic for its protective effects on ischemia/reperfusion injury and therapeutic effects on shock, acute myocardial infarction, chronic congestive heart failure and ischemic cardiomyopathy with heart insufficiency[1–7]. It can be used alone or integrated with other routine treatments.

SFI dosing used in clinical practice ranges from 20 mL to 200 mL, and occasionally higher. The curative effect of SFI depends on its dose[8–10]. It is necessary to investigate the pharmacokinetics of the SFI index components to define the relationship between dose and drug exposure. As a typical multiple-constituent Chinese herbal formulation, SFI contains multiple active ingredients, including ginsenosides and aconitum alkaloids. Ginsenosides are divided into 20(*S*)-protopanaxatriol (Ppt) and 20(*S*)-protopanaxadiol (Ppd) types based on their aglycone moieties. The half-life ($t_{1/2\beta}$) of Ppd ginsenoside (Rb1 and Rc) is longer than that of Ppt ginsenoside (Rg1), and Rg1 showed fast elimination *in vivo* with a short $t_{1/2\beta}$ of 0.45 h, while Rb1 and Rc had long $t_{1/2\beta}$ values of 58 h and 20 h, respectively[11–13]. Aconitum alkaloids are composed of diester-, monoester- and amine-diterpenoid alkaloids, with aconitine (CA), benzoylmesaconine (BMA) and fuziline (FN) the typical components of the three types of alkaloids, respectively. With proper processing the highly toxic diester-diterpenoid alkaloids can be easily hydrolyzed and converted to monoester-diterpenoid alkaloids, whose toxicity is 50- to 500-fold lower than that of diester-diterpenoid alkaloids[14,15]. Monitoring their plasma concentrations after administration is still of importance for safety and efficacy evaluation in clinical pharmacotherapy. A number of studies have evaluated the pharmacokinetic parameters of these components with different preparations[16–20]; however, only a few studies have evaluated the pharmacokinetics of "Shenfu": Li et al.[21] revealed the pharmacokinetic profiles of seven ginsenosides in rat plasma in a single dose study and Zhang et al.[22] carried out a pharmacokinetic study of six aconitum alkaloids in a phase I clinical trial, but only the ester-alkaloids were chosen as the index components without data on the amine-diterpenoid alkaloids. We propose that the pharmacokinetic behavior of multiple ingredients in a single herb would more accurately portray the pharmacokinetics of the entire medicinal compound *in vivo*. Moreover, comparison of pharmacokinetic profiles after various doses will be helpful for the rational use of this multiple-constituent TCM. We selected Rg1, Rb1, Rc, BMA and FN as the index components of SFI to gain a more comprehensive understanding of the pharmacokinetic behavior of SFI.

Towards this goal, a simple, sensitive yet reliable analytical method to determine BMA, FN, Rg1, Rb1 and Rc in plasma is important for illustrating the pharmacokinetic behavior of SFI. Various methods have been developed for the detection of ginsenosides and aconitum alkaloids[16–18,23,24], but it is still challenge to achieve simultaneous determination of both ginsenosides and aconitum alkaloids in biological samples because of their very different physicochemical properties and polarities[25]. Owing to its excellent selectivity and sensitivity, liquid chromatography coupled to tandem mass spectrum (LC–MS/MS) is becoming a useful technique for determination of these components in pharmaceutical and biological samples, with the ability to discriminate the different components based on their distinct molecular weights. In this work, a rapid and sensitive HPLC–MS/MS method was established for quantification of ginsenosides (Rg1, Rb1 and Rc) and aconitum alkaloids (BMA and FN) in plasma with different sample preparations. We applied the method to determine the pharmacokinetics of SFI in beagles after intravenous drip infusion injection of a single dose with ascending doses of 2, 4 and 8 mL/kg.

2. Materials and methods

2.1. Materials

The reference standards of BMA, FN, Rc (purity >98%) were purchased from Chengdu Pufei De Biotech Co., Ltd. (Chengdu, China). Rg1 and Rb1 (purity >93%), lappaconite hydrobromide (LA) and diazepam (DZP) were purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Methanol of HPLC grade was purchased from Merck (Darmstadt, Germany). Acetic acid of HPLC-LA grade was purchased from ROE (Newark, New Castle, DE, USA). Doubly deionized water was purified using a Millipore Simplicity System (Millipore, Bedford, MA, USA). Other reagents of analytical grade were obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Shenfu Injection was supplied by a Chinese pharmaceutical company (San-Jiu Pharmaceutical Company of Ya'an, China).

2.2. HPLC–MS/MS instruments and conditions

An API 3000 Qtrap triple quadrupole mass spectrometer with electrospray ionization (ESI) (Applied Biosystem/MDS SCIEX, Foster City, CA, USA) was used to determine concentrations of aconitum alkaloids and ginsenosides in blood. The main working parameters for mass spectrometry were set as follows: nebulizer gas, 12; curtain gas, 8; collision gas, 4; gas 2, 7500; ion source temperature, 500 °C; ion spray voltage: 5000 V. LA and DZP were used as the internal standards (IS) for quantification of aconite alkaloids and ginsenosides in plasma, respectively. The quantification was performed using multiple-reaction monitoring (MRM) in the positive-ion mode. The compound-dependent MS/MS parameters for analytes in the MRM mode are summarized in Table 1. HPLC conditions: column, Waters Atlantis T3 column (100 mm × 2.1 mm, 5 μm); column temperature, 30 °C; injection volume, 10 μL. The analysis was performed at a flow rate of 0.25 mL/min, and the mobile phase consisted of 0.1% formic acid: methanol (40:60, *v/v*).

2.3. Preparation of stock and working standard solutions

Stock solutions of BMA and FN were prepared in acetonitrile at a concentration of 400 μg/mL and ginsenoside Rg1, ginsenoside Rb1, ginsenoside Rc, DZP (IS for ginsenosides) and LA (IS for aconitum alkaloids) were prepared in methanol at a concentration of 400 μg/mL. All solutions were stored at 4 °C until analysis. The working solutions for aconitum alkaloids were prepared in methanol–water (1:1, *v/v*) at concentrations of 1000, 400, 200, 100, 40, 20, 10 and 4 ng/mL. The working solutions for ginsenosides were prepared in methanol–water (1:1, *v/v*) at different concentrations (the concentrations of Rg1 were 4, 2, 1, 0.4, 0.2, 0.1, 0.05, 0.02 μg/mL, Rb1 were 20, 10, 5, 2, 1, 0.5, 0.25, 0.1 μg/mL, and Rc were 10, 5, 2.5, 1, 0.5, 0.25, 0.125, 0.05 μg/mL).

Table 1 Optimized MS/MS parameters of analytes and internal standards in MRM mode.

Compound	Q1/Q3	DP (V)	FP (V)	EP (V)	CE (V)	CXP (V)
Rb1	1131.7/365.1	200	242	11	81	19
Rg1	823.6/643.4	78	304	9	51	14
Rc	1101.6/335.2	189	244	12	75	17
BMA	590.3/105.1	109	287	11	71	16
FN	454.2/436.4	109	224	13	48	51
LA	585.4/162.2	42	221	13	62	14
DZP	285.0/193.2	82	295	8	48	8

DP: declustering potential; EP: entrance potential; FP: focusing potential; CE: collision energy; CXP: cell exit potential.

2.4. Sample preparation

Since ginsenosides and aconitum alkaloids have different physicochemical properties and are present at different concentrations in plasma samples, two sample preparation procedures were employed in this work for their determination.

2.4.1. Sample preparation for the determination of alkaloids

To a 400 μL aliquot of plasma 40 μL of IS (LA, 100 ng/mL) and 40 μL of 50% methanol were added in a 4 mL EP tube. After vortex-mixing for 30 s the mixture was alkalinized with 40 μL of ammonium hydroxide and extracted with 2 mL of ethylacetate. After vortexing the resulting mixture was centrifuged at 4000 rpm (YINGTAI TD4A, China) for 5 min. One mL of supernatant was transferred into a 2 mL centrifuge tube and evaporated to dryness under a stream of nitrogen at 40 °C. The residue was reconstituted in 100 μL of mobile phase. An aliquot of 10 μL of the final testing sample was injected into LC−MS/MS system for analysis.

2.4.2. Sample preparation for the determination of ginsenosides

Aliquots (20 μL) of plasma were diluted with 180 μL water and spiked with 20 μL of IS, mixed by vortexing for 30 s, and extracted with 1 mL of ethyl acetate–isopropanol (1:1, *v/v*) by vortex-mixing for 3 min. After centrifugation at 13,000 rpm at 4 °C for 10 min, the organic phase was quantitatively transferred to a clean centrifuge tube and evaporated to dryness under a gentle stream of nitrogen at 40 °C. The residue was reconstituted with 200 μL of mobile phase and vortex-mixed for 3 min, and then centrifuged at 13,000 rpm (YINGTAI TGL16M, China) for 10 min. An aliquot of 10 μL of the final testing sample was injected into LC−MS/MS system for analysis.

Quality control (QC) samples for the validation were prepared daily by adding an appropriate volume of standard working solutions into the blank plasma and processed the same way as described above to obtain three different concentration levels (alkaloids-low, -medium and -high at 0.5, 5 and 40 ng/mL and ginsenosides-low, -medium and -high at 0.05/0.25/0.125, 0.4/2/1 and 3/15/7.5 μg/mL for Rg1/Rb1/Rc, respectively).

2.5. Method validation

2.5.1. Specificity and selectivity

The endogenous interference from a biological sample was assessed by comparing chromatograms of blank dog blood, blood spiked with BMA, FN, LA or Rg1, Rb1, Rc, DZP and plasma samples obtained after intravenous drip infusion of SFI to beagle dogs.

2.5.2. Linearity of calibration curve and LLOQ

The linearity was investigated by preparing calibration curves with blood spiked with standards at different concentration levels, using the peak area ratios of each analyte with comparison to the internal standard using $1/\chi^2$ as the weighing factor. The acceptable correlation coefficient (r^2) for calibration curves was 0.99 or higher. The lower limit of quantification (LLOQ) was defined as the concentration of the lowest calibration standard with the determined signal to noise ratio of least 10:1.

2.5.3. Accuracy and precision

The intra-day and inter-day accuracy and precision of measurement of the target compounds were determined (five replicates) on the QC samples on the same day or three sequential days, respectively. The acceptance criteria for the precision and accuracy were within ±15% (within ±20% for LLOQ).

2.5.4. Extraction recovery and matrix effect

The mean extraction recoveries (five replicates) were measured at three QC levels for analytes by comparing the peak areas of analytes from plasma spiked with a reference substance before sample processing to those of the pretreatment of blank plasma following by redissolution with standard solution.

The matrix effect was assayed to compare the peak areas of the analytes from blank blood extracts dissolved with standard solution to those from the standard solutions directly and reconstituted in mobile phase at equivalent concentrations.

2.5.5. Stability

Short-term (25 °C for 24 h), long-term (–20 °C for 30 days) and freeze–thaw (three freeze–thaw cycles) stability of alkaloids and ginsenosides in the samples were determined with QC samples (five samples for each concentration). An acceptable level of change of actual concentration was set at less than 15%.

2.6. Pharmacokinetic study

Beagle dogs, aged 4–5 years and weighing 10±2 kg (certificate No. SCXK 2011-0007) were provided by the Experimental Animal Center of Hunan province. The animal studies were approved by the Animal Ethics Committee of the Third Xiangya Hospital of Central South University. All experiments were conducted in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals.

In our previous study we quantified the 5 index components in SFI, the concentrations of BMA, FN, Rg1, Rb1, Rc are 1.7, 0.7, 44.8, 153.0 and 68.9 μg/mL, respectively. In this work, six beagle dogs (half male and half female) were given 2, 4, or 8 mL/kg of

SFI for single doses trial *via* a randomized 3 × 3 crossover design with a three-week washout period. The dosages in beagles are calculated by the weight according to the clinical doses in humans from 20 to 200 mL, so the results can be used to inform the use of SFI in a clinical setting. The animals were fasted overnight but with free access to water before dosing. On the day of experiment, 2, 4 or 8 mL/kg of SFI in 5% glucose injection with a total volume of 100 mL was administered by intravenous drip to dogs in 1 h with an infusion rate of approximately one drop every 2 s. Serial blood samples (3 mL) were drawn at 0, 0.33, 0.67, 1 (drip accomplished), 1.5, 2, 2.5, 3, 4, 6, 8, 12, 24, 48, 72 and 120 h into plastic whole blood tube with spray-coated K_2EDTA. Plasma samples were obtained following centrifugation at 3500 rpm for 10 min and kept frozen at –20 °C until analysis.

2.7. *Parameters calculation*

The pharmacokinetic analysis of the five analytes was performed by a non-compartmental approach using the DAS3.0 software to calculate area under the concentration–time curve ($AUC_{0-\infty}$), half-life ($t_{1/2}$) and mean retention time (MRT), etc. The maximum value of concentration (C_{max}) and time to reach C_{max} (T_{max}) were obtained directly from the experimental process. Statistical analyses among the three dosages were performed using SPSS 19.0 (Statistical Package for the Social Science). A P value <0.05 was considered as statistically significant for all the tests. All data were expressed as mean ± standard deviation (SD).

3. Results and discussion

3.1. *Optimization of HPLC–MS/MS method*

The ion intensities for all compounds were higher in positive ionization mode than in negative ionization mode. The ion pair and working parameters for the MRM method are listed in Table 1. In order to get better resolution and peak shape, methanol, acetonitrile, water, and 0.1% formic acid were tested as mobile phases, and methanol with 0.1% formic acid were found to yield better peak shape, suitable retention time and enhance the efficiency of ionization. The HPLC–MS/MS method is suitable for both ginsenosides and aconitum alkaloids, which greatly simplifies the determination. Since ginsenosides and aconitum alkaloids have different physicochemical properties and are present in different concentrations in plasma samples, they can't

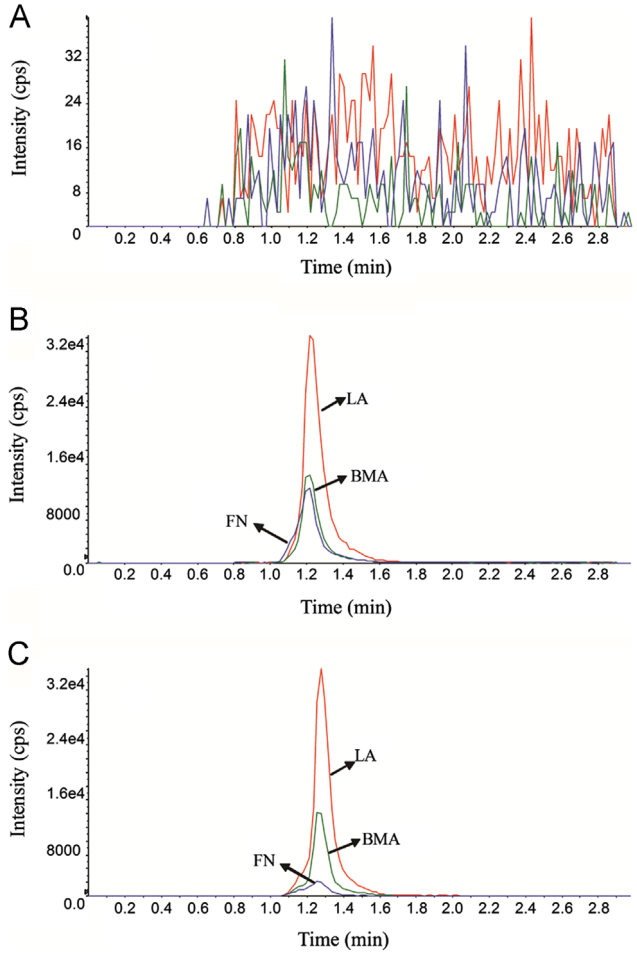


Figure 1 Representative chromatograms of (A) blank plasma; (B) blank plasma spiked with BMA (20 ng/mL, t_R = 1.25 min), FN (20 ng/mL, t_R = 1.25 min) and LA (100 ng/mL, t_R = 1.3 min); (C) plasma sample of 1 h after administration of SFI at a dose of 4 mL/kg.

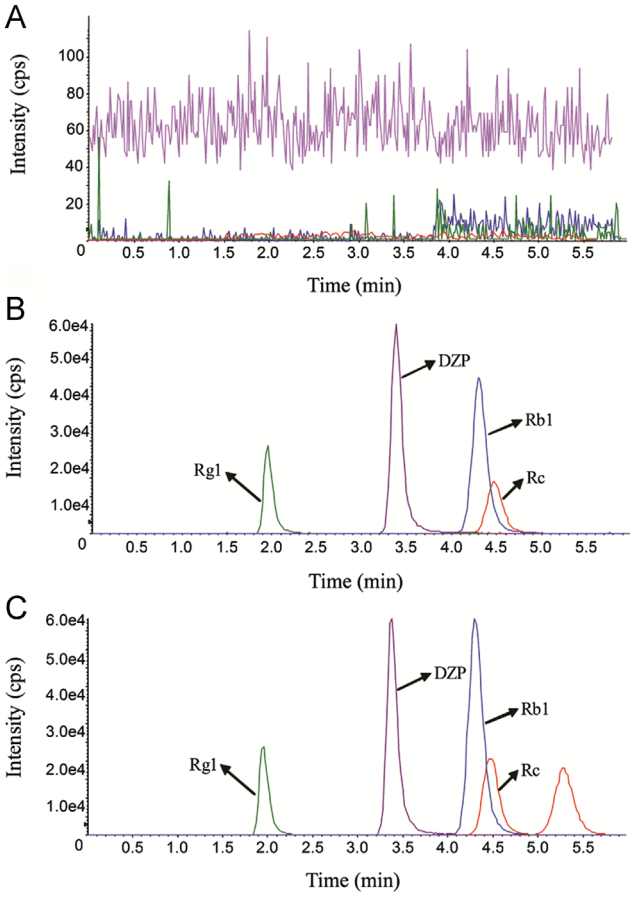


Figure 2 Representative chromatograms of (A) blank plasma; (B) blank plasma spiked with Rg1 (1 μg/mL, t_R = 2.0 min), Rb1 (5 μg/mL, t_R = 4.3 min), Rc (2.5 μg/mL, t_R = 4.5 min) and DZP (diazepam, 0.1 μg/mL, t_R = 3.4 min); (C) plasma sample of 1 h after administration of SFI at a dose of 4 mL/kg.

be extracted simultaneously in one preparation while maintaining good detection and good peak shape. For the preparation process of plasma samples for ginsenosides determination, we tried protein precipitation with methanol, liquid–liquid extraction with water–saturated butanol and ethyl acetate–isopropyl alcohol (1:1, *v/v*). The results showed that liquid–liquid extraction with ethyl acetate–isopropyl alcohol (1:1, *v/v*) displayed better peak shape, greater extraction recovery and fewer matrix effects. For aconitum alkaloids, extraction by organic solvents after alkalifying the plasma is necessary due to its very low content.

Table 2 Linear ranges, regression equation, correlation coefficient and LLOQ of five analytes.

Analyte	Regression equation	Linear range (ng/mL)	Correlation coefficient	LLOQ (ng/mL)[a]
BMA	$y=0.156x+0.046$	0.2–50	0.999	0.2
FN	$y=0.0994x+0.0579$	0.2–50	0.995	0.2
Rg1	$y=0.\ 000303x+0.0000642$	20–4,000	0.9978	20
Rb1	$y=0.\ 000203x+0.00399$	100–20,000	0.9995	100
Rc	$y=0.\ 000164x+0.000757$	50–10,000	0.9985	50

[a]$S/N > 10$.

Table 3 Intra-day and inter-day precision and accuracy of BMA, FN, Rg1, Rb1 and Rc in dog plasma ($n = 5$).

Compound		Conc.	Intra-day			Inter-day		
			Mean	Accuracy (%)	RSD (%)	Mean	Accuracy (%)	RSD (%)
Alkaloids (ng/mL)	BMA	0.5	0.54	107.8	6.1	0.51	102.7	4.8
		5	5.23	104.5	4.2	5.28	105.6	5.1
		40	41.4	103.6	1.5	41.6	103.9	6.9
	FN	0.5	0.44	87.3	2.4	0.47	94.3	7.6
		5	5.07	101.4	7.5	5.13	102.5	6.2
		40	39.80	99.5	2.5	40.70	101.8	3.8
Ginsenosides (μg/mL)	Rg1	0.05	0.045	90.4	3.5	0.050	99.3	10.1
		0.4	0.39	98.8	5.4	0.41	102.2	5.1
		3.0	2.91	97.0	11.1	2.98	99.3	6.9
	Rb1	0.25	0.27	106.8	7.1	0.26	104.1	5.8
		2.0	2.24	112.2	2.3	2.14	106.9	6.6
		15.0	13.60	90.5	4.2	14.10	94.0	5.7
	Rc	0.125	0.130	104.0	1.0	0.130	103.7	7.0
		1.0	1.08	108.3	6.4	1.02	102.3	7.6
		7.5	6.96	92.7	4.7	7.19	95.9	5.4

Table 4 The recovery and matrix effect of BMA, FN, Rg1, Rb1and Rc in dog plasma.

Compound		Spiked conc.	Recovery (%)[a]	Matrix effect (%)[a]
Alkaloids (ng/mL)	BMA	0.5	85.1 ± 11.8	113.0 ± 11.5
		5	95.0 ± 10.0	85.4 ± 4.2
		40	92.7 ± 4.0	85.0 ± 7.2
	FN	0.5	79.9 ± 7.0	106.0 ± 2.3
		5	77.5 ± 2.6	92.2 ± 6.3
		40	81.1 ± 7.2	83.1 ± 3.7
	LA (IS)	100	99.6 ± 5.4	95.4 ± 7.6
Ginsenosides (μg/mL)	Rg1	0.05	86.1 ± 9.4	106.0 ± 4.5
		0.4	78.3 ± 1.3	102.0 ± 1.4
		3	87.0 ± 2.8	103.0 ± 3.3
	Rb1	0.25	80.7 ± 8.3	95.3 ± 13.5
		2	68.8 ± 4.6	110.0 ± 7.2
		15	79.3 ± 4.2	97.7 ± 4.6
	Rc	0.125	76.9 ± 6.6	92.8 ± 4.8
		1	67.1 ± 5.3	109.0 ± 7.8
		7.5	82.3 ± 5.7	99.4 ± 5.5
	DZP (IS)	0.1	55.3 ± 4.0	105.0 ± 3.0

[a]Data are mean ± SD, $n = 5$.

3.2. Method validation

3.2.1. Specificity and selectivity

Representative chromatograms of blank plasma, blank plasma spiked with BMA, FN, and LA at concentrations of 20, 20 and 100 ng/mL, and a plasma sample taken 1 h after administration of SFI at a dose of 4 mL/kg are shown in Fig. 1. Similarly, chromatograms of blank plasma, blank plasma spiked with Rg1, Rb1, Rc and DZP at a concentration of 1, 5, 2.5 and 0.1 μg/mL individually and plasma sample 1 h after administration of SFI at a dose of 4 mL/kg are shown in Fig. 2. These results show that no interference by endogenous components, since no interfering peaks around the retention times of all analytes was observed.

3.2.2. Linearity of calibration curve and LLOQ

The standard calibration curves used for alkaloids and ginsenosides in dog plasma all showed good linearity. The results are shown in Table 2.

Table 5 The stability of BMA, FN, Rg1, Rb1 and Rc in dog plasma.

Conc.			Room temperature (24 h)		Storage at −20 °C (30 days)		Freeze–thaw cycles	
			Measured conc.[a]	Accuracy (RE, %)	Measured conc.[a]	Accuracy (RE, %)	Measured conc.[a]	Accuracy (RE, %)
Alkaloids (ng/mL)	BMA	0.5	0.51 ± 0.05	102.6	0.54 ± 0.02	107.3	0.54 ± 0.01	107.6
		5	5.00 ± 0.20	107.4	4.93 ± 0.20	98.7	5.29 ± 0.20	105.8
		40	40.7 ± 1.00	101.8	38.46 ± 0.80	96.1	42.1 ± 1.90	105.3
	FN	0.5	0.48 ± 0.02	95.6	0.52 ± 0.05	104.9	0.48 ± 0.05	96.4
		5	4.92 ± 0.40	98.4	4.72 ± 0.30	94.3	5.29 ± 0.30	105.6
		40	38.68 ± 1.40	96.6	36.4 ± 1.30	91.0	39.8 ± 1.20	99.5
Ginsenosides (μg/mL)	Rg1	0.05	0.049 ± 0.003	98.4	0.049 ± 0.005	97.2	0.049 ± 0.002	98.4
		0.4	0.41 ± 0.03	102.5	0.39 ± 0.03	97.5	0.37 ± 0.03	92.6
		3	3.07 ± 0.20	102.2	2.82 ± 0.20	94.1	2.92 ± 0.30	97.4
	Rb1	0.25	0.24 ± 0.02	96.0	0.25 ± 0.02	101.4	0.24 ± 0.02	97.7
		2	1.94 ± 0.09	97.0	2.02 ± 0.12	100.9	2.01 ± 0.12	100.4
		15	14.2 ± 0.80	94.7	15.80 ± 0.70	105.0	16.04 ± 0.80	106.9
	Rc	0.125	0.124 ± 0.005	99.4	0.129 ± 0.010	102.9	0.125 ± 0.010	100.1
		1	1.01 ± 0.06	101.4	0.99 ± 0.10	99.4	0.96 ± 0.07	96.1
		7.5	7.32 ± 0.36	97.6	7.62 ± 0.48	101.6	7.60 ± 0.44	101.6

[a]Data are mean ± SD, $n = 5$.

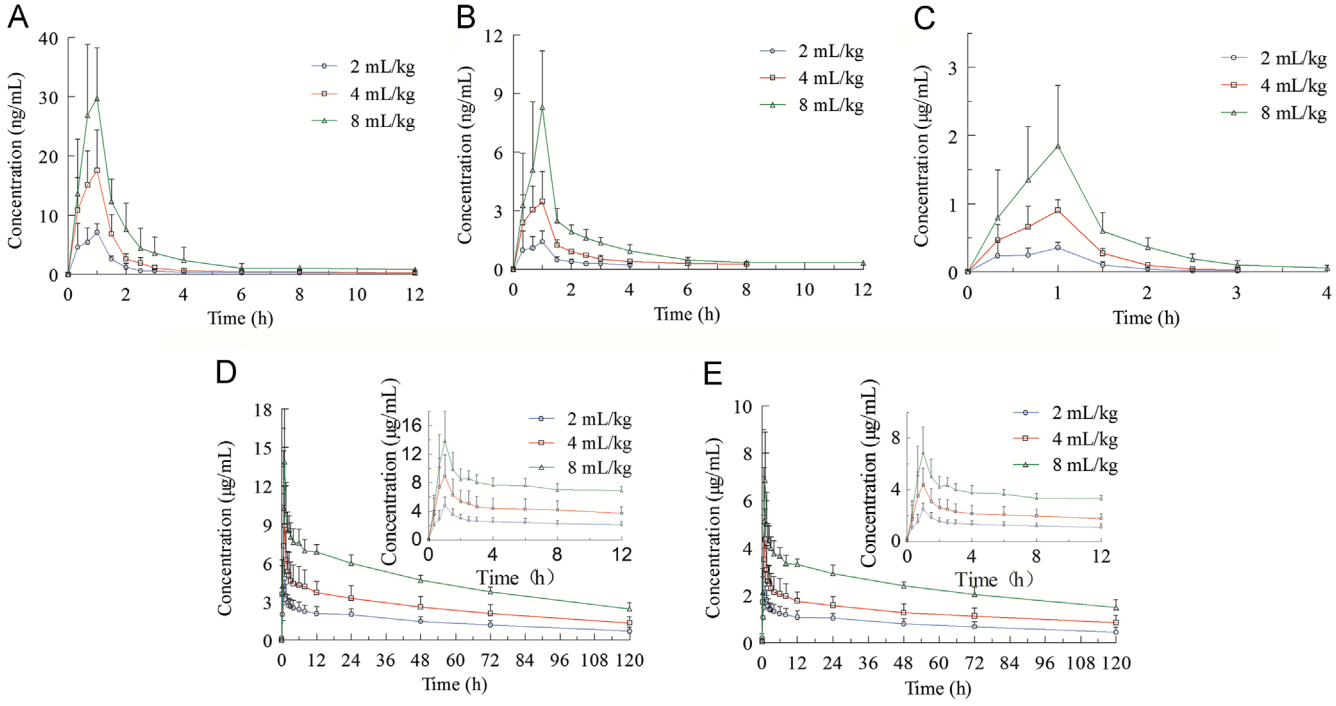


Figure 3 Mean plasma concentration–time curve of five components in beagle dogs after intravenous drip of different single dose (2, 4 and 8 mL/kg) of SFI: A, B, C, D and E represent BMA, FN, Rg1, Rb1 and Rc, respectively (mean ± SD, $n = 6$).

Table 6 Pharmacokinetic parameters of BMA, FN, Rg1, Rb1and Rc in dogs after intravenous drip of different single-dose (2, 4 and 8 mL/kg) of SFI ($n = 6$).

Parameter	BMA (mL/kg)			FN (mL/kg)		
	2	4	8	2	4	8
AUC_{0-t} (μg · h/L)	10.44 ± 3.91	27.09 ± 6.97	55.19 ± 18.19	2.13 ± 0.89	6.17 ± 1.04	13.43 ± 4.44
$AUC_{0-\infty}$ (μg · h/L)	10.44 ± 3.91	27.58 ± 7.47	58.18 ± 20.75	2.13 ± 0.89	6.17 ± 1.04	13.60 ± 4.74
C_{max} (μg/L)	7.18 ± 1.38	17.58 ± 6.78	29.75 ± 8.45	1.42 ± 0.54	3.48 ± 1.41	6.14 ± 3.30
T_{max} (h)	1	1	1	1	1	1
$t_{1/2}$ (h)	4.12 ± 2.63	5.77 ± 4.69	5.33 ± 4.11	1.55 ± 0.48	2.08 ± 1.11	2.15 ± 0.24
MRT_{0-t} (h)	6.44 ± 3.80	8.83 ± 4.69	8.47 ± 5.94	2.74 ± 0.69	3.50 ± 1.61	3.61 ± 0.35
V_d (L)	18.91 ± 5.88	21.65 ± 13.65	16.43 ± 9.10	19.92 ± 13.62	13.99 ± 7.12	14.76 ± 4.73
CL (L/h)	3.70 ± 1.31	2.65 ± 0.72	2.74 ± 1.31	8.51 ± 4.65	4.94 ± 0.86	4.77 ± 1.47

Parameter	Rg1 (mL/kg)			Rb1 (mL/kg)			Rc (mL/kg)		
	2	4	8	2	4	8	2	4	8
AUC_{0-t} (mg · h/L)	0.39 ± 0.16	0.96 ± 0.22	2.13 ± 0.94	173.50 ± 43.34	303.40 ± 91.54	543.50 ± 25.51	96.80 ± 23.77	155.41 ± 42.09	282.27 ± 18.11
$AUC_{0-\infty}$ (mg · h/L)	0.39 ± 0.16	0.96 ± 0.22	2.17 ± 0.93	249.96 ± 89.30	444.46 ± 140.88	780.10 ± 66.40	154.05 ± 52.23	259.48 ± 83.03	473.09 ± 81.66
C_{max} (mg/L)	0.36 ± 0.08	0.75 ± 0.16	1.56 ± 0.57	4.81 ± 1.17	8.90 ± 3.02	13.87 ± 4.12	2.49 ± 0.49	4.38 ± 1.26	6.87 ± 2.02
T_{max} (h)	1	1	1	1	1	1	1	1	1
$t_{1/2}$ (h)	0.38 ± 0.09	0.43 ± 0.11	0.53 ± 0.16	68.11 ± 17.80	73.36 ± 8.38	70.85 ± 11.71	81.91 ± 19.15	92.00 ± 9.22	93.01 ± 24.04
MRT_{0-t} (h)	1.05 ± 0.13	1.12 ± 0.16	1.27 ± 0.24	98.78 ± 25.68	106.36 ± 12.10	102.74 ± 16.89	118.70 ± 27.64	133.25 ± 13.31	134.72 ± 34.68
V_d (L)	3.08 ± 0.94	2.44 ± 0.42	2.35 ± 0.60	1.25 ± 0.28	1.57 ± 0.44	1.60 ± 0.17	1.10 ± 0.20	1.50 ± 0.34	1.55 ± 0.18
CL (L/h)	2.58 ± 0.94	1.94 ± 0.42	1.85 ± 0.60	0.02 ± 0.00	0.02 ± 0.01	0.02 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00

Data are expressed as mean ± SD; AUC_{0-t}, area under the concentration–time curve from 0 to t (t stands for 24, 12, 3, 120 and 120 h to BMA, FN, Rg1, Rb1, Rc, respectively); $AUC_{0-\infty}$, area under the concentration–time curve from 0 h to time infinite; C_{max}, the maximum value of concentration; $t_{1/2}$, elimination half-life; MRT, mean residence time; V_d, volume of distribution; CL, clearance.

Table 7 Statistics results between doses in single-dose pharmacokinetics trial.

Parameter		Compound				
		BMA	FN	Rg1	Rb1	Rc
$LnAUC_{0-t}$/Dose	*F*	1.365	3.565	1.285	1.438	2.857
	p	0.285	0.054	0.306	0.268	0.089
$LnAUC_{0-\infty}$/Dose	*F*	1.500	3.556	1.498	0.855	0.945
	p	0.255	0.054	0.255	0.445	0.411
LnC_{max}/Dose	*F*	0.209	0.202	0.060	1.810	2.844
	p	0.814	0.819	0.942	0.198	0.090
$t_{1/2}$	*p*	0.846	0.115	0.042	0.607	0.135

*$P < 0.05$, a significant statistical difference among three dosage.

3.2.3. *Accuracy and precision*

For all five compounds, the intra-day and inter-day precision and accuracy measured at three concentrations are shown in Table 3. These results demonstrate that the precision and accuracy values are well within the 15% acceptance range.

3.2.4. *Extraction recovery and matrix effect*

The recoveries of all compounds showed no significant differences across the three concentrations (Table 4). Ionization suppression or enhancement caused by co-eluting compounds originating from the matrix is also an important problem in HPLC–MS/MS. At the three QC levels, the observed matrix effects were within the acceptable limits with the results shown in Table 4.

3.2.5. *Stability*

No significant variability (within $\pm 15\%$) of QC concentration was observed in the short-term stability tests (25 °C for 24 h) and three freeze–thaw and long-term stability tests (-20 °C for 30 days), indicating that the five analytes were stable in plasma during the sample preparation process and storage. The results are shown in Table 5.

3.3. *Pharmacokinetic study*

In our study the validated method was applied to the comparative pharmacokinetic analysis of SFI *via* intravenous drip administration. BMA, FN, Rg1, Rb1 and Rc were chosen as the index components to determine the pharmacokinetics of SFI. The mean plasma concentration–time profiles of the five analytes after a single dose administration are illustrated in Fig. 3. The T_{max} equaled the duration of intravenous drip, *i.e.* 1 h, and the maximum plasma concentrations were achieved at the point of drip accomplishment. The pharmacokinetic parameters obtained from DAS 3.0 based on a non-compartmental model are summarized in Table 6. The pharmacokinetics showed a short $t_{1/2}$ for the two aconitum alkaloids, which were approximately 5 and 2 h for BMA and FN, respectively. In comparison of Rb1 and Rc (Ppd type ginsenoside with $t_{1/2}$ of 70 and 90 h, respectively), Rg1 (Ppt type ginsenoside) had the shortest $t_{1/2}$ (less than 30 min) which was in accordance with literature reports[11]. The results showed that plasma concentration increased proportionally to the dosages.

By comparing the dose-normalized pharmacokinetic parameters ($AUC_{0-\infty}$/dose, C_{max}/dose and $t_{1/2}$) among the three dosages with SPSS19.0 statistical software, no statistically remarkable differences were observed (Table 7). The results indicated that all the five analytes exhibited linear kinetics over the dosage range of 2–8 mL/kg after intravenous drip infusion of SFI on beagles.

Due to the light toxicity of BMA and FN, the relative short $t_{1/2}$ makes them relatively safe in clinical use as they can be eliminated completely in the dosing interval. For Rb1 and Rc, the longer $t_{1/2}$ may facilitate maintained effective plasma levels duration dosing and achieve better therapeutic effect.

4. Conclusions

In present study, a rapid, specific and sensitive HPLC–MS/MS method was established for the quantitation of BMA, FN, Rg1, Rb1 and Rc in dog plasma samples. We demonstrated the simultaneous pharmacokinetic evaluation of aconitum alkaloids and ginsenosides after intravenous drip infusion of Shenfu Injection on beagle dogs. In conclusion, exposure to the five components was proportional over the therapeutic dose range of 2–8 mL/kg used in this study. Based on these results, a predictable and linear increase in the systemic exposure of SFI can be expected.

Acknowledgments

The work is supported by the China Resources Sanjiu (Ya'an) Pharmaceutical Co., Ltd., Sichuan, China.

References

1. Zheng CD, Min S. Cardioprotection of Shenfu Injection against myocardial ischemia/reperfusion injury in open heart surgery. *Chin J Integr Med* 2008;**14**:10–6.
2. Song WT, Cheng FF, Xu L, Lin CR, Liu JX. Chinese medicine Shenfu Injection for heart failure: a systematic review and meta-analysis. *Evid Based Complement Altern Med* 2012;**2012**:713149.
3. Liu CX, Hou YZ, Wang XL, Zhao ZQ, Liu Z, Zhai JB, et al. Clinical assessment of Shenfu Injection loading in the treatment of patients with exacerbation of chronic heart failure due to coronary heart disease: study protocol for a randomized controlled trial. *Trials* 2015;**16**:23–8.
4. Ke DZ, Chen QW, Li CL, Li GQ. Cytokines mechanism of Shenfu Injection in treatment of cardiogenic shock in canine. *China J Chin Mater Med* 2007;**32**:2273–7.

5. Li MQ, Pan CG, Wang XM, Mo X, Shi ZX, Xu JY, et al. Effect of the Shenfu Injection combined with early goal-directed therapy on organ functions and outcomes of septic shock patients. *Cell Biochem Biophys* 2015;**72**:807–12.
6. Ji XF, Yang L, Zhang MY, Li CS, Wang S, Cong LH. Shenfu Injection attenuates postresuscitation myocardial dysfunction in a porcine model of cardiac arrest. *Shock* 2011;**35**:530–6.
7. Gu W, Li CS, Yin WP, Guo ZJ, Hou XM, Zhang D. Shen-fu Injection reduces postresuscitation myocardial dysfunction in a porcine model of cardiac arrest by modulating apoptosis. *Shock* 2012;**38**:301–6.
8. Li ZP, Chen SQ, Zhang J, Cheng JY, Huang WJ, Wang WT, et al. Protective effect of different doses of Shenfu Injection on myocardial injury after cardiopulmonary resuscitation in rats. *Chin J TCM WM Crit Care* 2007;**14**:162–5.
9. Yan ZT, Luo WL, Huang JM, Ding H, Qiu Q. Clinical efficacies of different doses of Shenfu Injection in the treatment of chronic heart failure. *China J Tradit Chin Med Pharm* 2015;**30**:636–40.
10. Cui JQ, Li NY, Ding HM. The clinical observation of different doses of Shenfu Injection against shock. *J New Chin Med* 2004;**36**:29–30.
11. Zhan SY, Guo WJ, Shao Q, Fan XH, Li Z, Cheng YY. A pharmacokinetic and pharmacodynamic study of drug–drug interaction between ginsenoside Rg1, ginsenoside Rb1 and schizandrin after intravenous administration to rats. *J Ethnopharmacol* 2014;**152**:333–9.
12. Sun JH, Wu W, Guo YY, Qin QJ, Liu SY. Pharmacokinetic study of ginsenoside Rc and simultaneous determination of its metabolites in rats using RRLC–Q-TOF-MS. *J Pharm Biomed Anal* 2014;**88**:16–21.
13. Kim H. Pharmacokinetics of ginsenoside Rb1 and its metabolite compound K after oral administration of Korean Red Ginseng extract. *J Ginseng Res* 2013;**37**:451–6.
14. Huang QA, Zhang YM, He Y, Lu J, Lin RC. Studies on hydrolysis of aconitine. *China J Chin Mater Med* 2007;**32**:2143–5.
15. Wang Y, Shi L, Song FR, Liu ZQ, Liu SY. Exploring the ester-exchange reactions of diester-diterpenoid alkaloids in the aconite decoction process by electrospray ionization tandem mass spectrometry. *Rapid Commun Mass Spectrom* 2003;**17**:279–84.
16. Tang L, Gong Y, Lv C, Ye L, Liu L, Liu ZQ. Pharmacokinetics of aconitine as the targeted marker of Fuzi (*Aconitum carmichaeli*) following single and multiple oral administrations of Fuzi extracts in rat by UPLC/MS/MS. *J Ethnopharmacol* 2012;**141**:736–41.
17. Zhang Q, Ma YM, Wang ZT, Wang CH. Pharmacokinetics difference of multiple active constituents from decoction and maceration of Fuzi Xiexin Tang after oral administration in rat by UPLC–MS/MS. *J Pharm Biomed Anal* 2014;**92**:35–46.
18. Liu JJ, Li Q, Yin YD, Liu R, Xu HR, Bi KS. Ultra-fast LC–ESI-MS/MS method for the simultaneous determination of six highly toxic *Aconitum* alkaloids from aconiti kusnezoffii radix in rat plasma and its application to a pharmacokinetic study. *J Sep Sci* 2014;**37**:171–8.
19. Long W, Zhang SC, Wen L, Mu L, Yang F, Chen G. *In vivo* distribution and pharmacokinetics of multiple active components from Danshen and Sanqi and their combination *via* inner ear administration. *J Ethnopharmacol* 2014;**156**:199–208.
20. Sun JG, Zhang FY, Peng Y, Liu JH, Zhong YX, Wang GJ. Quantitative determination of diterpenoid alkaloid Fuziline by hydrophilic interaction liquid chromatography (HILIC)–electrospray ionization mass spectrometry and its application to pharmacokinetic study in rats. *J Chromatogr B* 2013;**913-914**:55–60.
21. Li ZG, Zhang R, Wang XP, Hu XF, Chen YG, Liu QF. Simultaneous determination of seven ginsenosides in rat plasma by high-performance liquid chromatography coupled to time-of-flight mass spectrometry: application to pharmacokinetics of Shenfu Injection. *Biomed Chromatogr* 2015;**29**:167–75.
22. Zhang F, Tang MH, Chen LJ, Li R, Wang XH, Duan JG, et al. Simultaneous quantitation of aconitine, mesaconitine, hypaconitine, benzoylaconine, benzoylmesaconine and benzoylhypaconine in human plasma by liquid chromatography–tandem mass spectrometry and pharmacokinetics evaluation of "SHEN-FU" injectable powder. *J Chromatogr B* 2008;**873**:173–9.
23. Lv CX, Li Q, Zhang YW, Sui ZY, He BS, Xu HR, et al. A UFLC-MS/MS method with a switching ionization mode for simultaneous quantitation of polygalaxanthone III, four ginsenosides and tumulosic acid in rat plasma: application to a comparative pharmacokinetic study in normal and Alzheimer's disease rats. *J Mass Spectrom* 2013;**48**:904–13.
24. Liu X, Li H, Song XQ, Qin KM, Guo H, Wu L, et al. Comparative pharmacokinetics studies of benzoylhypaconine, benzoylmesaconine, benzoylaconine and hypaconitine in rats by LC–MS method after administration of Radix Aconiti Lateralis Praeparata extract and Dahuang Fuzi Decoction. *Biomed Chromatogr* 2014;**28**:966–73.
25. Yang H, Liu L, Gao W, Liu K, Qi LW, Li P. Direct and comprehensive analysis of ginsenosides and diterpene alkaloids in Shenfu Injection by combinatory liquid chromatography–mass spectrometric techniques. *J Pharm Biomed Anal* 2014;**92**:13–21.