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Short communication

Determination of Raddeanin A in rat plasma by liquid chromatography–tandem mass spectrometry: Application to a pharmacokinetic study

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ABSTRACT

A simple, rapid and sensitive LC–MS/MS analysis method was developed and validated for the determination of Raddeanin A (RA) in rat plasma. Protein precipitation with three volumes of methanol as the precipitation reagent was used as the sample preparation method. The analysis process was performed on a Thermo Syncronis C18 column with the mobile phase of methanol–water (containing 5 mM ammonium formate, pH 2.2) (85:15, v/v). RA and glycyrrhetinic acid (internal standard) were monitored under negative electrospray ionization in multiple reaction monitoring (MRM) mode. Retention time of RA and IS were 2.1 min and 3.5 min, respectively. The limit of detection was 5 ng/mL and the linear range was 50–50,000 ng/mL. The intra-day and inter-day precision was 1.87–2.94% and 3.25–5.36%, and the intra-day and inter-day accuracy ranged from 5.9% to 10.5% and 5.6% to 11.1%, respectively. The absolute recovery was above 90.3%. The method has been successfully translated to the pharmacokinetic study of RA in rats after intravenous and intraperitoneal administration (0.75 mg/kg).

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1. Introduction

Anemone raddeana Regel, also known as “Liangtoujian” in China, is widely distributed in the northeast of China, Japan, Korean peninsula, and the far east of Russia [1]. *A. raddeana*, which has been included in Chinese Pharmacopeia, is a traditional Chinese medicinal herb for the treatment of cancer, rheumatism, neuralgia [2,3]. The main constituents of *A. raddeana* include triterpenoids, steroids, lactones, fats and oils, saccharide and alkaloid [1–8], and the oleanane-type triterpenoid saponins have been revealed to be the main bioactive principles through extensive phytochemical and pharmacological research [1].

Among all the identified oleanane-type saponins from *A. raddeana* [11], Raddeanin A (RA) have received more attention with respect to its attractive antitumor activity both *in vitro* and *in vivo*. RA could effectively inhibit the DNA, RNA and protein synthesis of sarcoma S180 and ascitic hepatoma cells [9,10]. It also showed inhibitory effect on human gastric cancer cell line BGC823, human leukemia cell line K562, and human non-small cell lung cancer H460 in a dose-dependent manner [11,12]. RA could also inhibit the growth of sarcoma S180, liver cancer H22, and cervical carcinoma U14 xenografts in mice [13]. In addition, RA has been designated in Chinese Pharmacopeia as the standard to monitor the quality of *A. reddeana*. These proofs demonstrate that RA is a promising antitumor agent and a potential leading compound for translational research.

In contrast to the encouraging pharmacodynamic results, the pharmacokinetic profile of RA has not been investigated before and there is still lack of *in vivo* validated analytical methods for RA determination. The high-performance liquid chromatography (HPLC) with UV detection has ever been used to determine the concentration levels of RA in plant extracts [14]. But the low sensitivity and long detection time of HPLC–UV method cannot meet the need of concentration monitoring *in vivo*. In other studies, ESI-MS/MS was utilized to investigate the fragmentation pathways of RA [15] or the structures of the crude saponins extracts isolated from *A. raddeana* [16]. Compared with HPLC–UV method, LC–MS/MS has the advantages in both qualitative and quantitative basis. The qualitative advantages come from the better specificity due to resolving co-eluting species by mass. The quantitative advantage is afforded by the low noise, and thus high s/n, observed at specific m/z values for different compounds. In this study, a new, specific, sensitive, and rapid LC/MS/MS method was developed, validated and successfully

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used in the study of RA pharmacokinetics in rats after intravenous and intraperitoneal administration (0.75 mg/kg).

2. Experimental

2.1. Reagents and chemicals

RA (purity ≥ 98%) and glycyrrhetinic acid (purity ≥ 98%) (internal standard, IS) were purchased from Pure-one Bio Technology Co. Ltd. (Shanghai, China). Ammonium formate and methanol (MeOH) of HPLC-grade were purchased from Tedia (Fairfield, OH, USA). Formic acid of HPLC-grade was purchased from J&K Scientific Ltd. (Shanghai, China). Water was purified with the Mili-Q Plus system (Millipore, Bedford, MA, USA). Freshly obtained drug free rat plasma was collected from six healthy male Sprague–Dawley (SD) rats in our lab and stored at −70 °C prior to use. The heparinized plastic tubes (5 mL, 13 mm × 100 mm) were obtained from Shanghai Kehua Bio-engineering Co. Ltd. (China). The syringes (1 mL, 0.4 mm × 13 mm) were purchased from Kindly Enterprise Development Group (Shanghai, China).

2.2. Animals

Male SD rats (190–210 g) were supplied by the SLAC Laboratory Animal Co. Ltd. (Shanghai, China), and were kept in an environmentally controlled quarters (20–22 °C, relative humidity 55–65%, 12:12 h light–dark cycle) for 3 days before experiment. The rats were fasted overnight before dosing, but the water was available all the time. Animal experiments were approved by the Animal Ethics Committee of Shanghai Jiao Tong University School of Medicine (Shanghai, China).

2.3. Instrument and conditions

Analyses were performed on an Agilent 6410 triple quadrupole mass spectrometer (Agilent Technologies, Inc., USA) equipped with electrospray ionization (ESI) and an Agilent 1200 HPLC system (Agilent Technologies, Inc., USA). The instrumental components of Agilent 1200 HPLC system are G1322A Degasser, G1311A Quat Pump, G1329A ALS Auto Sampler, and G1316A Colcom Column Oven. A Thermo Syncronis C18 column (2.1 mm × 100 mm, 5 μm), equipped with a Thermo BDS-Hypersil-C18 (2.1 mm × 10 mm, 3 μm) Drop-In Guards, was used for analyte separation. Isocratic elution with a mobile phase consisting of methanol and water (85:15, v/v, the aqueous phase contained 5 mM ammonium formate, was adjusted to pH 2.2 using formic acid) was used for the separation. The flow rate was 0.45 mL/min, the run time of each sample was 5 min, the injection volume was 15 μL and the column temperature was kept at 35 °C. Data were collected under the negative ionization mode with multiple reaction monitoring (MRM). Two MRM transitions, RA (m/z 895.1–455.4, fragmentor 200 eV, collision energy 66 eV) and IS (m/z 469.3–425.2, fragmentor 160 eV, collision energy 40 eV) were monitored. The working parameters of the ESI source were set as follows: temperature 350 °C, capillary voltage −4000 V, drying-gas flow rate 9 L/min and nebulizer pressure 45 psi. Data processing of MS was performed on the MassHunter software package (VersionB.04.00, Agilent Technologies, Inc., USA).

2.4. Preparation of calibration standards and quality control (QC) samples

The stock solutions of RA and IS each with the exact concentration of 1 mg/mL were prepared in methanol and stored at −70 °C. Working solutions for spiking plasma ranging from 250 ng/mL to 250 μg/mL were all freshly prepared by step wise dilution of the stock solution with methanol. A series of calibration standards were prepared by spiking blank plasma (100 μL) with 20 μL aliquots of standard solutions to yield the concentrations of 50, 100, 500, 1000, 5000, 10,000 and 50,000 ng/mL. QC samples were prepared at three levels (low: 100 ng/mL, medium: 1000 ng/mL, high: 40,000 ng/mL) independently in the same way. All QC samples were stored at −70 °C. The IS working solution (1 μg/mL) was made by diluting the stock solution with methanol.

2.5. Sample preparation

All frozen standards and samples were thawed at room temperature (25 °C) and homogenized by vortex. Fifty microliter methanol and 100 μL methanol containing IS (1 μg/mL) were added to 50 μL plasma. The mixed solution was homogenized by vortex for 1 min and centrifuged at the condition of 15,000 × g for 5 min with the supernatant (15 μL) injected into the system.

2.6. Method validation

The method was validated for selectivity, linearity, precision, accuracy, recovery, matrix effect and stability.

Blank plasma from six different rats with and without RA and IS were used to evaluate the selectivity. Calibration curves were generated at the range of 50–50,000 ng/mL by using the peak area ratio (y) of RA to IS *vs.* normal concentration of RA (x) with the weighting factor of $1/x$. The lower limit of quantification (LLOQ) was defined as the lowest concentration point of the calibration curve. QC samples were processed in five replicates at three concentration levels (low, middle, and high) for RA. The precision was expressed as intra- and inter-day relative standard deviation (%RSD, calculated from the standard deviation divided by the mean) of the QC and LLOQ samples. The accuracy (defined as relative error, %RE) was determined by comparing the calculated concentration to the theoretical concentration of the QC and LLOQ samples. The intra-day accuracy was determined on three separate days. The inter-day precision was determined on 20 operating days as the approach outlined in the document EP05-A2 from Clinical and Laboratory Standards Institute [17]. Matrix effects ($n = 5$) were defined by calculating the ratio of the peak area for RA in deproteinized plasma relative to the peak area for RA in methanol. Absolute recoveries ($n = 5$) at QC and LLOQ levels were evaluated by determining the peak area ratios of RA in the post-deproteinized spiked samples to that acquired from pre-deproteinized spiked samples. Long-term stability (60 days), room-temperature (4 h) stability, auto-sampler (24 h) stability and three freeze/thaw stability were studied at three levels of QC samples in five replicates.

2.7. Pharmacokinetic study

Twelve SD rats (190–210 g) were randomly divided into two groups, one group for intravenous administration through caudal vein and the other for intraperitoneal injection, using plastic syringes (1 mL, 0.4 mm × 13 mm). Blood samples (no more than 0.2 mL) were obtained *via* retro-orbital puncture with a glass capillary at 0 h (pre-dose) and 0.083, 0.167, 0.33, 0.5, 1, 2, 4, 6, 9 and 12 h after administration of 0.75 mg/kg RA dissolved in saline water. The blood sample was collected into the commercial heparinized plastic tubes and separated by 4000 r/min centrifugation (Centrifuge 5417R, Eppendorf, Hamburg, Germany) at 4 °C for 10 min and stored in the refrigerator at −70 °C before the analysis. The pharmacokinetic parameters were calculated with the WinNonlin software (Version 6.1 Pharsight, Mountain View, CA, USA) according to non-compartmental model.

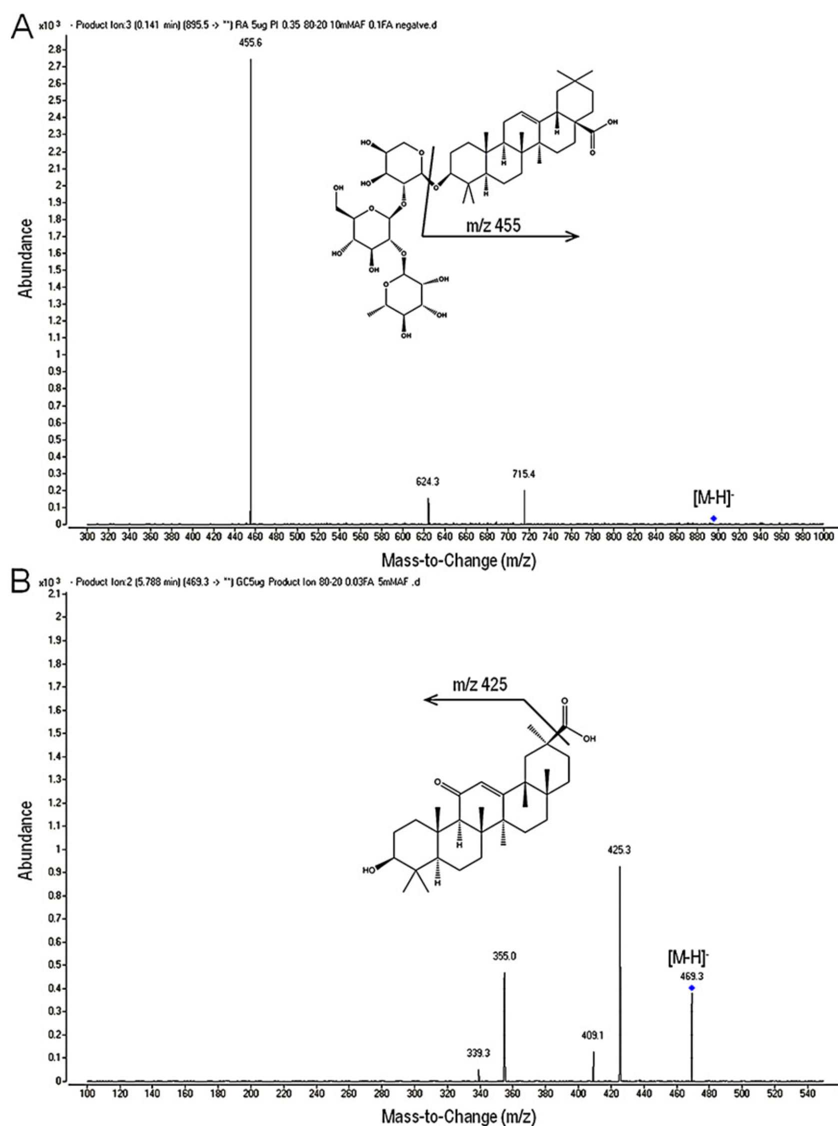

Fig. 1. Product ion mass spectra of RA (A) and IS (B) and the chemical structures of both compounds.

3. Results and discussion

3.1. Method development

The picking of IS is very important for the analysis of target compound. Glycyrrhetinic acid was chosen as the IS because of its similar basic structure, physicochemical property and mass spectrometric behavior to those of RA. MS/MS operation parameters have been carefully optimized for determination of RA. It was shown that RA could be monitored under both positive and negative mode, whereas the mass response of RA under positive mode is not sensitive enough for pharmacokinetic research. Thus $[M-H]^-$ (895.1) was chosen for further fragmentation, and the MassHunter optimizer software (Version B.03.01, Agilent Technologies, Santa Clara, CA, USA) was used to obtain the product ion $[M-H-rha-glc-ara]^-$ (455.4) and optimize ESI source parameters such as fragmentor voltage and collision energy (CE). The product ion spectra of RA (455.4) and IS (425.2) and their allocation are shown in Fig. 1.

RA belongs to the oleanane-type saponin with the structure containing acidic group (—COOH), so the pH of solvent is the key for the mass response of RA. We found that when the pH of the mobile phase was adjusted to 2.2, the peaks of both RA and IS was symmetrical and sharp. A Thermo Syncronis C18 column was used due to its tolerance to the pH range from 2 to 8. The high flow rate (0.45 mL/min) and the high ratio of methanol in mobile phase (85%, v/v) were adopted to obtain an ideal retention time (RT < 5 min) for the purpose of rapid analysis, and it was observed that higher percentage of organic phase can enhance the peak signal by better ionization. A Thermo BDS-Hypersil-C18 Drop-In Guards was adopted as the guard column to filter the remaining granular precipitates which may not be completely removed during the sample preparation process and extend the operational life span of the chromatography column.

Sample preparation method should be optimized to be reproducible, minimized matrix effects and as inexpensive as possible. Protein precipitation with three volumes of methanol was adopted for sample preparation. After centrifugation, the supernatant fluid of the sample can be directly injected into the analysis system because of its similar composition to that of the mobile phase.

3.2. Method validation

The method validation includes several respects such as selectivity, linearity, precision, accuracy, matrix effect, recovery and stability. All the contents were examined according to the US Food

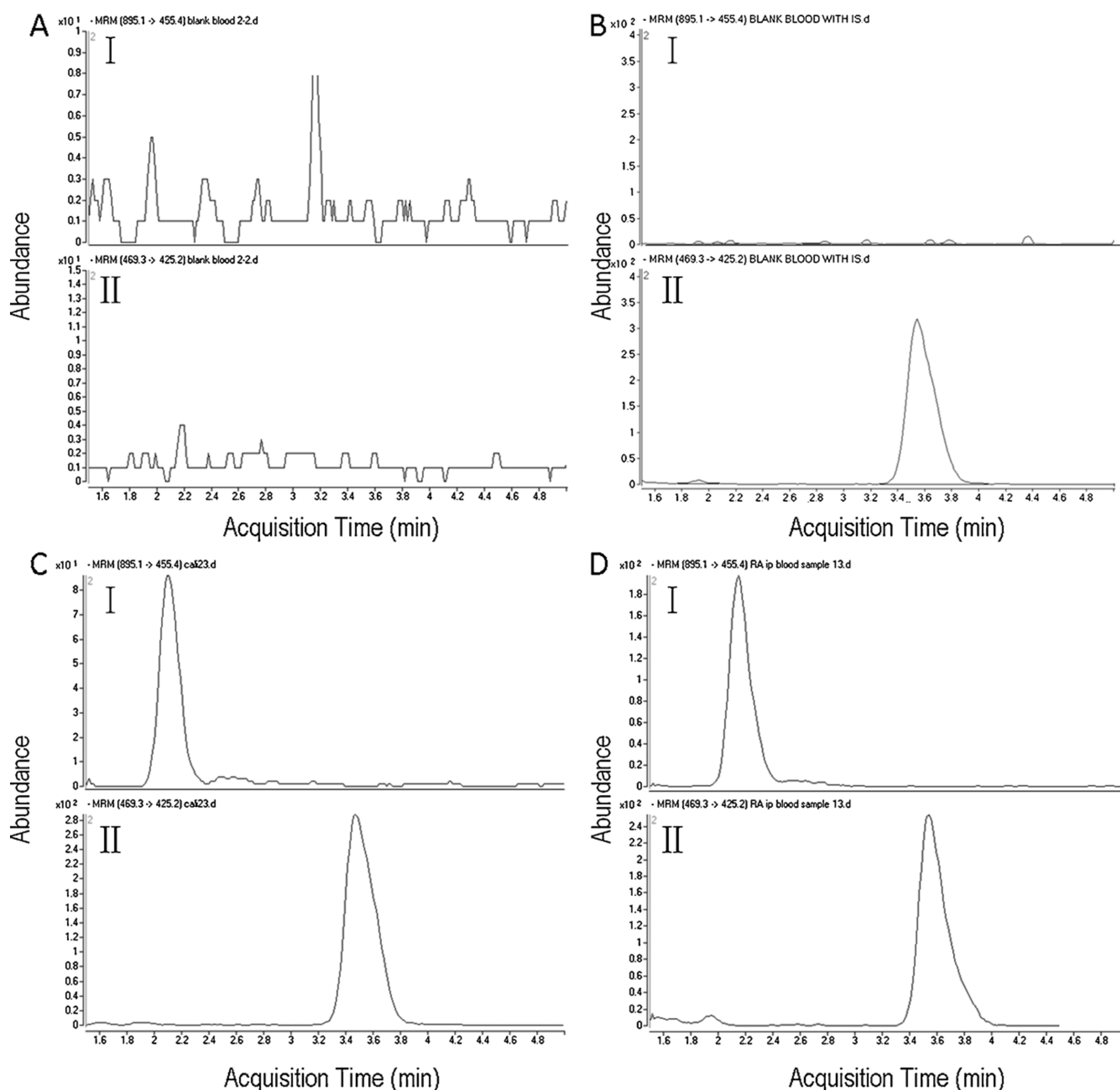

Fig. 2. Representative MRM chromatograms of a double-blank rat plasma sample (A), a blank plasma sample spiked with IS (500 ng/mL) (B), a blank rat plasma sample spiked with RA at LLOQ level (50 ng/mL) and IS (500 ng/mL) (C), and a rat plasma sample obtained at 0.167 h after intraperitoneal injection of RA (0.75 mg/kg) (D). Peak I, RA; peak II, IS.

and Drug Administration (FDA) bioanalytical method validation guidance [18].

No endogenous interference was observed during the retention time of RA and IS from the chromatograms of six lots of control blank rat plasma. The MRM chromatograms of double-blank rat plasma, plasma sample spiked with IS (500 ng/mL), blank plasma spiked with RA at LLOQ level (50 ng/mL) and IS (500 ng/mL), and the plasma sample obtained at 0.167 h after the intraperitoneal injection of 0.75 mg/kg RA were shown in Fig. 2.

The calibration curve displayed a good linear correlation within the concentration range of 50–50,000 ng/mL ($y = 0.580591x + 0.138381$) with a weighting factor of $1/x$ to reduce the effect of large concentrations on the calculation of regression statistics. The coefficient of determination (R^2) was higher than 0.999. The limit of detection (LOD) for RA, which requires a signal-to-noise ratio above 3, was 5 ng/mL. LLOQ was established at 50 ng/mL. The results of intra-day and inter-day precision, accuracy matrix effect and absolute recovery of the method for the quantification of RA in rat plasma were summarized in Table 1. The values for intra- and inter-day precision were 1.87–2.94% and 3.25–5.36%, and the values for intra- and inter-day accuracy ranged from 5.9% to 10.5% and 5.6% to 11.1%, respectively. The matrix effect of QC samples were 98.3% (100 ng/mL), 111% (1000 ng/mL) and 113% (40,000 ng/mL) with no statistical difference. Absolute recoveries of RA were 91.7% (50 ng/mL), 90.3% (100 ng/mL), 94.1% (1000 ng/mL) and 97.3% (40,000 ng/mL), respectively.

The plasma containing RA was stored at −70 °C, and showed good stability for 60 days and after three freeze/thaw cycles. RA was also stable during the processed samples at room temperature for 4 h and in the auto sampler for at least 24 h. All the values of RSD and RE ranged from 2.7% to 10.8% and −3.4% to 8.7%, respectively.

Table 1
Intra-day and inter-day precision, accuracy, matrix effect and absolute recovery of the method for RA quantification in rat plasma.

Concentration (ng/mL)	Intra-day		Inter-day		Matrix effect		Absolute recovery	
	Precision (%RSD)	Accuracy (%RE)	Precision (%RSD)	Accuracy (%RE)	Mean	RSD (%)	Mean	RSD (%)
50	2.56	10.5	5.36	11.1	NA[a]	NA	91.7	5.7
100	2.94	5.9	4.19	5.6	98.3	2.1	90.3	7.9
1000	1.87	6.6	3.25	8.1	111.6	3.4	94.1	6.1
40,000	2.66	9.5	5.29	10.3	113.0	4.7	97.3	2.0

[a] NA, not available.

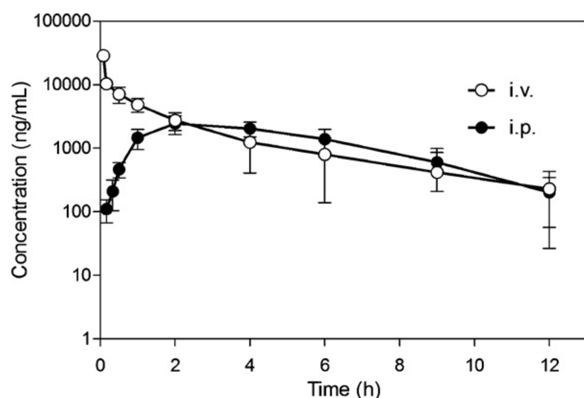

Fig. 3. Concentration time profiles of RA in rats after intravenous (i.v.) or intraperitoneal (i.p.) administration at 0.75 mg/kg (mean ± SD, $n = 6$).

The method was claimed to be credible and robust under our experiment conditions.

3.3. Application to a pharmacokinetic research

The analysis method was successfully used in the determination of RA plasma concentration in rats after intravenous and intraperitoneal administration (0.75 mg/kg). The plasma concentration time curves were shown in Fig. 3.

The main pharmacokinetic parameters of RA were summarized in Table 2. The apparent volume of distribution at terminal phase (V_Z) is calculated from the total amount of drug divided by the plasma concentration during the terminal phase. V_z was 0.11 L/kg, much smaller than the total body water volume (0.67 L/kg), indicating that RA may largely distributed in blood compartment, not in the extravascular tissues. The half-life ($T_{1/2}$) is the time required for RA plasma concentration to fall to half its value as measured at the beginning of the time period. $T_{1/2}$ were 2.6 h and 2.0 h for intravenous and intraperitoneal administration, respectively, with no statistical difference. The characteristic distribution and elimination performance of RA can be explained at least from the two aspects: (1) limited membrane permeability: the three hydrophilic sugar moieties of RA increase the hydrogen binding potential and polar molecular surface area. These features combined with its large molecular mass (>500 Da) may result in poor membranepermeability and compromise transmembrane transport. (2) Biliary excretion [19]: most saponins were susceptible to rapid and extensive biliary excretion through active transport, which may lead to short $T_{1/2}$, low systemic exposure, and small V_z.

Table 2
The pharmacokinetic parameters of RA in rats after intravenous or intraperitoneal administration at 0.75 mg/kg ($n = 6$, mean ± SD).

Parameters	Intravenous	Intraperitoneal
$AUC_{(0–12\,h)}$ (h.μg/mL)	24.7 ± 6.6	14.7 ± 4.9
$AUC_{(0\to\infty)}$ (h.μg/mL)	25.6 ± 7.6	15.3 ± 5.4
$MRT_{(0–12\,h)}$ (h)	1.9 ± 0.5	4.4 ± 0.6
$T_{1/2}$ (h)	2.6 ± 0.4	2.0 ± 0.5
T_{max} (h)	/	2.0 ± 0.0
C_{max} (μg/mL)	28.8 ± 2.6	2.5 ± 0.7
V_Z (L/kg)	0.11 ± 0.01	0.15 ± 0.03
CL (mL/h/kg)	31.8 ± 10.4	55.3 ± 17.3

4. Conclusion

In this study, a sensitive, rapid and specific method was firstly developed for the analysis and quantification of RA, an important ingredient with attractive antitumor activity from *A. raddeana*, in rat plasma. The method exhibited excellent performance with wide range (50–50,000 ng/mL), small sample volume (15 μL), short running time (5 min), and easy sample preparation process. It was successfully translated to the pharmacokinetic study of RA in rats, and may be easily extended to the pharmacokinetic study in other species of animal or biological matrixes.

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