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Simultaneous determination of three *Panax notoginseng* saponins at sub-nanograms by LC–MS/MS in dog plasma for pharmacokinetics of compound Danshen tablets

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ABSTRACT

Compound Danshen tablets are composed of *Panax notoginseng*, *Salvia miltiorrhiza* and *Borneol*. The tablets are prescribed for treatment of cardiovascular diseases in China. The present study aimed at developing a specific and sensitive LC–MS/MS method to simultaneously determine three bioactive *P. notoginseng* saponins, i.e., notoginsenoside R1, ginsenoside Rg1 and Rb1, in dogs after a single oral administration of the compound tablets in order to obtain the clinically relevant saponin-related pharmacodynamics of the tablets in patients. The R1, Rg1 and Rb1 were extracted from dog plasma with acetone–methanol (80:20, v/v), separated by reversed phase liquid chromatography and determined by tandem mass spectrometry (LC–MS/MS) with positive electrospray ionization (ESI). The developed method reached lower limit of quantitation (LLOQ) at 0.10 ng/ml for the three saponins. The method was validated in terms of selectivity, matrix effects, linearity, precision and accuracy, and then was applied to a pharmacokinetic study of the three bioactive saponins simultaneously in dogs after a single oral administration of compound Danshen tablets at a clinical equivalent dose. The C_{max} and $AUC_{(0-\infty)}$ for R1, Rg1 and Rb1 were 1.91, 3.34 and 28.6 ng/ml, and 7.5, 11.0, and 1712 (h ng/ml), respectively.

1. Introduction

Compound Danshen tablets are formulated with traditional Chinese medicines, i.e., Radix *Panax notoginseng,* Sanqi in Chinese; Radix *Salviae miltiorrhizae,* Danshen in Chinese; and Borneol, Bingpian in Chinese [1]. It has been prescribed for treatment of cardiovascular diseases in China [2] and documented in the Chinese Pharmacopoeia [3]. *P. notoginseng* contains saponins (or sapogenin glycosides), a type of glycoside that widely distributes in plants. Each saponin consists of a sugar and a sapogenin, the latter constitutes the aglucon moiety. The sapogenin may be a steroid or a triterpene. The sugar moiety may be a glucose, maltose, fructose, galactose, pentose, or methylpentose. Ginsenosides are considered as the main active principals of saponins and often used as a marker for the quality control of related commercial products [4]. Saponins from *P. notoginseng* are effective for antagonizing thrombosis, dilating blood vessel and protecting cardiac microvessels [5–8], therefore they have substantial contribution to the effectiveness of compound Danshen tablets in the treatment of coronary heart disease, cardiac angina and atherosclerosis [9,10].

In compound Danshen tablets, there are three main bioactive saponins from *P. notoginseng*, known as notoginsenoside R1 (R1), ginsenoside Rg1 (Rg1) and ginsenoside Rb1 (Rb1) (Fig. 1) [4,11,12]. However, due to the low contents (only several milligrams of these bioactive saponins contain in each tablet [13]) and the low oral bioavailability of these saponins (single-digit percentage) [14,15], the plasma concentrations of these saponins after oral administration are in the low nanogram per millilitre levels. In recent, pharmacokinetic studies of compound Danshen prescriptions focused mainly on the bioactive phenolic acids or diterpenoid quinones derived from Radix *S. miltiorrhizae* [16,17]. To date, only a few of pharmacokinetic studies were focused on saponins using HPLC–UV [18,19], enzyme-linked immunosorbent assays (ELISA) [20], MS [21–25] or MS/MS detection [26,27] with insufficient selectivity and/or sensitivity. As a result, the studies were often carried out at much higher doses than those clinically relevant to compromise the assay sensitivity [14,24,28]. Pharmacokinetic studies with 20(*S*)-protopanaxadiol (PPD) [29,30], the aglycone of ginsenoside Rb1, Rg3, Rh2 and other PPD containing saponins have been reported. However, these studies could only reflect the saponins in total and cannot directly depict the pharmacokinetic profiles of each individual saponin simultaneously.

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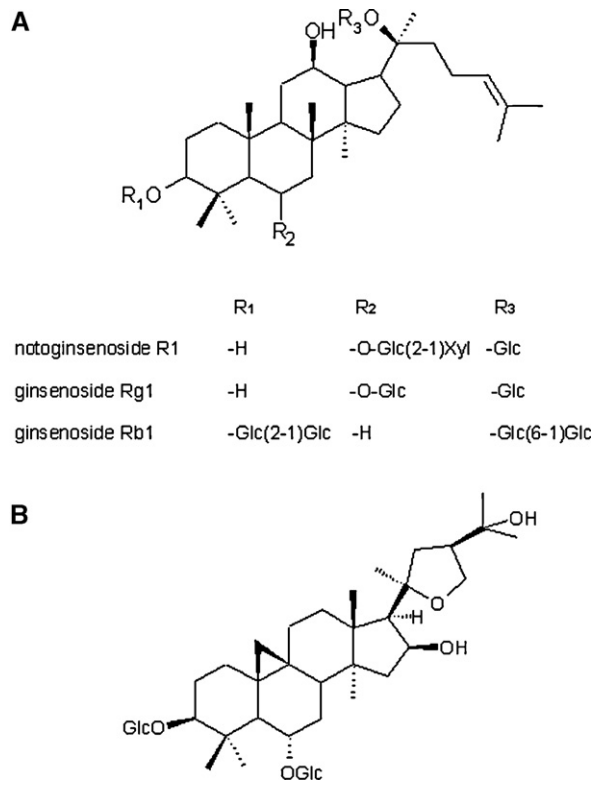

Fig. 1. Chemical structures of: (A) notoginsenoside R1, ginsenoside Rg1 and Rb1 and (B) astragaloside IV (IS).

This study focused on developing an LC–MS/MS method that is specific and sensitive enough to allow to determine the concentrations of the three saponins, i.e., R1, Rg1 and Rb1, simultaneously in clinical trials. To achieve the goal, the method was validated and then applied the method to the pharmacokinetic study with the tablets in dogs at a clinical equivalent dose to demonstrate the feasibility of the method for human study.

2. Materials and methods

2.1. Materials and reagents

Reference standards of ginsenoside Rg1 and astragaloside IV (the internal standard, IS) were purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Notoginsenoside R1 and ginsenoside Rb1 were obtained from Chengdu Scholar bio-Tech. Co., Ltd. (Chengdu, China). The chemical structures of the saponins and internal standard (IS) are shown in Fig. 1. Compound Danshen tablets used in this study were the products of BYS Co., Ltd. (Guangzhou, China, Batch No. A8A024). Contents of the major components in each tablet were quantified by HPLC–ELSD for saponins from Radix *P. notoginseng* [13] and HPLC–UV for phenolic acids and diterpenoid quinones from Radix *S. miltiorrhizae* [31]. Methanol and methyl tert-butyl ether (Merck, Germany) were of HPLC grade. Formic acid and acetone (Nanjing Reagent, China) were of analytical grade. Doubly distilled water was used throughout the study.

2.2. Equipments and chromatographic conditions

The LC–MS/MS system consisted of a Surveyor LC pump, a Surveyor auto-sampler, and a Finnigan TSQ Quantum Ultra AM triple-quadrupole tandem mass spectrometer with an ion max source, Xcalibur 1.2 software for data acquisition and analysis (Thermo Finnigan, San Jose, CA, USA).

The HPLC separation was performed on a Lichrospher ODS-3 analytical column (150 mm × 4.6 mm, 5 μm) with a linear gradient elution by a mobile phase consisting of water (A) and methanol (B) both containing 0.1% formic acid with following steep steps: 0 min (A 50%–B 50%)–1.0 min (A 0%–B 100%)–4.5 min (A 0%–B 100%)–4.6 min (A 50%–B 50%)–6.5 min (A 50%–B 50%), with the flow rate of 1.0 ml/min. The injection volume was 20 μl. Post column split was used and 30% of the effluent from time 2.7 to 6.5 min was delivered into the mass spectrometer. The detection was made with electrospray positive ionization and multiple reactions monitoring (MRM) with following parameters used: spray voltage, 5 kV; capillary temperature, 350 °C; nitrogen sheath gas and auxiliary gas pressures, 241 and 34 kPa, respectively. The prominent parent ions produced were all of sodium adducts $[M+Na]^+$. Argon gas collision-induced dissociation were operated with a pressure of 0.2 Pa and the collision energies of 45 eV for both R1 and Rg1, 53 eV for both Rb1 and IS. The ion transitions chosen for multiple reactions monitoring (MRM) were m/z 955.3 → 775.5 for R1, m/z 823.2 → 643.3 for Rg1, m/z 1131.5→365.1 for Rb1 and m/z 807.2→627.4 for IS.

2.3. Preparation of standards, IS and QCs solution

Stock solutions of R1, Rg1 and Rb1 were prepared separately by dissolving an accurately weighted amount of each reference standard in a mixture of water–methanol (1:1, v/v) to yield a concentration of 5 μg/ml, respectively. The combined working standard solutions were generated by serial dilution of the stock solutions with the same solvent. The calibration plasma standards were prepared by spiking aliquots of 0.5 ml dog blank plasma with 50 μl of each of the combined working standard solution covering the ranges from 0.1 to 10.0 ng/ml for R1 and Rg1, and from 0.1 to 100 ng/ml for Rb1. The IS (astragaloside IV) solutions were prepared in the same way as described above at a concentration of 1 μg/ml. All the stock and working standard solutions were stored under refrigeration at about 10 °C.

QC samples were prepared at three concentrations of 0.22, 2.25 and 11.2 ng/ml for R1; 0.21, 2.10 and 10.5 ng/ml for Rg1; and four concentrations of 0.22, 2.17, 10.8 and 54.2 ng/ml for Rb1, with five replicates for each concentration.

2.4. Sample preparation

An aliquot of 0.5 ml plasma sample was mixed with 50 μl IS solution (astragaloside IV, 1 μg/ml) and 50 μl water–methanol (50:50, v/v). 2.5 ml acetone–methanol (80:20, v/v) was then added for protein precipitation. After vortexing for 3 min and centrifuging at 1500 × *g* for 10 min, the supernatant was separated and evaporated to dryness under a gentle stream of nitrogen at 40 °C. The residue was reconstituted with 0.15 ml of methanol–water (50:50, v/v) by vortexing for 1 min. The supernatant obtained after centrifugation (15,000 × *g*, 10 min) was transferred to an auto-sampler vial and an aliquot of 20 μl was injected into the LC–MS/MS system.

2.5. Method development

2.5.1. Assay specificity and matrix effects

The specificity was investigated by analyzing six individual dog blank plasma samples. Each blank sample was tested for potential interferences. To evaluate the matrix effects, chromatographic peak of R1, Rg1, Rb1 and IS from the spike-after preparation samples were compared with those of the neat standards in mobile phase at the QC concentrations.

2.5.2. Linearity and lower limit of quantification (LLOQ)

The linearity of the method was determined by analyzing a series of standard plasma samples at concentrations from 0.1 to

Table 1
Contents of the major components in each compound Danshen tablet ($n = 3$).

Component	Mean ± SD (mg)
Danshensu[a]	0.96 ± 0.003
Protocatechuic aldehyde[a]	0.065 ± 0.0002
Lithospermic acid[a]	0.47 ± 0.01
Rosmarinic acid[a]	0.41 ± 0.003
Salvianolic acid B[a]	6.41 ± 0.03
Salvianolic acid A[a]	0.79 ± 0.003
Tanshinone II_A[a]	0.47 ± 0.01
Cryptotanshinone[a]	0.37 ± 0.01
Notoginsenoside R1[b]	0.39 ± 0.02
Ginsenoside Rg1[b]	1.53 ± 0.03
Ginsenoside Rb1[b]	1.81 ± 0.04

[a] Components from *Salvia miltiorrhiza*, quantitated by HPLC–UV.
[b] Components from *Panax notoginseng*, quantitated by HPLC–ELSD.

10 ng/ml for R1 and Rg1, and from 0.1 to 100 ng/ml for Rb1 by least-squares linear regression of the peak area ratios of each analyte to IS obtained against the corresponding concentrations (C) with a weighting factor of $1/C^2$. The LLOQ was defined as the lowest concentration on the calibration curve with acceptable precision and accuracy. The criteria for the calibration included a correlation coefficient (r) of 0.99 or better and the found value for plasma calibration standards being within ±15% deviation (expressed as RE%) from the nominal value except for the LLOQ within ±20%. Concentrations in the QCs and unknown plasma samples were quantified by using the internal standard calibration method.

2.5.3. Accuracy, precision and recovery

The intra-assay accuracy and precision were evaluated by analyzing five replicates at the QC concentration levels on the same day. The inter-assay precision was determined by analyzing the respective QC samples on three different days. The accuracy expressed as RE% was set within ±20% at the LLOQ and ±15% at all other concentrations, while the results of precision expressed as RSD% should below 20% for LLOQ and less than 15% for the others.

Recoveries of R1, Rg1, Rb1 and IS from dog plasma were determined by comparing of the responses of the saponins and IS in plasma carried through the complete preparation procedure to those spiked into the prepared blank plasma of the same concentrations as those of QCs, respectively.

2.5.4. Stability test

Freeze–thaw stability was assessed by conducting three freeze–thaw cycles of the dog plasma samples containing the three saponins separately at 0.5 and 5.0 ng/ml. Each cycle consisted of removing the samples from the freezer at −20 °C, thawing them unassisted to room temperature, keeping them at room temperature for 2 h and refreezing them at −20 °C for 12 h.

Long-term stability was evaluated on duplicate samples at 0.5 and 5.0 ng/ml stored at −80 °C for 10, 20 and 30 days, respectively. The time period is considered long enough to disclose any problems of sample stability from the date of collection to the date of determination.

Deviation of the stability results was set within ±15% of the nominal values.

2.6. Pharmacokinetic study in dogs

The method was applied to a pharmacokinetic study of R1, Rg1 and Rb1 in beagle dogs after a single oral administration of three compound Danshen tablets which were equivalent to a clinical single oral dose. The contents of the major components of the compound Danshen tablets used in this study are shown in Table 1. The contents of the saponins administrated were determined as 1.17 ± 0.05, 4.59 ± 0.08 and 5.43 ± 0.11 mg of R1, Rg1 and Rb1, respectively.

Five male and five female beagle dogs of 9–10 months old with an average weight of 10.2 ± 0.7 kg purchased from Nanjing Animal Technology (Nanjing, China) were included in the study. Each dog was randomly given a single oral dose of three tablets, equivalent to a typical clinical dose after an overnight fast. Blood samples about 2 ml were withdrawn from the dog forelimb veins into heparinized polythene tubes prior to, 0.25, 1, 1.5, 2, 3, 4, 5, 6, 8, 12, 24, 48, 72, 96 and 120 h after the oral administration. The plasma samples were prepared and stored at −80 °C until analysis. The study protocol was approved by the Institutional Animal Care and Use Committee of Nanjing Medical University. The pharmacokinetic parameters of R1, Rg1 and Rb1 were calculated by using the non-compartmental method with the aid of the DAS program (Drugs and Statistics version 2.0, Chinese Pharmacological Society, China).

3. Results and discussion

3.1. Sample preparation and LC–MS/MS optimization

Different liquid–liquid extraction and protein precipitation methods by using *n*-butanol, acetonitrile, methanol, or acetone as a single organic phase were tested. And no single organic solvent mentioned above could give a satisfactory recovery for all the three saponins. We then tested mixtures of organic solvents at different ratios, and found that a mixture of acetone and methanol at 80:20 (v/v) was most suitable for both the solubility of the saponins and the protein precipitation. The average recoveries of the three saponins from the dog plasma were above 80% when the solvent mixture was used.

To optimize the MS conditions, we tested both electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI). When APCI was used, no specific and abundant ions for the saponins were observed in either positive or negative mode probably because the saponins possess high polarity and/or weak lipophilicity. In the negative mode of ESI, $[M-H]^-$ for R1, Rg1, Rb1 and $[M+HCOO]^-$ for IS were formed only at low abundance. In contrast, the positive ESI showed abundant and stable sodium adduct ions of $[M+Na]^+$ and therefore we selected $[M+Na]^+$ as the precursor ions for the multiple reactions monitoring (MRM) detection of the three analytes and IS with positive ESI. The product ion spectra of the three saponins and IS are shown in Fig. 2. The precursor-product ion reactions selected were m/z 955.3 → 775.5 for R1, m/z 823.3 → 643.3 for Rg1, m/z 1131.5 → 365.1 for Rb1 and m/z 807.2 → 627.4 for IS. Other conditions such as spray voltage, desolvation gas flow, capillary temperature, and argon gas pressure and collision energy were then optimized. Although no sodium ions were added to the samples and chromatographic system, the ions may come from sodium chloride in plasma as reported by others [32]. The sensitivity obtained in this study with the LLOQs at 0.1 ng/ml for the three saponins was much better than previously reported MS methods using either chlorinated adduct ions $[M+Cl]^-$ [21,25], de-protonated ions of $[M-H]^-$ [33] or $[M+HCOO]^-$ [23].

To separate these saponins, we tried different stationary phases, mobile phase compositions and elution modes. Diverse retentions and poor peak shapes were often produced with isocratic elution. To improve peak shapes, sensitivity and selectivity, the gradient elution on Lichrospher ODS-3 analytical column (150 mm × 4.6 mm, 5 μm) with a mobile phase consisted of water (A) and methanol (B) both containing 0.1% formic acid was applied. The linear gradient steps was optimized as follows: 0 min (A 50%–B 50%)–1.0 min (A 0%–B 100%)–4.5 min (A 0%–B 100%)–4.6 min (A 50%–B 50%)–6.5 min (A 50%–B 50%). All the analytes were eluted from 4 to 5 min with increasing methanol which was necessary for

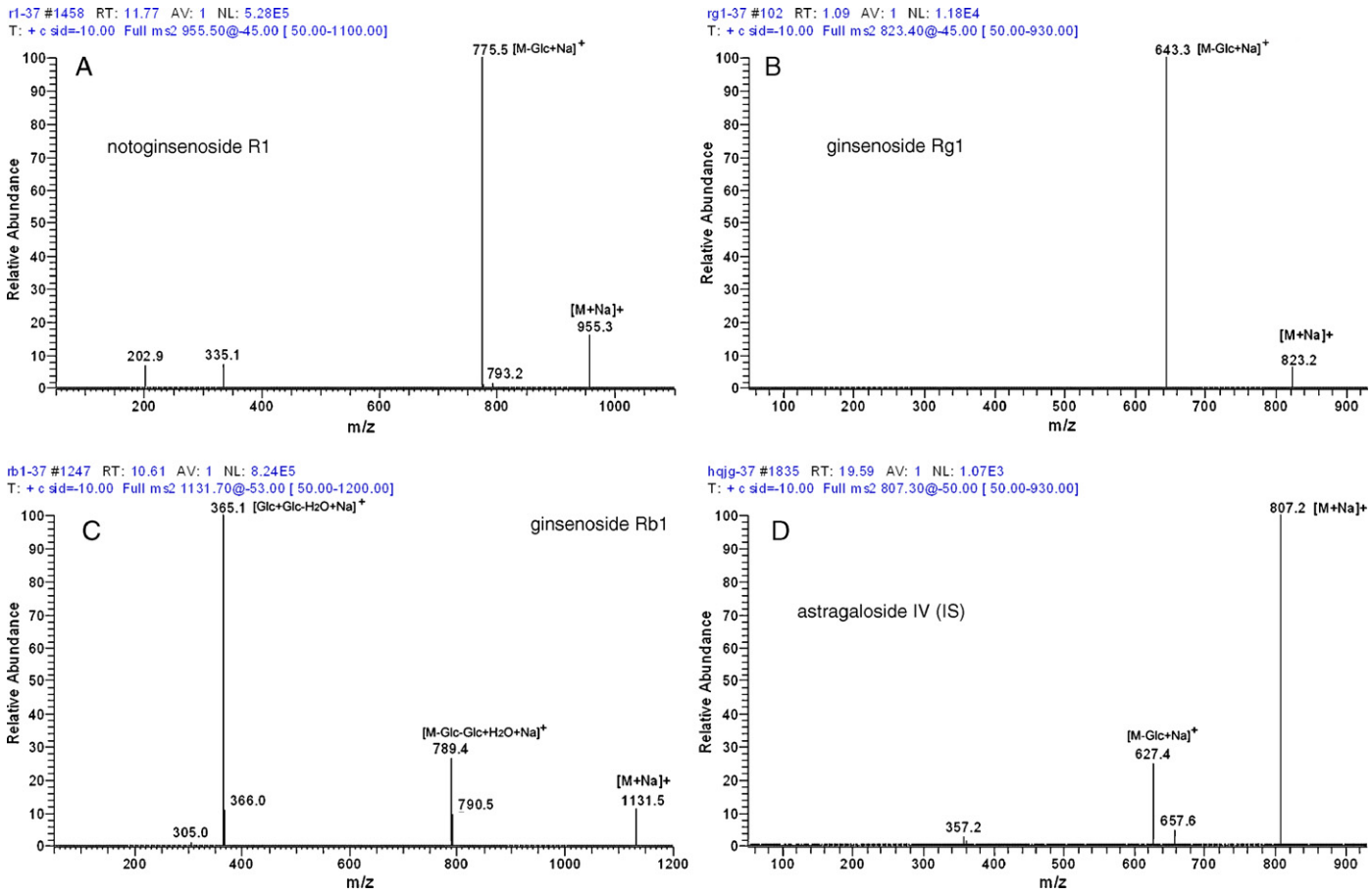

Fig. 2. The product ion spectra of: (A) notoginsenoside R1; (B) ginsenoside Rg1; (C) ginsenoside Rb1; (D) astragaloside IV (IS).

effective ionization and sufficient separation of the three saponins from the endogenous polar matrix components as shown in Fig. 3. The retention time of R1, Rg1, Rb1 and IS was 4.06, 4.14, 4.44 and 4.93 min, respectively.

3.2. Method validations

3.2.1. Specificity

Six different blank plasmas were checked for any erroneous positive MS responses. The blank plasma spiked with analytes and IS was tested as well. Under the described LC–MS/MS conditions, a good separation of the analytes was achieved and no obvious interferences from endogenous plasma substances were observed.

3.2.2. Extraction recovery, precision, accuracy and matrix effects

High efficiency of recovery was found for each analyte with the mean values of absolute recovery for R1, Rg1 and Rb1 above 80% as shown in Table 2. The intra- and inter-assays precision values were ranged from 4.7% to 11.4% and 4.6% to 19.0%, respectively, for all the analytes at the corresponding QC levels tested (Table 3). The accuracy expressed as deviation percentage was found to be within the limits of criteria for the three saponins. No obvious matrix effects were found for all the analytes as the test results ranged from 86% to 117% (Table 4), which were within the acceptable limit. The same matrix effect evaluation was performed on the IS and no significant matrix effects were observed as well (data not shown).

3.2.3. Stability

The three saponins in stock and working standard solutions were stable under the specified conditions for at least 2 months. R1, Rg1 and Rb1 in dog plasma were stable after three freeze–thaw cycle tests, and stable at room temperature for 8 h and at −80 °C for 30 days since no significant degradations were observed in all the cases and the deviations in the concentrations tested were all within 10% of their nominal values with RSD < 15% (data not shown).

3.2.4. Linearity and LLOQ

The linear calibration curves were established from concentration ranges of 0.10–10 ng/ml for both R1 and Rg1, and 0.10–100 ng/ml for Rb1 in dog plasma, respectively. Typical equations were as follows: $R_{R1} = 0.04994 \times C_{R1} - 0.00451$ (0.10–10 ng/ml, $r = 0.9981$), $R_{Rg1} = 0.07554 \times C_{Rg1} - 0.00550$ (0.10–10 ng/ml, $r = 0.9987$), $R_{Rb1} = 0.06407 \times C_{Rb1} - 0.00437$

Table 2
Extraction recoveries of notoginsenoside R1, ginsenoside Rg1 and Rb1 from dog plasma ($n = 5$).

Analytes	Spiked concentration (ng/ml)	Recovery (%)	RSD (%)
Notoginsenoside R1	0.22	91.0	12.2
	2.25	100.9	4.0
	11.2	97.8	6.7
Ginsenoside Rg1	0.21	98.1	14.4
	2.10	91.4	3.6
	10.5	90.1	5.4
Ginsenoside Rb1	0.22	88.2	5.2
	2.17	81.1	4.0
	10.8	81.3	3.6
	54.2	80.4	11.3

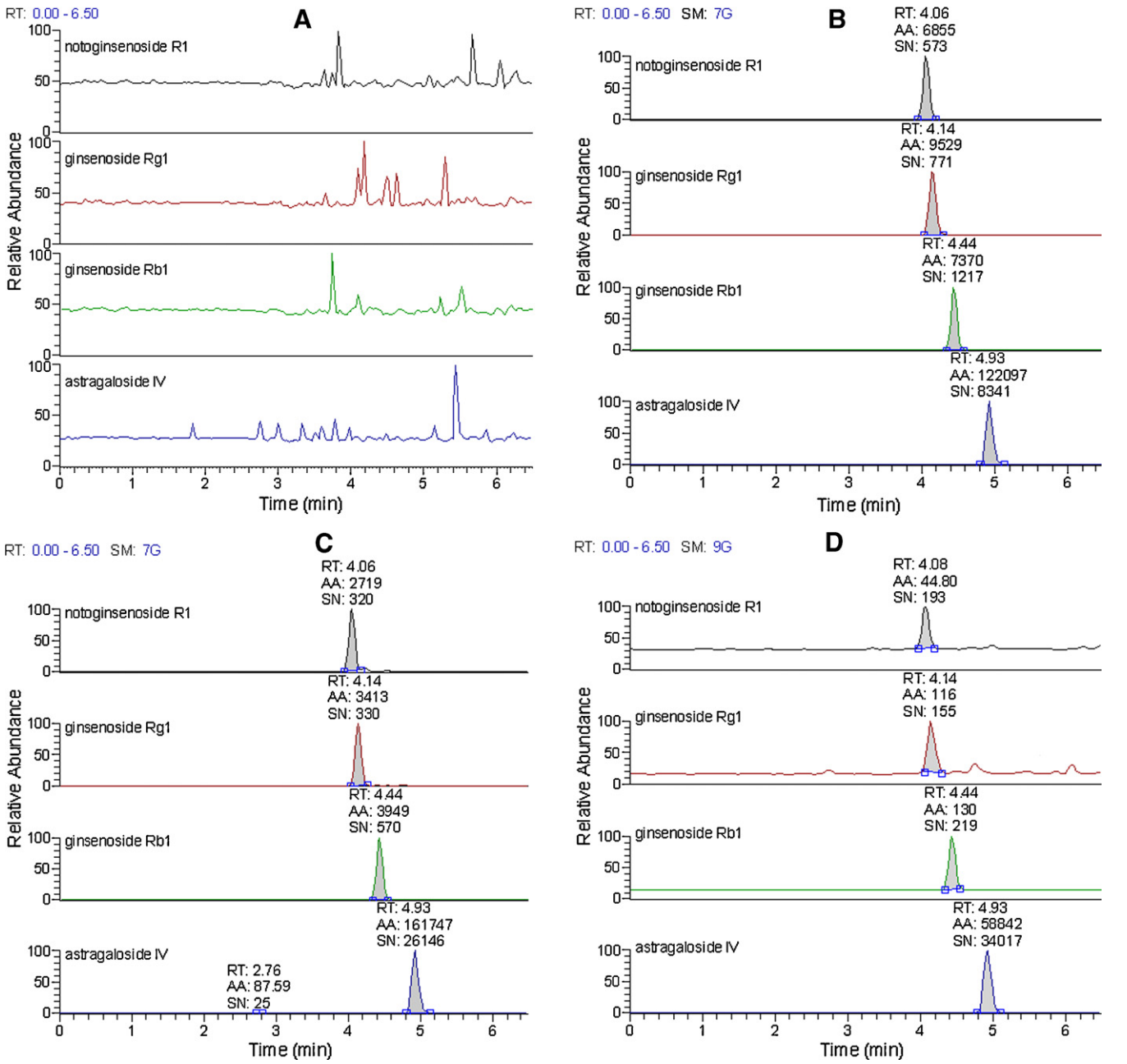

Fig. 3. LC–MS/MS chromatograms of: (A) Blank plasma; (B) blank plasma spiked with notoginsenoside R1 (t_R = 4.06 min, C = 1.12 ng/ml), ginsenoside Rg1 (t_R = 4.14 min, C = 1.05 ng/ml), ginsenoside Rb1 (t_R = 4.44 min, C = 1.08 ng/ml) and astragaloside IV (IS, t_R = 4.91 min, C = 100 ng/ml). (C) Plasma sample obtained 2 h after oral administration of compound Danshen tablets; (D) plasma sample at LLOQ concentrations of the saponins (C = 0.10 ng/ml, respectively).

Table 3
Precision and accuracy for the analysis of notoginsenoside R1, ginsenoside Rg1 and Rb1 in dog plasma (n = 3 runs, 5 replicates per run).

Analytes		Notogisenoside R1			Ginsenoside Rg1			Ginsenoside Rb1			
Spiked concentration (ng/ml)		0.22	2.25	11.2	0.21	2.10	10.5	0.22	2.17	10.8	54.2
Measured concentration (ng/ml)	Run 1	0.22	2.03	11.5	0.23	2.13	11.1	0.23	2.08	11.2	53.6
	Run 2	0.21	2.16	10.3	0.22	2.19	10.7	0.20	2.06	10.0	55.9
	Run 3	0.21	2.02	11.3	0.22	2.11	11.7	0.20	1.99	10.3	55.4
Grand mean		0.21	2.07	11.0	0.22	2.14	11.2	0.21	2.04	10.5	55.0
(RE%)		−3.03	−8.00	−1.49	6.35	2.06	6.35	−4.55	−5.84	−2.78	1.41
Inter-assay precision (RSD%)		8.5	8.5	12.8	5.2	4.6	10.1	19.0	5.0	13.6	5.0
Intra-assay precision (RSD%)		11.0	5.6	5.7	11.4	4.8	4.7	6.3	5.6	7.2	7.5

Table 4
Matrix effects of notoginsenoside R1, ginsenoside Rg1 and Rb1 ($n = 2$).

Analytes	Spiked concentration (ng/ml)	Matrix effects (%)	Mean ± SD (%)
Notoginsenoside R1	0.22	89.8	
	2.25	86.0	89.6 ± 3.5
	11.2	92.9	
Ginsenoside Rg1	0.21	103.1	
	2.10	90.2	95.8 ± 6.6
	10.5	94.2	
Ginsenoside Rb1	0.22	107.0	
	2.17	115.1	109.0 ± 5.3
	10.8	117.0	
	54.2	97.0	

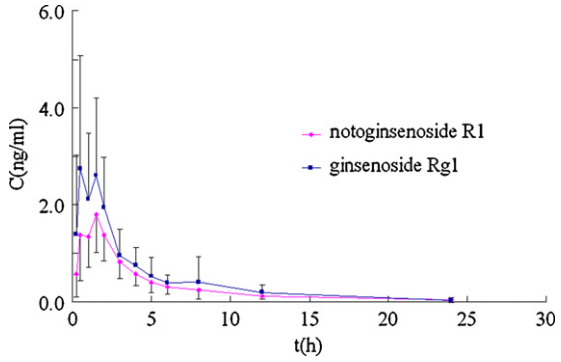

Fig. 4. Plasma concentration–time profiles of notoginsenoside R1 and ginsenoside Rg1 in beagle dogs after a single oral dose of three compound Danshen tablets (means ± SD, $n = 10$).

(0.10–100 ng/ml, $r = 0.9983$) for R1, Rg1 and Rb1, respectively. The LLOQs were found to be 0.10 ng/ml for each of the three analytes as shown in Table 5. Fig. 3D depicts a typical chromatogram obtained at the LLOQ level. The sensitivity achieved has made the method feasible for the pharmacokinetic study of saponins in beagle dogs at clinically equivalent dosage of compound Danshen tablets.

3.3. Pharmacokinetic study

The mean plasma concentration–time profiles of the three saponins in beagle dogs following a single oral dose of three compound Danshen tablets are illustrated in Figs. 4 and 5. The pharmacokinetic parameters of R1, Rg1 and Rb1 estimated are shown in Table 6.

Significant differences were found between the pharmacokinetic behaviors of Rb1, a glycoside of 20(*S*)-protopanaxadiol, and those of R1 and Rg1, both are categorized as glycosides of 20(*S*)-protopanaxatriol [4,12]. The peak concentration and AUC of Rb1 were more than 10 times higher than those of R1 and Rg1 even if the differences in doses among the three saponins were taken into consideration (Table 1). The T_{max} and $t_{1/2}$ of Rb1 was much longer than those of R1 and Rg1. For instance, the terminal elimination half life ($t_{1/2}$) of Rb1 is about 13 times longer than those of R1 and Rg1, and the clearance of Rb1 was correspondingly much slow. These results were consistent with other reports about the saponins [15,27].

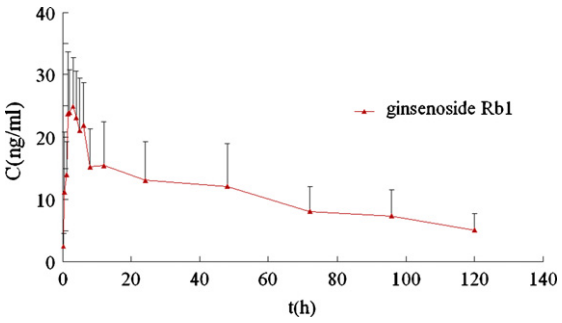

Fig. 5. Plasma concentration–time profile of ginsenoside Rb1 in beagle dogs after a single oral dose of three compound Danshen tablets (means ± SD, $n = 10$).

Table 6
Pharmacokinetic parameters of notoginsenoside R1, ginsenoside Rg1 and Rb1 in beagle dogs after a single oral dose of three compound Danshen tablets (mean ± SD, $n = 10$).

Parameters	Notoginsenoside R1	Ginsenoside Rg1	Ginsenoside Rb_1
C_{max} (ng/ml)	1.91 ± 0.81	3.34 ± 2.13	28.6 ± 8.7
t_{max} (h)	1.17 ± 0.52	1.08 ± 0.67	4.08 ± 2.5
$MRT_{(0-t)}$ (h)	4.01 ± 1.21	3.70 ± 1.05	46.3 ± 3.6
$MRT_{(0-\infty)}$ (h)	5.80 ± 2.46	5.27 ± 1.91	87.2 ± 20.2
$t_{1/2z}$ (h)	4.82 ± 3.13	4.59 ± 2.95	60.9 ± 17.5
CL_z/F (L/h)	187 ± 85	560 ± 301	3.94 ± 1.96
V_z/F (L)	1116 ± 566	3276 ± 2050	336 ± 183
$AUC_{(0-t)}$ (ng h/ml)	6.84 ± 3.07	10.10 ± 6.33	1270 ± 570
$AUC_{(0-\infty)}$ (ng h/ml)	7.50 ± 3.38	10.95 ± 6.60	1712 ± 863

Previous studies demonstrated that R1, Rg1 and Rb1 were metabolized to other ginsenosides and/or aglycones mainly by intestinal microflora after oral administration [34–39]. Therefore, only a small proportion of the intact R1, Rg1 and Rb1 can reach the systemic circulation from the gastrointestinal tract [14]. In addition, various intestinal bacteria species may be responsible for metabolizing different saponins [40–45], which might result in the observed differences in the pharmacokinetic behaviors of R1, Rg1 and Rb1.

4. Conclusion

A selective and sensitive LC–MS/MS method for the simultaneous determination of notoginsenoside R1, ginsenoside Rg1 and Rb1 in dog plasma was established and validated, and successfully applied to the pharmacokinetic study of the major saponins after a single oral administration of three compound Danshen tablets in beagle dogs. The separation was carried out on a C-18 column with steep steps linear gradient elution that was important for sharp peak shapes and high-throughput analysis. Significant differences in pharmacokinetics were found between glycosides of 20(*S*)-protopanaxadiol (Rb1) and 20(*S*)-protopanaxatriol (R1 and Rg1). These differences might be drug–drug interactions among the chemical constituents in the compound Danshen tablets and/or the transformation or multiple metabolic pathways related to the saponins investigated. Therefore, comparative pharmacokinetic study may give more profound understanding [46,47].

Table 5
LLOQ for the analysis of notoginsenoside R1, ginsenoside Rg1 and Rb1 in dog plasma ($n = 5$).

Analytes	Spiked concentration (ng/ml)	Measured concentration (ng/ml)					Mean ± SD	RSD (%)	RE (%)
Notoginsenoside R1	0.11	0.11	0.11	0.10	0.10	0.10	0.10 ± 0.004	4.1	−6.8
Ginsenoside Rg1	0.10	0.10	0.11	0.09	0.11	0.11	0.10 ± 0.01	9.7	−1.1
Ginsenoside Rb1	0.11	0.10	0.10	0.09	0.11	0.11	0.10 ± 0.005	5.0	−5.0

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