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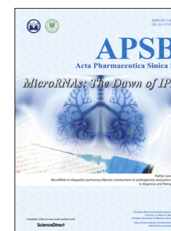

ORIGINAL ARTICLE

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

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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.

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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

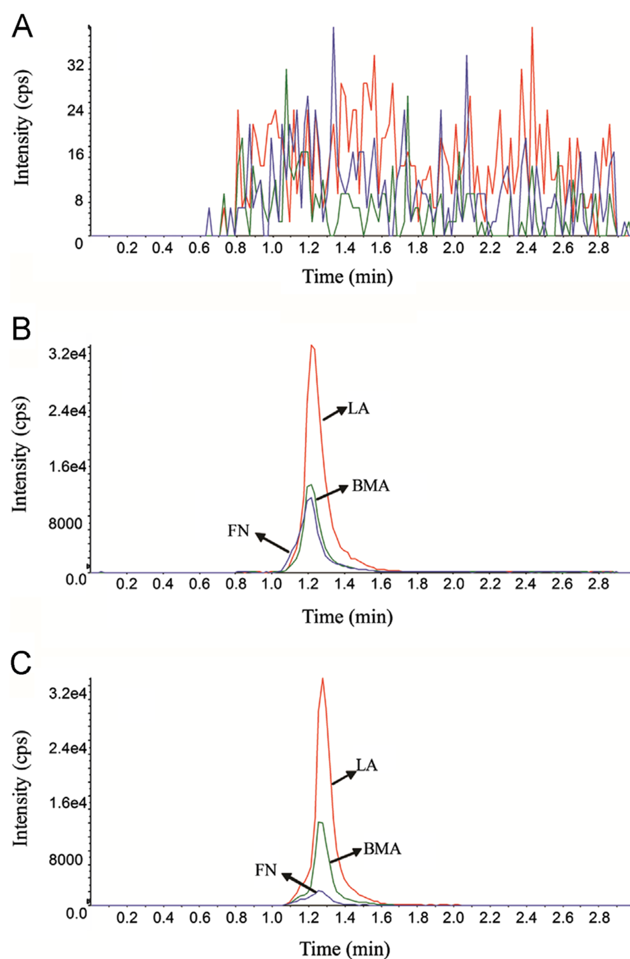

Figure 1 Representative chromatograms of (A) blank plasma; (B) blank plasma spiked with BMA (20 ng/mL, $t_R=1.25$ min), FN (20 ng/mL, $t_R=1.25$ min) and LA (100 ng/mL, $t_R=1.3$ min); (C) plasma sample of 1 h after administration of SFI at a dose of 4 mL/kg.

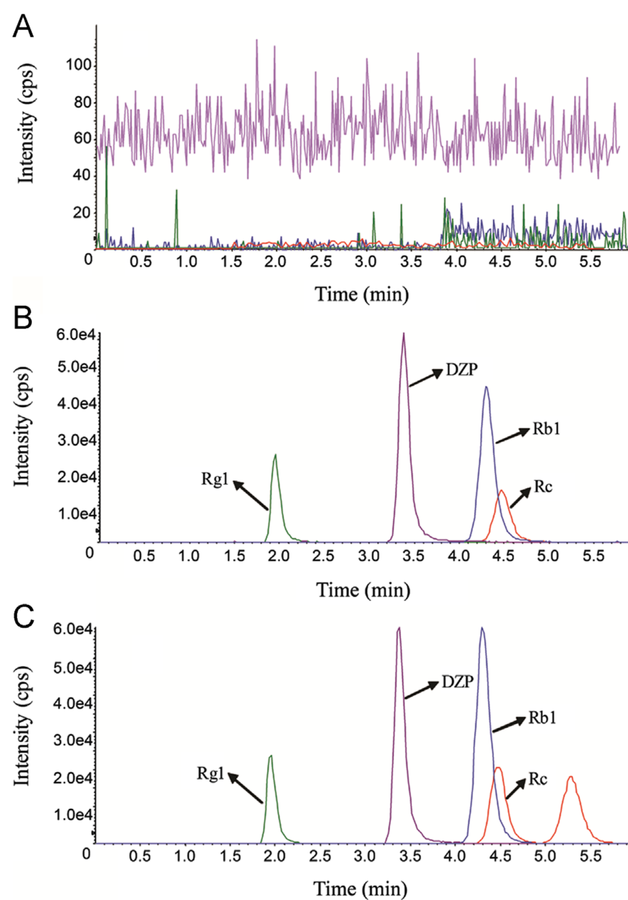

Figure 2 Representative chromatograms of (A) blank plasma; (B) blank plasma spiked with Rg1 (1 μg/mL, $t_R=2.0$ min), Rb1 (5 μg/mL, $t_R=4.3$ min), Rc (2.5 μg/mL, $t_R=4.5$ min) and DZP (diazepam, 0.1 μg/mL, $t_R=3.4$ min); (C) plasma sample of 1 h after administration of SFI at a dose of 4 mL/kg.

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, $n = 5$.

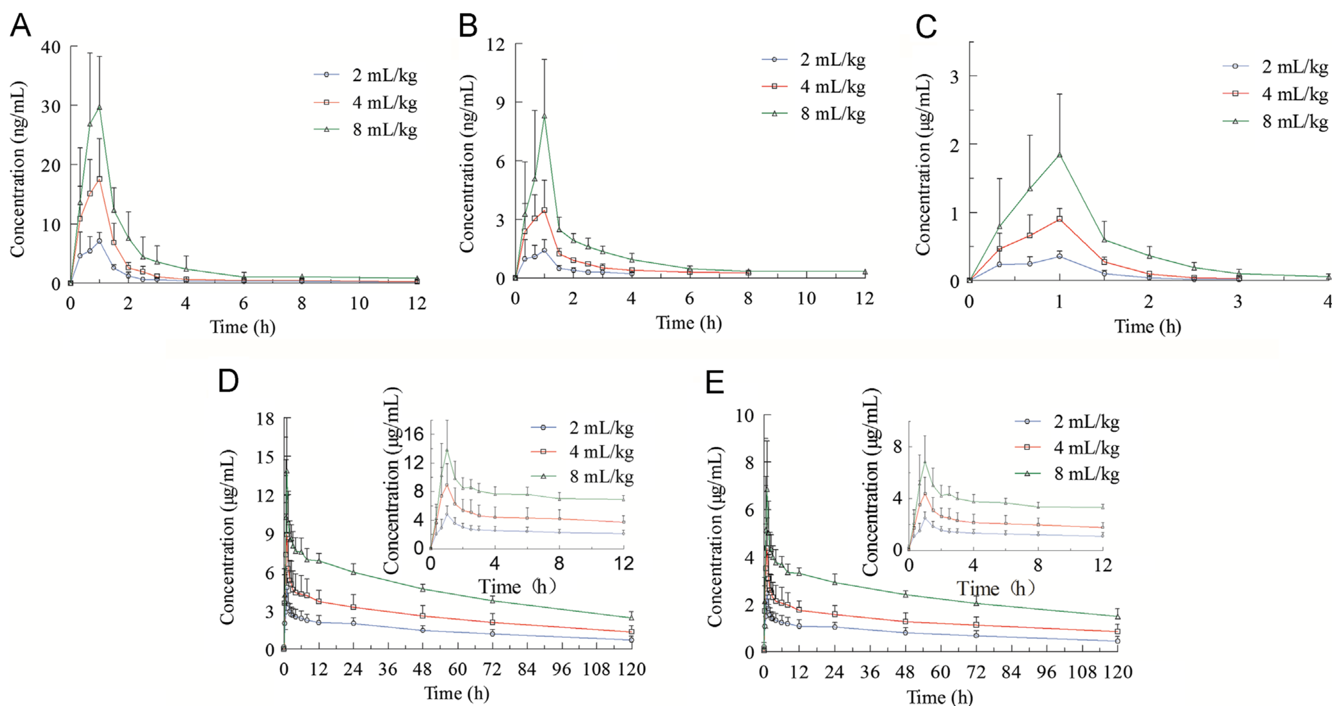

Figure 3 Mean plasma concentration–time curve of five components in beagle dogs after intravenous drip of different single dose (2, 4 and 8 mL/kg) of SFI: A, B, C, D and E represent BMA, FN, Rg1, Rb1 and Rc, respectively (mean±SD, n=6).

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 ±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.

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