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Determination of ginsenoside Rd in dog plasma by liquid chromatography–mass spectrometry after solid-phase extraction and its application in dog pharmacokinetics studies

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Abstract

A sensitive liquid chromatography–mass spectrometric (LC/MS) method for the quantification of ginsenoside Rd in dog plasma was developed and validated after solid-phase extraction (SPE).Chromatographic separation was achieved on a reversed-phase Cromosil C_{18} column with the mobile phase of acetonitrile–ammonium chloride (500 μmol/L) and step gradient elution resulted in a total run time of about 5.5 min. The analytes were detected by using an electrospray negative ionization mass spectrometry in the selected ion monitoring (SIM) mode. A good linear relationship was obtained in the concentration range studied (0.005–2.500 μg/mL) ($r = 0.9998$). Lower limit of quantification (LLOQ) was 5 ng/mL by using 500 μL plasma sample. Average recoveries ranged from 70.71 to 75.89% in plasma at the concentrations of 0.010, 0.100 and 2.500 μg/mL. Intra- and inter-day relative standard deviations were 8.49–11.71 and 5.71–16.48%, respectively. This method was successfully applied to the pharmacokinetic studies on dogs. The absolute bioavailability of Rd in dogs was 0.26%.
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Keywords: Ginsenoside Rd; LC/MS; Solid-phase extraction (SPE); Pharmacokinetics; Bioavailability

1. Introduction

Panax ginseng C.A. Meyer, one of the most widely used traditional Chinese medicine, possesses various biological and pharmacological activities, such as anti-aging, anti-inflammation and anti-oxidation effects on the central nervous system, cardiovascular system and immune system. In most ginseng species, ginsenosides are mainly considered as the active constituents. Ginsenoside Rd, which is one of the main protopanaxadiol saponins in its leaf, berry and root, proved to have effective anti-inflammatory function, obvious vasodilating effect, tranquilizing function to central nervous system and it can also inhibit tumor cell proliferation, induce differentiation and apoptosis and inhibits metastasis [1–4].

Pharmacokinetic studies suggest that ginsenosides are very poorly absorbed following oral administration to rats and humans [19,20]. Qian et al. [13] reported that ginsenoside Rb1 is metabolized to ginsenoside Rd, Rg3 or F2, Rh2 or C-K, and the deglycosylated metabolites have activities comparable to or higher than that of Rb1. This suggests that active metabolites might be responsible, at least in part, for the observed pharmacodynamics; hence, research on the main metabolic pathways is being undertaken in our laboratory.

Various analytical methods were applied to the assay of Rd in vitro [5,6], such as thin-layer chromatography (TLC), high performance liquid chromatography (HPLC), as well as gas chromatography coupled with mass spectrometry (GC/MS). Because of its structure (see Fig. 1), Rd has poor ultraviolet radiation in its maximum absorption wavelength of 203 nm. Thus, using HPLC–UV is hard to detect the concentration in biological fluids. Due to high polarity of Rd, the GC methods required a complex and time-consuming derivatization procedure. And HPLC methods also have several disadvantages, such as lack of sensitivity and requiring long chromatographic times [12–15]. LC/MS has been successfully applied for qualitatively analyzing ginsenosides extracted from raw *P. ginseng* plant material [7–9,16]. There are a few reports on LC/MS [10] and LC/MS/MS

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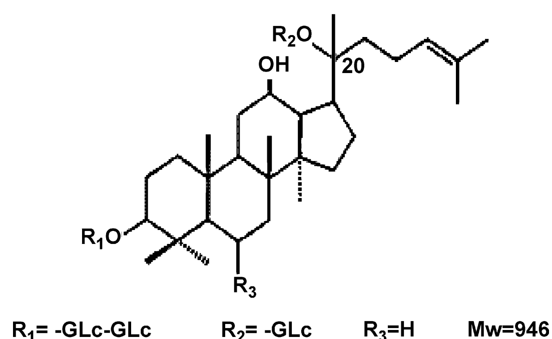

Fig. 1. Structure of ginsenoside Rd.

[11,17] analysis of ginsenosides extracted from biological samples. Such analyses are essential for pharmacokinetic studies and for other fundamental or practical purposes, where it is necessary to analyze the main metabolic products of the target analyte in biological samples at low concentrations [18–20].

In this paper, we reported a relatively simple and rapid liquid chromatography–mass spectrometry method to determine Rd in dog plasma after solid-phase extraction (SPE). The lower limit of quantification (LLOQ) of Rd was 5 ng/mL, which, compared with existing methods,was more sensitive. It has been successfully applied to a pharmacokinetic study after six dogs were given a 0.2 mg/kg dose intravenously or 2 mg/kg dose by oral administration.

2. Experimental

2.1. Chemicals and reagents

Ginsenoside Rd (99.0% purity) and digoxin (internal standard, IS, 99.0% purity) was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (NICPBP). Acetonitrile and methanol were of HPLC-grade and obtained from Fisher (USA), and other chemicals used were of analytical grade. Purified water from a Milli-Q system (Millipore, Bedford, MA, USA) was used throughout.

2.2. Animal

Six dogs were provided by the Experimental Animal Feeding Center of China Pharmaceutical University and studies were approved by the University's Animal Ethic Committee.

2.3. Instrumentation

A Shimadzu 2010 LC–MS (including two LC-10ADvp pumps, an online vacuum deaerator, a constant temperature automatic sampler, a quadruple mass spectrometer equipped with an electrospray ionization interface (ESI) source and LC–MS solution (Version 2.02) was used for data processing, Japanese, Kyoto) and a six-port switching valve was used to direct HPLC elute to a waste container in the first 1.5 min of the chromatographic run and afterwards to the ionization source.

2.4. Chromatographic conditions

Liquid chromatographic separations were achieved using a Cromosil 5 μm C18 column (150 mm × 2.1 mm). The column and autosampler tray temperature were kept constant at 40 and 4 °C, respectively. The mobile phase consisted of a mixture of 500 μmol/L ammonium chloride in water (A) and acetonitrile (B) and was delivered at a flow-rate of 0.2 mL/min. The gradient program was from 40 to 95% B within 1.5 min and hold on for 2.25 min at a flowrate of 0.2 mL/min. The sample injection volume was 10 μL.

2.5. Mass spectrometric conditions

Samples were ionized by negative-ion electrospray ionization mode under the following source conditions: gas flow: 4.5 L/min; curve dissolution line (CDL) voltage was fixed as in tuning, CDL temperature: 250 °C; and block temperature: 200 °C. Mass spectra were obtained at a dwell time of 0.2 and 1 s for SIM and scan mode accordingly. Analysis was carried out using selected ion monitoring (SIM) for specific *m/z* 981.55 for Rd $[M+Cl]^-$ and *m/z* 815.35 for digoxin $[M+Cl]^-$. Peak areas for all components were automatically integrated using LC/MS solution Version 2.04 (© 1997–2002 Shimadzu Corp.).

2.6. Preparation of stock and sample solutions

Stock solution of Rd was prepared by dissolving the accurately weighed reference compound in dimethyl sulphoxide (DMSO) to give a final concentration of 2 mg/mL, stored at 4 °C until it is used. The solution was then serially diluted with methanol and mixed with blank dog plasma to achieve standard working solutions at concentrations of 0.005, 0.010, 0.025, 0.050, 0.100, 0.250, 0.500, 1.000 and 2.500 μg/mL for Rd, respectively. A 5 μg/mL internal standard working solution was prepared by diluting the 20 μg/mL stock solution of digoxin with ultrapure water.

2.7. Sample preparation

Venous blood samples (2.5 mL) were withdrew to the heparinized tubes and were at once centrifuged at 3500 rpm for 10 min at 4 °C. A 1.0 mL volume of plasma was finally obtained and stored at −20 °C until analysis.

Plasma samples (0.5 mL) from dogs were first added to 10 μl of IS solution (5 μg/mL) and then mixed with equal volume of 60% methanol aqueous solution and vortexed for 1 min and then centrifugated at 4000 rpm for 5 min. All supernatant fluid was loaded and drawn through by gravity on a SPE cartridge (C_{18}, HLB 1 cm^3, Oasis™, Waters, Milford, MA, USA), which was pre-conditioned by passing through 2 mL of methanol followed by 2 mL of water before loading and drawn through by gravity. Then, the solid-phase cartridge was washed with 2 mL of water and finally the SPE cartridge was slowly eluted by 1 mL of 90% (v/v) methanol aqueous solution. The eluent was centrifugated at 20,000 rpm for 10 min, and 10 μL of the supernatant fluid was injected into the HPLC/MS system.

2.8. Assay validation

2.8.1. Sensitivity and specificity

The lower limit of quantification was determined as the minimum concentration that could be accurately and precisely quantified (lowest data point of the standard curve). The specificity of the assay for the analytes versus endogenous substances in the matrix was assessed comparing the lowest concentration in the calibration curves with reconstitutions prepared with drug-free plasma from five different dogs.

2.8.2. Accuracy and precision

The accuracy and precision (presented as relative standard deviation, R.S.D.) of the assay were determined using quality control (QC) samples (at 0.010, 0.100 and 2.500 μg/mL). Accuracy (%) was determined by the percentage ratio of measured over spiked QC concentration (mean of measured/spiked × 100%). Intra-day precision was determined by analyzing replicate aliquots of QCs ($n = 5$ per each concentration) on the same day. Inter-day precision was determined by repetitive analysis of QC samples (each concentration) on 5 consecutive days.

2.8.3. Recovery and ionization

To investigate the recovery of Rd by the SPE method, plasma samples were spiked with Rd at concentrations of 0.025, 0.250 and 2.500 μg/mL. The resulting peak–area ratios (analyte: internal standard) were compared with that of the standards prepared in mobile phase to provide the recovery values. Ion suppression of ionization was evaluated by comparing the absolute peak areas of control plasma extracted and then spiked with a known amount of drug, to neat standards injected directly in the same reconstitution solvent.

2.8.4. Stability

To evaluate sample stability after three freeze–thaw cycles and at room temperature, five replicates of QC samples at each of the low, medium and high concentrations were subjected to three freeze–thaw cycles or were stored at room temperature for 4 h before sample processing, respectively. Five replicates of QC samples at each of the low and high concentrations were processed and stored under autosampler conditions for 24 h. Stability was assessed by comparing the mean concentration of the stored QC samples with the mean concentration of freshly prepared QC samples.

2.8.5. Application of the assay

The developed LC/MS assay method was used in the pharmacokinetic study after intravenous (i.v.) (0.2 mg/kg) and intragastic (i.g.) (2 mg/kg) administration of Rd to dogs. Animals were fasted for 12 h before dosing and 4 h afterwards, with free access to water. For intravenous bolus, Rd powder was dissolved in isotonic saline containing 10% ethanol and dosing solution was delivered by intravenous injection of 5 mL on a dog. For oral route, dosing solutions were prepared by dissolving Rd powder in isotonic saline containing 10% ethanol and mixed well. The preparations were made immediately before drug administration. Blood samples (2.5 mL) were collected immediately before and at 0.083, 0.25, 0.5, 1, 2, 3, 4, 6, 8, 12, 24, 36, 48, 60 and 72 h for i.v. group and 0.5, 1, 1.5, 2, 3, 4, 6, 8, 12, 24, 36, 48, 60 and 72 h for i.g. group after Rd administration. The blood sample was transferred into a heparinized eppendoff tube and mixed gently and then centrifuged (1000 × *g*, 5 min) to obtain 1.00 mL plasma, which was kept at −20 °C until analysis.

3. Results and discussion

3.1. Method validation

3.1.1. Specificity

The full scan mass spectra of Rd after direct injection in mobile phase are presented in Fig. 2. Protonated molecules $[M-H]^-$ of Rd were not detected. The predominant protonated molecules found for Rd were $[M+Cl]^-$ *m/z* 981.55. The mass spectrometric parameters were optimized to obtain the higher signal for the selected ion 981.55, which also showed less internal interference. The Cl^- adduct was $[M+Cl]^-$ detected for digoxin (IS) at *m/z* 815.35.

While using the gradient programme, observed retention time were about 3.0 and 3.1 min for Rd and digoxin, respectively. No additional peaks due to endogenous substances that could have interfered with the detection of the compounds of interest was observed. Representative chromatograms for Rd and digoxin in actual plasma sample are presented in Fig. 3.

3.1.2. Linearity and lower limit of quantification

The linear regression analysis of Rd constructed by plotting the peak–area ratio of Rd to the internal standard (y) versus analyte concentration (μg/mL) in spiked plasma samples (x). The calibration curves were fluctuated in the range of 0.005–2.500 μg/mL. The average regression equation of these curves and their correlation coefficients (r) were calculated as follows: $y = 0.5585x + 0.0007$ ($r^2 = 0.9999$, $n = 5$), weighting coefficient: $1/x$; it showed good linear relationship between the peak areas and the concentrations. The lower limit of quantitation, defined as the lowest concentration analyzed with accuracy

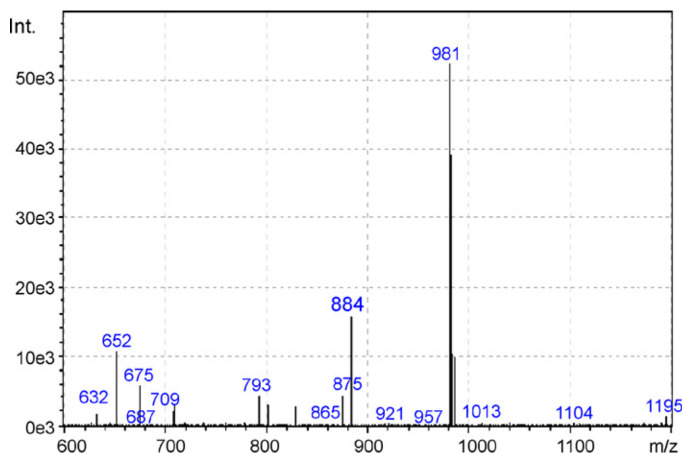

Fig. 2. Full scan mass spectra of Rd (16 ng per injection).

within ±15% and a precision ±15%, was 5 ng/mL for determination of Rd in plasma. The limit has already been sufficient for pharmacokinetic studies of Rd.

3.1.3. Precision

The intra-day precision (presented as relative standard deviation) is shown in Table 1. The precision for concentrations of 0.010, 0.100 and 2.500 μg/mL Rd were 11.71, 10.01 and 8.49%, respectively. And the accuracy, defined as (measured concentration/spiked concentration) × 100%, reached from 100.25 to 109.77% throughout the three concentrations examined.

The inter-day precision was studied over 5 days, and the results were also given in Table 1. The precision ranged from 5.71 to 16.48%, and the accuracy, reached from 91.85 to 100.08% throughout the three concentrations examined. The lower limit of quantification was 5 ng/mL for Rd.

3.1.4. Recovery and stability

The absolute recoveries of Rd at concentrations of 0.025, 0.250 and 2.500 μg/mL ($n = 5$) were 75.56 ± 8.85, 70.71 ± 7.08 and 75.89 ± 7.44%, respectively. Stability of Rd during sample handling (freeze–thaw and short-term temperature) and the sta-

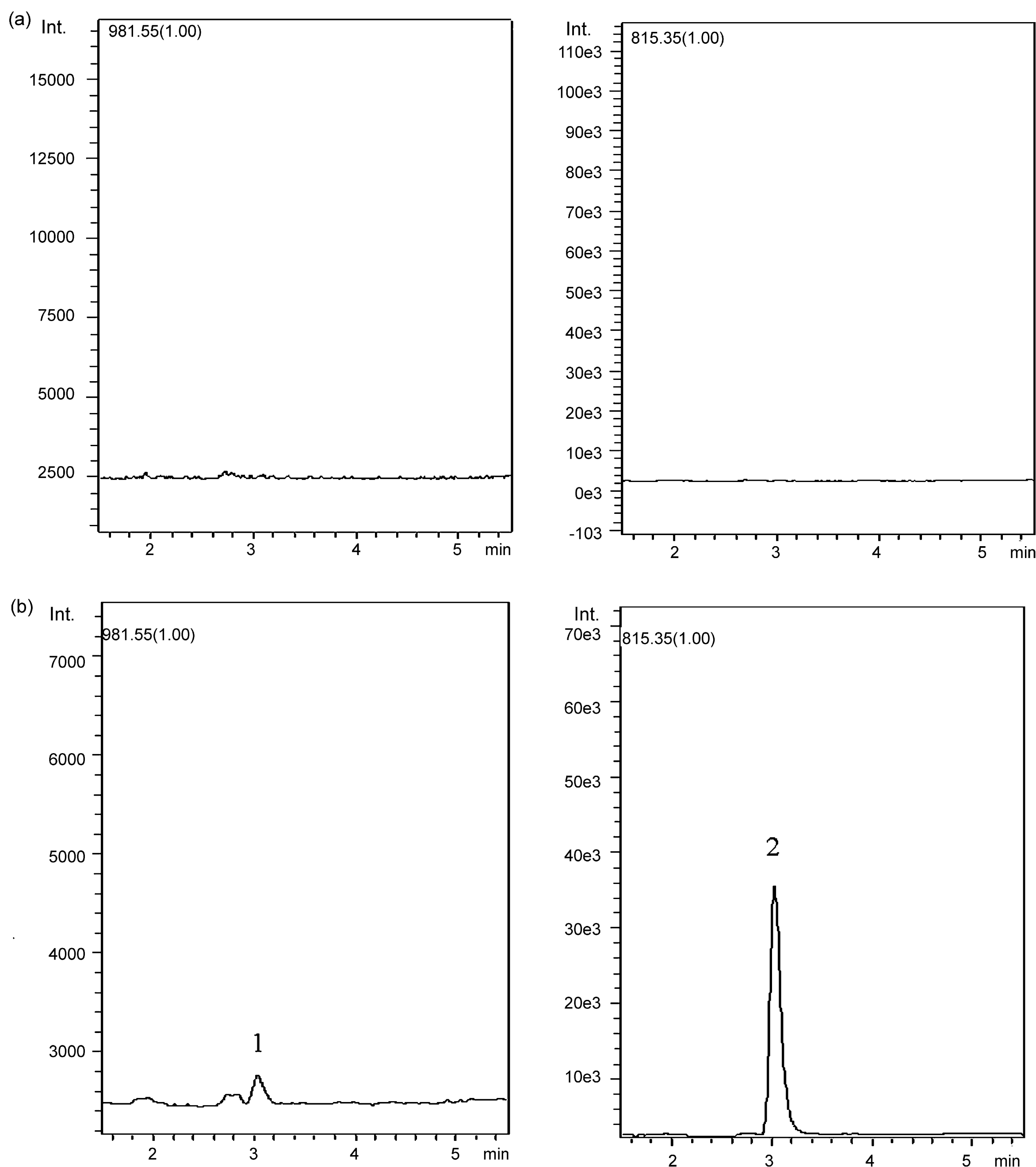

Fig. 3. LC–MS chromatograms for the determination of Rd in dog plasma: (a) blank plasma; (b) blank plasma spiked with Rd (5 ng/mL) and digoxin (5 μg/mL); (c) plasma sample 3 h after i.g. administration of Rd saline solution to dogs (C_{Rd} = 102.1 ng/mL); (d) plasma sample 72 h after i.v. administration of Rd saline solution to dogs (C_{Rd} = 556.0 ng/mL). (1) Rd (m/z = 981.55) and (2) digoxin (internal standard, m/z = 815.35).

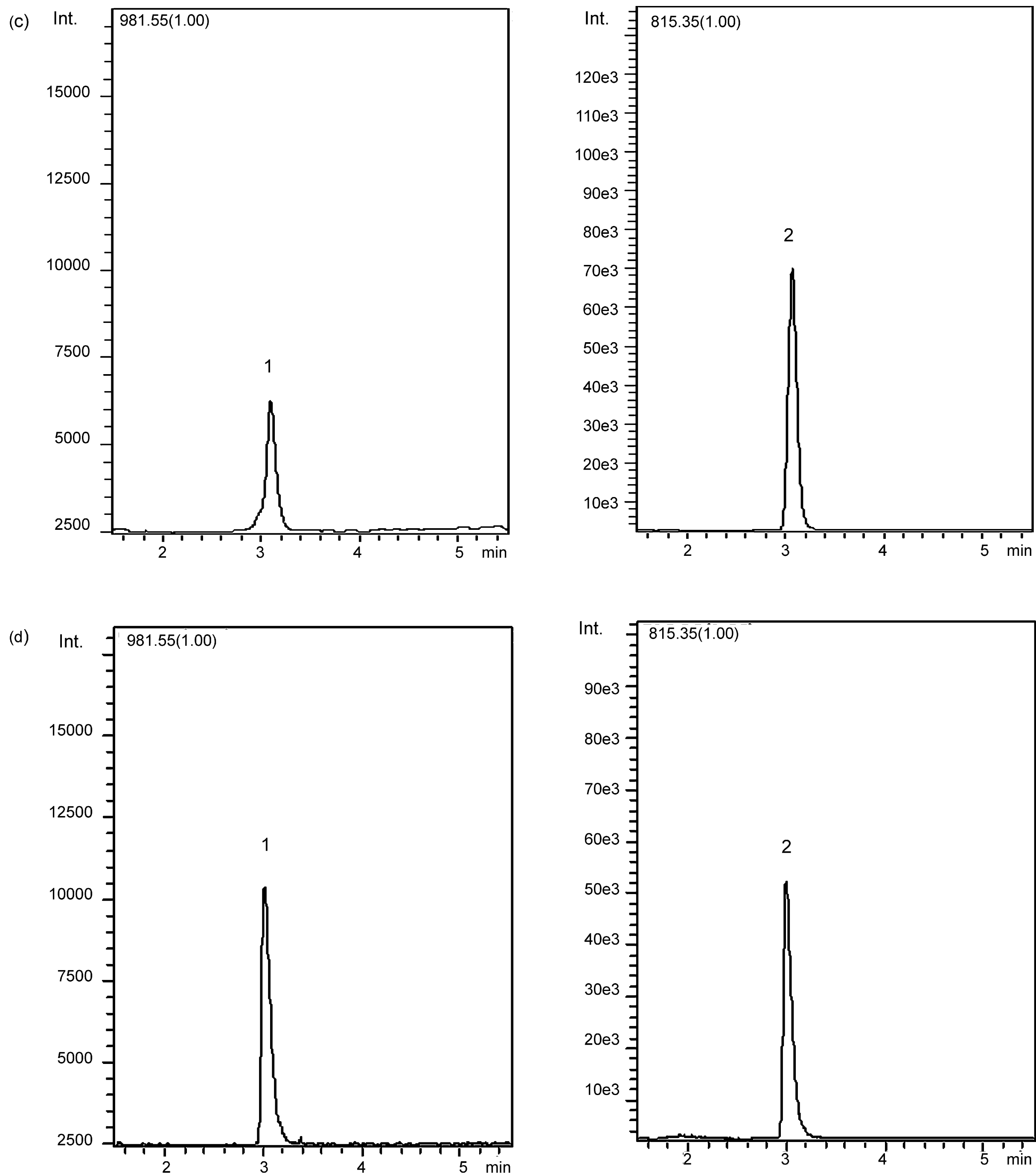

Fig. 3. (*Continued*).

bility of processed samples were evaluated and Rd was stable for at least 4 h at room temperature in plasma samples, for 24 h in autosampler conditions and in plasma samples following three freeze–thaw cycles.

3.1.5. Ionization

It was shown that SPE improves the sample clean-up to remove internal substances from plasma and thereby decrease the amount of matrix injected onto the column, thus the ion suppression effect was minimized. The results indicated that there was no significant difference between the signals of analytes extracted from dog plasma and the mobile phase, which proves that there were no matrix effects.

3.2. Pharmacokinetic study

The assay was conducted to obtain pharmacokinetic data for Rd in dog plasma after i.v. administration (0.2 mg/kg) and i.g. administration (2 mg/kg). Figs. 4 and 5a and b show application of the LC/MS method developed here to in vivo pharmacokinetic studies in dogs. The area under the plasma concentration (AUCs curve) of Rd after i.v. and i.g. administra-

Table 1
Precision of Rd in dog plasma

C (ng/mL)	Intra-day (n = 5)		
	10	100	2500
Mean ± S.D.	0.007 ± 0.001	0.058 ± 0.006	1.4 ± 0.119
Accuracy (%)	109.77	102.87	100.25
R.S.D. (%)	11.71	10.01	8.49
C (ng/mL)	Inter-day (n = 5)		
	10	100	2500
Mean ± S.D.	0.006 ± 0.001	0.052 ± 0.005	1.398 ± 0.080
Accuracy (%)	94.90	91.85	100.08
R.S.D. (%)	16.48	8.96	5.71

tions were 76451.1 ± 15874.8 and 1930.3 ± 647.4 ng h mL^{-1}, respectively. The absolute bioavailability (F, %) of Rd, was found to be 0.26%.

As for i.v. administration, the half-lives for the distribution and exterminate phase ($t_{1/2\alpha}$ and $t_{1/2\beta}$) were 0.873 ± 0.543 and 39.4 ± 12.0 h, the volume of the central compartment (V_c) was 0.0020 ± 0.0005 L/kg. As for oral administration, the mean C_{max} value was 81.0 ± 24.6 ng/mL (range: 56.1–109.8 ng/mL), corresponding mean t_{max} value was 2.67 ± 1.17 h (range: 1.5–4.0 h). The mean plasma elimination half-life was 24.2 ± 2.85 h (range: 15.4–34.8 h). Rd concentration time profile conformed to a two-compartment pharmacokinetic model after intravenous administration and a one-compartment pharmacokinetic model after oral administration (Fig. 5).

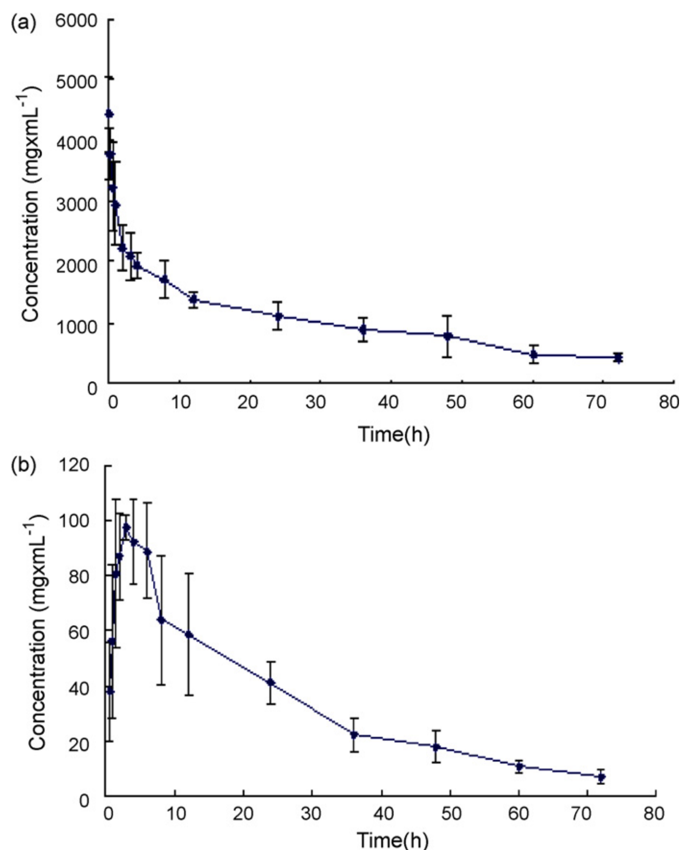

Fig. 4. (a) Serum concentration of ginsenoside Rd after i.v. administration of 0.2 mg/kg to dogs (each point represents the mean ± S.D. of six animals). (b) Serum concentration of ginsenoside Rd after oral administration of 2 mg/kg to dogs (each point represents the mean ± S.D. of six animals).

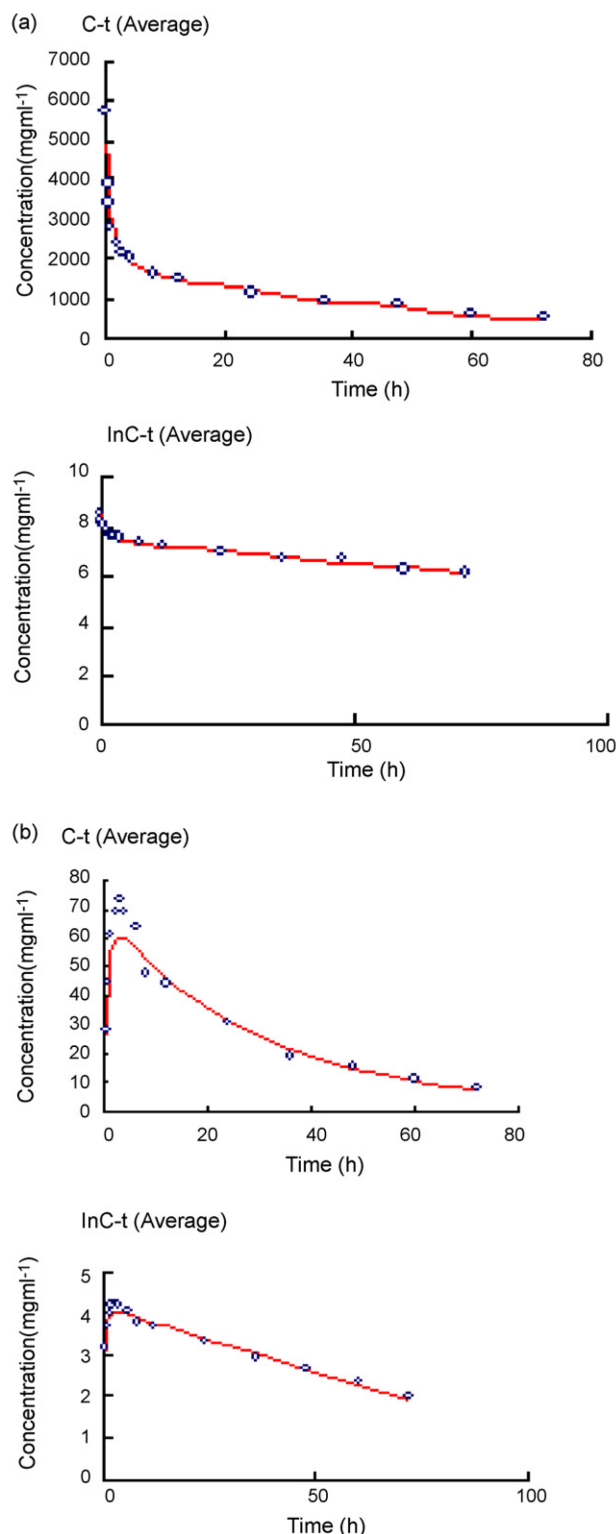

Fig. 5. (a) Simulation of mean plasma concentration–time curve after i.v. administration of 0.2 mg/kg Rd by DAS software (two comparments). (b) Simulation of mean plasma concentration–time curve after oral administration of 2 mg/kg Rd by DAS software (one compartment).

Table 2
Pharmacokinetic parameters after i.v. dose of 0.2 mg/kg Rd and oral administration of 2 mg/kg to dogs

Pharmacokinetic parameters	i.v. Administration	Oral administration
$t_{1/2}$ (h)	39.4 ± 12.0	24.2 ± 2.85
MRT (h)	26.7 ± 1.63	25.5 ± 3.84
AUC_{0-t} (ng h mL^{-1})	76,403.4 ± 15,880.6	1890.2 ± 668.6
$AUC_{0\to\infty}$ (ng h mL^{-1})	76,451.1 ± 15,874.8	1930.3 ± 647.4
CL (L/kg h^{-1})	0.0020 ± 0.0005	1.14 ± 0.40
F (%)	0.26 ± 0.10%	

Other pharmacokinetic parameters in this study are shown in Table 2. The present method could be applied to pharmacokinetic studies after a lower dose administration of Rd (2 mg/kg).

4. Conclusions

In order to measure Rd selectively and precisely in dog plasma, the method for the determination of Rd in dog plasma after SPE has been established and applied for pharmacokinetic studies. This is the most sensitive method for simultaneous determination of ginsenoside Rd in dog plasma by LC–MS. We have compared SPE with liquid–liquid extraction using ether, etc. and found that SPE is an appropriate extraction procedure. Choosing 1 mL of 90% methanol aqueous solution for eluting Rd and digitoxin from SPE cartridge showed satisfactory results. Concerning the mobile phase, 500 μM/L ammonium chloride in water was preferred to maintain abundant and stable signals. Plasma concentrations of Rd could be quantified from 0.5 to 250 ng/100 μL, using 100 μL plasma sample, making it suitable for pharmacokinetic studies when blood can be taken from a single animal many times.

This method guarantees a relatively short analysis time and the acceptable sensitivity, precision, accuracy, selectivity, recovery and stability be achieved. The method was successfully applied to a pharmacokinetic study of Rd in dogs. And as far as we know, it is the first report of LC/MS method on the determination of Rd concentration in vivo.

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