Journal of Chromatography B, 912 (2013) 16–23

Contents lists available at SciVerse ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Short communication

Development and validation of a sensitive liquid chromatography/tandem mass spectrometry method for the determination of raddeanin A in rat plasma and its application to a pharmacokinetic study

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ARTICLE INFO

Article history:
Received 27 July 2012
Accepted 22 September 2012
Available online 4 October 2012

Keywords:
Raddeanin A
LC–MS/MS
Pharmacokinetics
Absolute bioavailability

ABSTRACT

A simple and sensitive high-performance liquid chromatography–electro-spray ionization tandem mass spectrometry (LC–ESI-MS/MS) method was developed and validated to determine raddeanin A in rat plasma. After precipitation of rat plasma samples with methanol, chromatographic separation was achieved on a BDS Hypersil C18 column (100 × 2.1 mm, 2.4 μm) using the mobile phase consisted of acetonitrile and 2 mM ammonium acetate with 0.05% formic acid (60:40, v/v). The detection was performed in a triple quadrupole tandem mass spectrometer by multiple reaction monitoring (MRM) mode using negative ionization. The transition monitored were m/z 895.6 → 455.0 for raddeanin A and m/z 359.3 → 329.0 for IS, respectively. The method was linear over the concentration range of 2–1000 ng/mL for raddeanin A. The intra-day and inter-day assay variations were <9.46%, and the accuracy values were between −2.04% and −6.52% relative error. The extraction recovery of raddeanin A was more than 70%, and the relative matrix effect ranges from 108.52% to 112.36%. The validated method has been successfully applied to determine the pharmacokinetic profile of raddeanin A in rat plasma following oral and intravenous administration.

1. Introduction

Radde anemone Rhizome, as a traditional Chinese medicine, is the rhizome of buttercup *Anemone raddeana Regel* and has been shown to exhibit anti-inflammation, anti-cancer and anti-rheumatism activities [1,2]. Raddeanin A (Fig. 1), a major triterpenoid saponin in Radde Anemone Rhizome, has strong inhibition on proliferation of human nasopharyngeal carcinoma KB cells, non-small cell lung cancer (NSCLC) H460 cancer cells and ovarian cancer SKOV3 cells in vitro as well as S180, H22 and U14 cell in vitro [2,3]. Although many studies have implicated the effect of raddeanin A on cancer, few researches about pharmacokinetic profiles have been published so far.

The qualitative and quantitative analyses of raddeanin A in Radde anemone Rhizome have been studied using ultra violet (UV), Fourier transformed infrared spectrum (FTIR) and high-performance liquid chromatography (HPLC) methods [4,5]. The fragmentation pathway of raddeanin A has been studied by electro spray ionization mass spectrometry (ESI-MS/MS) together with chemical derivatization [6]. To our knowledge, reported detection methods are not suitable for quantification in rat plasma due to lack of sensitivity and specificity. In order to characterize the precise pharmacokinetic profile of raddeanin A, a simple and sensitive analytical method is required for routine analysis in biological samples.

This paper describes a fast, selective and highly sensitive approach, which enables the determination of raddeanin A using LC–MS/MS by precipitation protein with 100 μl plasma volume for sample preparation. This method was validated and applied to the pharmacokinetic and bioavailability study in rats after oral or intravenous administration of 2 mg/kg raddeanin A, and that also could be further used for the analysis of raddeanin A physiological disposition and drug–drug interaction studies in the future.

2. Experimental

2.1. Material and reagents

Raddeanin A (purity >98%) were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, PR China). The prednisolone as internal standard (IS) was

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http://dx.doi.org/10.1016/j.jchromb.2012.09.038

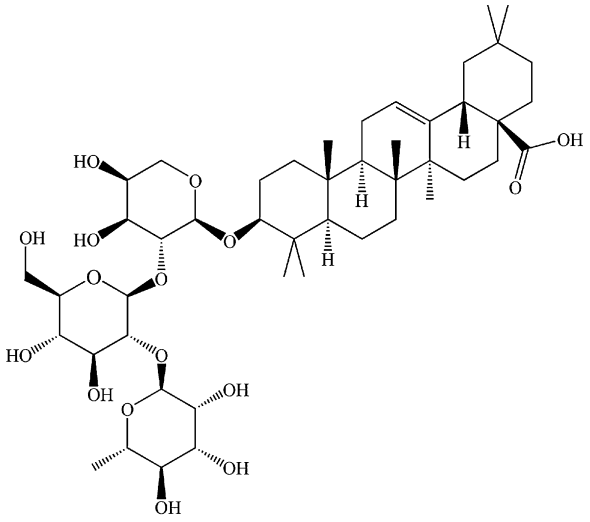

Fig. 1. The chemical structure of raddeanin A.

obtained from Sigma (St. Louis, MO). Methanol and acetonitrile of HPLC grade were provided by Merck (Darmstadt, Germany) and LC–MS grade formic acid was obtained from Sigma (St. Louis, MO). De-ionized water was purified by a Milli-Q water purification system from Millipore (Milford, MA, USA). The blank rat blood was collected from healthy, drug-free rats and stored at −80 °C until needed.

2.2. Preparation of standard solutions and quality control (QC) samples

Primary stock solution of raddeanin A and IS was prepared by dissolving 2.2 mg raddeanin A and 13.5 mg prednisolone in 10 mL methanol, respectively. Standard working solutions of raddeanin A ranging from 20 ng/mL to 10 μg/mL were prepared by serially dilution of the stock solutions with methanol. Finally, the plasma calibration standards were prepared at concentrations of 2, 5, 10, 20, 50, 100, 200, 500, 1000 ng/mL by spiking 10 μL working solution of corresponding concentrations to 100 μL of blank rat plasma. And, the three QC samples at concentrations of low (5 ng/mL), medium (50 ng/mL), high (800 ng/mL) for raddeanin A were prepared in a similar way. The IS working solution at concentration of 1 μg/mL was obtained by dilution of IS stock solution with methanol. All solutions were stored at 4 °C.

2.3. Sample preparation

The frozen plasma samples were thawed at room temperature and vortex-mixed thoroughly. Plasma samples, including calibration standards and QC samples, were extracted by protein precipitation with methanol. 10 μL IS (1 μg/mL) solution was added to 100 μL rat plasma in 1.5 mL Eppendorf tubes, and immediately vortex-mixed 30 s. Then, 400 μL methanol was added and vortex-mixed 5 min, followed by centrifugation for 10 min at 20,000 × *g* at 4 °C. A volume of 300 μL of the upper organic layer was directly transferred into new Eppendorf tubes and evaporated to dryness under vacuum in speedvac concentrator. The residue was re-dissolved in 100 μL of mobile phase and 5 μL was injected onto analytical column.

2.4. Instrumentation and LC–MS/MS analytical conditions

A Shimadzu HPLC system consisting of two LC-20AD pump, a DGU-20A3 degasser, a SIL-20AC auto sampler and a CTO-20AC column oven (Shimadzu Corporation, Kyoto, Japan) was used. Chromatographic separation was carried out using a BDS Hypersil C18 column (100 × 2.1 mm, 2.4 μm, Thermo, USA) protected by a C18 Security guard (4 mm × 3.0 mm ID 5 μm) and it was maintained at 40 °C. A binary isocratic elution was performed at a flow rate of 0.2 mL/min and the mobile phase consisted of acetonitrile and 2 mM ammonium acetate with 0.05% formic acid (60:40, v/v). The total period for one sample was about 5 min.

Mass spectrometric analysis preformed on an API 4000 Q-trap MS/MS system (Applied Biosystems, Foster City, CA, USA) equipped with a Turbo Ion Spray inlet in the negative ion mode and multiple reaction monitoring (MRM) at unit resolution was employed to monitor the transitions of the molecular ions ($[M-H]^-$) of raddeanin A and IS at m/z 895.6 → 455.0, and m/z 359.3 → 329.0, respectively. The optimal MS parameters obtained were as follows: ion spray (IS) voltage at −4500 V, temperature at 450 °C, curtain gas, gas 1 and gas 2 (nitrogen) were set at 10, 50, 60 psi, respectively. The optimized declustering potential (DP) for raddeanin A and IS were −206 and −100 V, and collision energy (CE) for raddeanin A and IS were −90 and −14 eV, respectively. The product ions of these compounds are shown in Fig. 2. Analyst 1.5.1 software (Applied Biosystems, Foster City, CA, USA) was applied for instrumental control, data acquisition and quantitative analysis.

2.5. Method validation

2.5.1. Specificity

The specificity of the LC–MS/MS method was investigated by comparing blank rat plasma from six different sources with the corresponding spiked plasma samples at the LLOQ level and actual plasma samples. The influences of endogenous substances in rat plasma were evaluated in the specificity validation experiment.

2.5.2. Linearity and lowest limit of quantification (LLOQ)

The linearity of each calibration curve was determined by plotting the peak area ratio (y) of raddeanin A versus IS with the nominal concentrations (x) of raddeanin A in plasma. The calibration curves were constructed by linear regression with a weighting factor of the reciprocal of the concentration squared ($1/x^2$). The acceptable correlation coefficients were 0.99 or better, and the accuracy and precision each back-calculated standard concentration must be within ±15% relative standard deviation (R.S.D.) from the nominal value. Meanwhile, LLOQ was investigated by analyzing five replicates of spiked samples at the concentration of 2 ng/mL with acceptable precision. The signal-to-noise ratio (S/N) of LLOQ was at least 10 and the values for precision and accuracy were less than 20%.

2.5.3. Recovery and matrix effect

The extraction recoveries of raddeanin A from rat plasma were carried out in plasma QC samples at low, medium and high levels. The absolute percentage recovery was determined by comparing the mean peak area of five replicates of extracted samples (A) with mean peak areas of the analyte in spiked plasma post-extraction (B). The ratio (A/B × 100) is defined as the absolute extraction recovery (ER). The relative matrix effect was calculated by comparing the peak area ratios of raddeanin A and IS dissolved in the post-extracted blank plasma samples solution (C) with that of raddeanin A and IS spiked in mobile phase at corresponding concentrations (D). The ratio (C/D × 100) is defined as the relative matrix effect

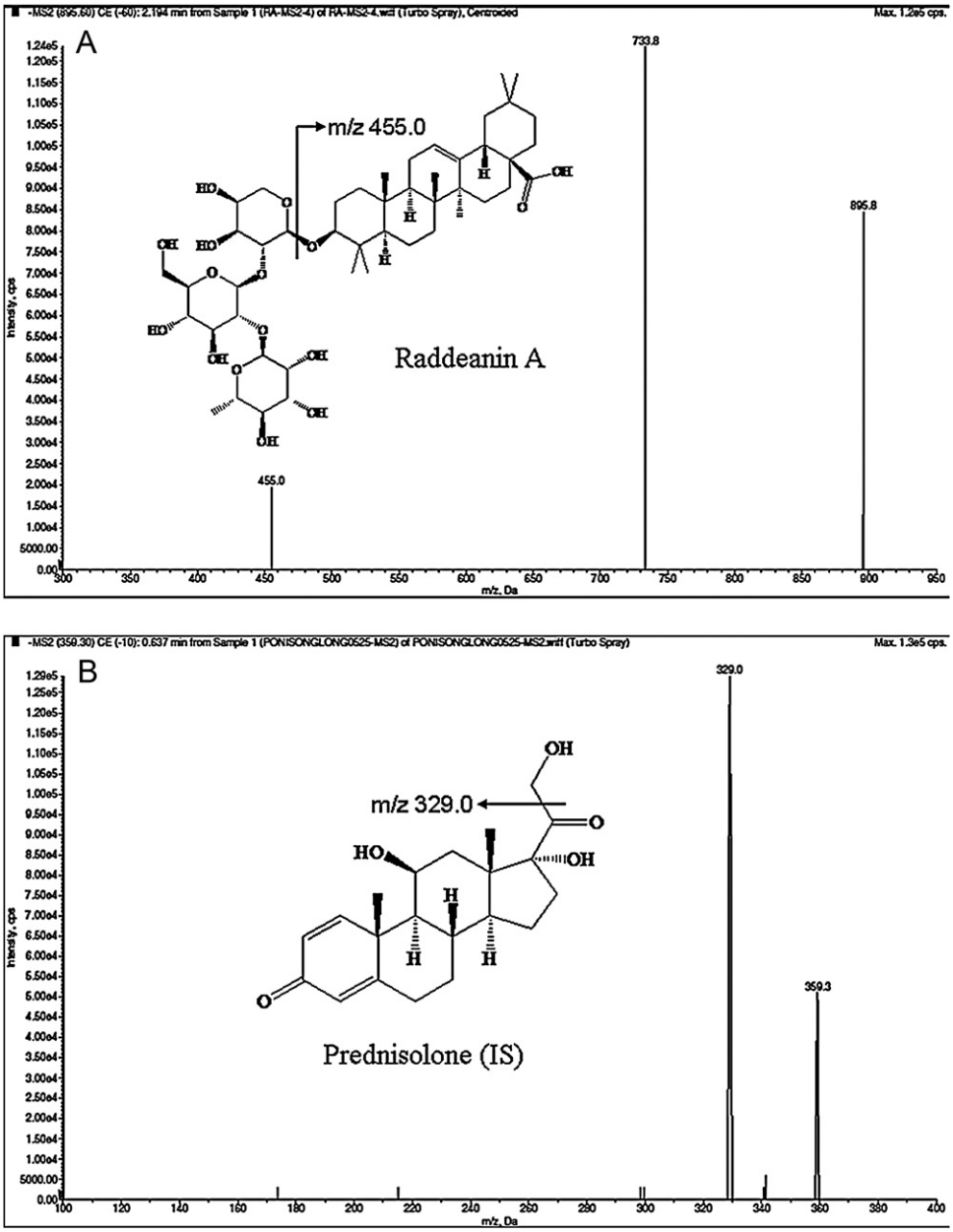

Fig. 2. Full-scan product ion spectra of $[M-H]^-$ for raddeanin A (A) and IS (B).

(ME). The acceptance criterion for the precision of the recovery and matrix effect samples at each level was ±15% R.S.D.

2.5.4. Precision and accuracy

Accuracy and precision of intra-day and inter-day were evaluated from replicate analysis ($n = 5$) of QC samples at three different concentrations on the same day and three consecutive days, respectively. The criteria for acceptability of the data included accuracy within ±15% relative error (R.E.) from the nominal values and a precision of within ±15% R.S.D.

2.5.5. Stability and dilution

Stability tests were performed for spiked plasma QC levels samples ($n = 5$) under various conditions: room temperature storage (around 24 h), three freeze/thaw cycles, auto-sampler storage (at 4 °C for 24 h) and long-term storage (frozen for 15 days). The analytes were considered stable if assay values were within the acceptable limits of accuracy (±15% R.E.) and precision (±15% R.S.D.).

In additional, some samples by intravenous injection were observed to have raddeanin A levels exceeding the highest concentration of the calibration curve. In order to evaluate precision and accuracy of the dilution integrity, 10-fold and 20-fold high concentration QC samples (800 ng/mL × 10, 800 ng/mL × 20) were diluted by 10-fold and 20-fold with blank matrix in sample preparation and analyzed on LC–MS/MS [7]. Accuracy should be within ±15% and the precision ≤20% were acceptable.

2.5.6. Carry-over

A blank sample was placed right after the upper limit of quantification (ULOQ) standard to evaluate the carry-over of the LC–MS/MS method [8,9].

2.6. Pharmacokinetic and statistical analysis

Twelve Sprague-Dawley rats with specific-pathogen-free (SPF) (six males and six females, body weight 220 ± 11.0 g) were obtained from the Animal Center of Nanjing Medical University (NJMU, Nanjing, China) in this study. They were housed under standard conditions and had ad libitum access to water prior to the experiment. This study was reviewed and approved by the Animal Ethical Committee of Nanjing University of Technology. After fasted for 12 h, six rats (three males and three females) were given a dose of 2.0 mg/kg raddeanin A via the tail vein, and the other six rats (three males and three females) were administered by oral with the same dose. Blood sampling (200 μL) were collected at 0, 0.083, 0.25, 0.5, 1, 1.5, 2, 4, 6, 8, 12, 24, 36 and 48 h after intravenous administration and at 0, 0.25, 0.75, 1, 1.5, 2, 4, 6, 8, 12, 24 h after oral administration. Plasma was separated by centrifugation at 3000 × *g* for 10 min and stored at −80 °C until analysis.

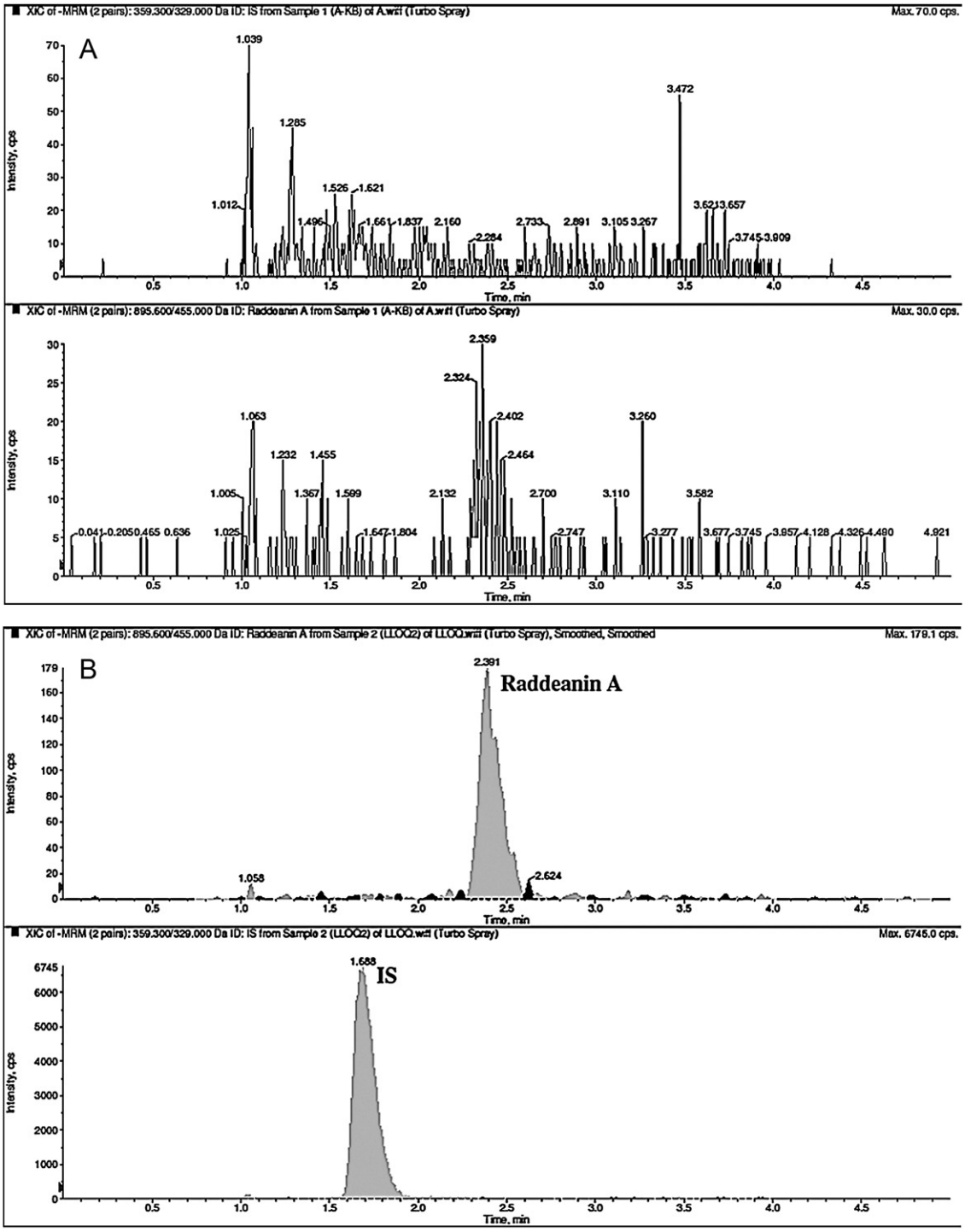

Fig. 3. Representative MRM chromatograms of (A) blank rat plasma, (B) rat plasma spiked with 2 ng/ml (LLOQ) raddeanin A, (C) rat plasma spiked with 100 ng/ml raddeanin A and (D) a rat plasma sample obtained 4 h after an intravenous administration of raddeanin A.

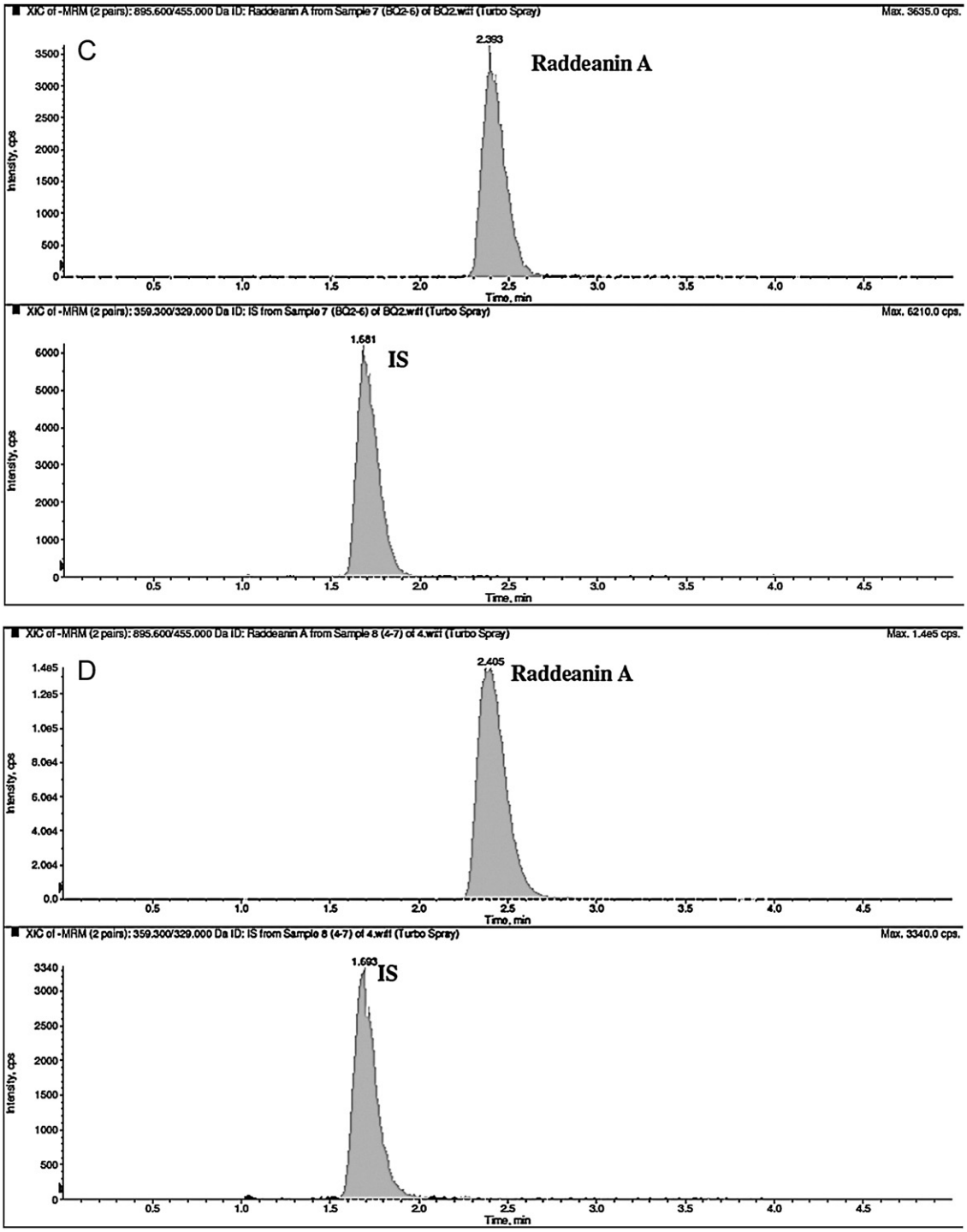

Fig. 3. (*Continued*).

The pharmacokinetic parameters calculations were performed by non-compartmental method using DAS (Drug and Statistics) 2.0 software (Mathematical Pharmacology Professional Committee of China, Shanghai, China). The peak plasma concentration (C_{max}) and the time (T_{max}) to reach C_{max} were directly obtained from the experimental data, and other parameters including area under the concentration–time curve from zero to the last measurable plasma concentration point (AUC_{0-t}), AUC from 0 to infinity ($AUC_{0-\infty}$), elimination rate constant (K), terminal elimination half-life ($T_{1/2}$), mean residence time (MRT), and clearance (CL) were calculated systematically.

3. Results and discussion

3.1. Optimization of mass spectrometric and chromatographic conditions

Under the electro spray ionization (ESI) operation, raddeanin A and IS showed higher sensitivity in the negative mode than that in the positive mode. The most abundant molecular ions for raddeanin A and IS are $[M-H]^-$ for raddeanin A (m/z 895.6) and IS (m/z 359.3), respectively. The fragment ions (m/z 455.0, m/z 733.9) of raddeanin A (m/z 895.6) were obtained by fragmentation in the

MS2 condition, and fragment ion (m/z 329.0) of IS (m/z 359.3) was also obtained in similar way. Through optimize mass spectrometry conditions (source temperature, ion spray voltage, collision energy and fragmentor voltage), the ion pairs m/z 895.6 → 455.0 for raddeanin A and m/z 359.3 → 329.0 for IS exhibited high responsivity in MRM mode.

Proportion of mobile phase, flow rate and peak shape were optimized by changing several combinations of acetonitrile, methanol, acetic acid and formic acid. Peak shape was improved by using 0.05% formic acid in the aqueous phase. 60% organic phase and the flow rate of 0.2 mL/min were performed in the chromatographic separation. Under a total analysis time of 5 min, the retention time of raddeanin A and IS was 2.4 min and 1.7 min, respectively.

3.2. Sample preparation

One-step protein precipitation was selected for plasma sample preparation due to the low recovery of liquid-liquid extraction using various kinds of organic solvents including diethyl ether, methyl-tert-butyl ether, acetic ether, dichloromethane et al. Methanol as a suitable protein precipitation solvent was adopted due to excellent precipitation, preferable recovery, little matrix effects and economic cost. By comparison, 400 μL methanol for 100 μL plasma protein precipitation was the optimal method for the sample preparation.

3.3. Validation of the method

3.3.1. Specificity

The specificity of the LC–MS/MS method was assessed by comparing the chromatograms of six different sources of blank rat plasma with the corresponding spiked plasma. No obvious endogenous interferences from blank plasma were observed at the retention time of raddeanin A (2.4 min) and IS (1.7 min). Representative chromatographs including blank plasma and spiked plasma was showed in Fig. 3.

Table 1
Extraction recovery and matrix effect of raddeanin A in rat plasma (n = 5).

Nominal concentrations (ng/mL)	Extraction recovery (%)	CV (%)	Matrix effect (%)	CV (%)
5	72.76	3.67	109.11	3.46
50	70.27	5.12	112.36	2.39
800	71.58	7.52	108.52	4.20

3.3.2. Linearity of calibration curve and LLOQ

All calibration curves were linear over the validated concentration range described in Section 2.3 using weighted linear least square regression ($1/x^2$). The mean linear regression equation of calibration curves (n = 5) was: $y = 0.00592x + 0.0003$ ($r = 0.9978$), where x was the concentration of raddeanin A and y was the peak area ratio of raddeanin A to IS. The precision and accuracy of LLOQ (n = 5) was 8.42% (R.S.D.) and −4.30% (R.E.), respectively.

3.3.3. Recovery and matrix effect

The extraction recoveries of raddeanin A at three QC concentrations (n = 5) were 72.76%, 70.27% and 71.58%, respectively. The relative matrix effects of raddeanin A at three QC concentrations (n = 5) were 109.11%, 112.36% and 108.52%, respectively. These results indicated that there was no obvious relative matrix effect for raddeanin A and IS for this LC/MS/MS determination. All the detail information is summarized in Table 1.

3.3.4. Precision and accuracy

The results of precision and accuracy for raddeanin A at three levels of QC samples (n = 5) were summarized in Table 2. The intra- and inter-day precisions ranged from 2.63% to 9.46% and from 3.96% to 6.49% (R.S.D.), respectively. The intra- and inter-day accuracy ranged from −2.47% to −6.52% and from −2.04% to −4.30% (R.E.), respectively. All assay values indicated that the acceptable accuracy and precision of the method have been developed.

Table 2
Summary of the inter-day as well as inter-day accuracy and precision of raddeanin A in rat plasma (n = 15).

Nominal concentrations (ng/mL)	Inter-day			Intra-day		
	Measured concentration (ng/mL)	Precision R.S.D. (%)	Accuracy R.E. (%)	Measured concentration (ng/mL)	Precision R.S.D. (%)	Accuracy R.E. (%)
5	4.90	3.96	−2.04	4.67	9.46	−6.52
50	48.18	5.98	−3.60	48.72	2.63	−2.56
800	765.2	6.49	−4.30	780.07	6.04	−2.47

Table 3
Stability of raddeanin A in rat plasma (n = 5).

Sample condition	Nominal concentration (ng/mL)	Mean measured concentration (ng/mL)	Precision R.S.D. (%)	Accuracy R.E. (%)
Autosampler (24 h, 4 °C)	5	5.09	101.80	1.80
	50	47.32	94.64	−5.36
	800	782.6	97.83	−2.18
Three freeze/thaw cycles	5	4.816	96.32	−3.68
	50	52.88	105.76	5.76
	800	810.8	101.35	1.35
Room temperature (24 h)	5	4.712	94.24	−5.76
	50	55.06	110.12	10.12
	800	866.2	108.28	8.28
Long term (15 days, −80 °C)	5	4.532	90.64	−9.36
	50	52.6	105.20	5.20
	800	789.8	98.73	−1.28

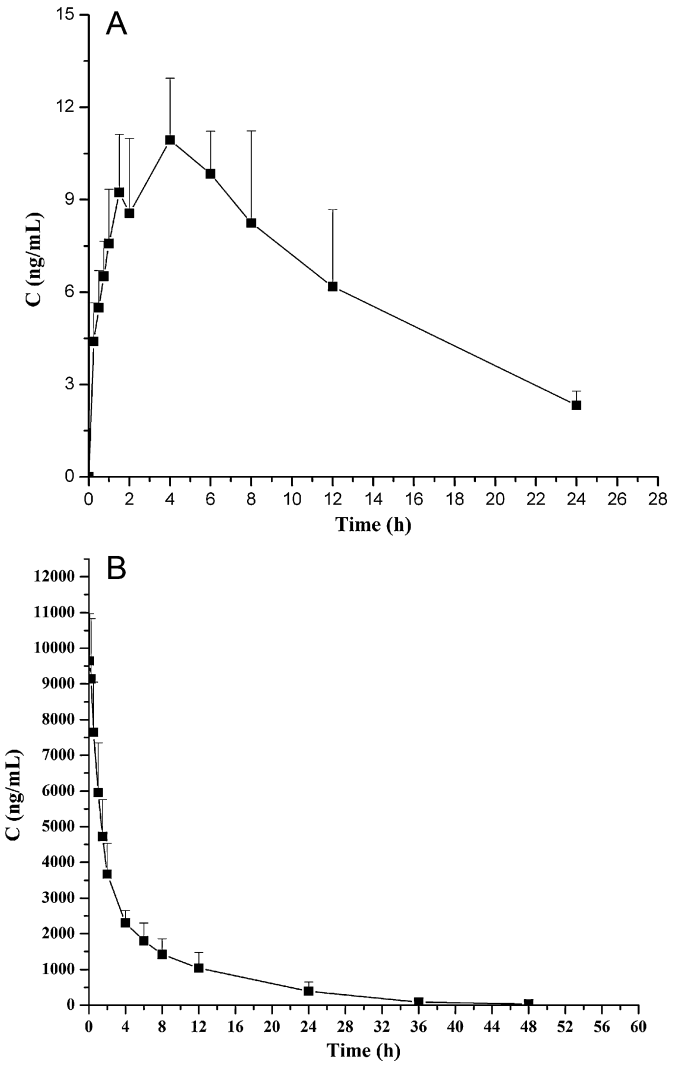

Fig. 4. Mean plasma concentration–time curve of raddeanin A after a single oral administration (A) and a single intravenous administration (B).

Table 4
Non-compartmental pharmacokinetic parameters for raddeanin A given as single-dose (2 mg/kg) in rats ($n = 6$).

Pharmacokinetic parameters	Unit	Oral	I.V.
		Values (mean ± SD)	Values (mean ± SD)
Area under concentration–time curve (AUC_{0-t})	mg/L h	0.126 ± 0.0528	42.6 ± 12.7
$AUC_{0-\infty}$	mg/L h	0.150 ± 0.0583	42.9 ± 12.9
Mean residence time (MRT_{0-t})	h	6.97 ± 2.64	7.96 ± 1.35
Half-life ($T_{1/2}$)	h	5.88 ± 3.24	7.12 ± 1.07
Time to peak concentration (T_{max})	h	5 ± 2	–
Clearance (CL)	L/h/kg	7.74 ± 3.69	0.025 ± 0.008
Apparent volume of distribution (V)	L/kg	55.0 ± 12.8	0.257 ± 0.091
Peak concentration (C_{max})	μg/L	11.0 ± 1.87	–
Bioavailability (F)	%	0.295	

3.3.5. Stability and dilution

The stability of raddeanin A in rat plasma was investigated under a variety of storage and process conditions. The data for stability tests was listed in Table 3, which included all the detail parameters about the stability experiments at three QC levels. The result indicated that raddeanin A was stable at the anticipated conditions including three freeze/thaw cycles, at room temperature for 24 h, at 4 °C in the autosampler for 24 h and in a freezer set to −80 °C for 15 days.

The precision (R.S.D.) and accuracy (R.E.) for raddeanin A concentrations at 8000 ng/mL following a 10-fold and 20-fold dilution with blank matrix were 3.71% and −5.28%, 8.45% and −6.59%, respectively, which suggested that samples whose concentrations exceeded the highest concentration of the calibration curve could be reanalyzed by dilution to obtain acceptable data.

3.3.6. Carry-over

In this assay, 50% methanol (containing 0.1% formic acid) was used to wash syringe and injection port multiple times before and after each injection to reduce carryover. No significant carryover was observed in the carry-over test.

3.4. Pharmacokinetic application

The sensitive and specific LC–MS/MS method of raddeanin A was applied to the pharmacokinetic study in rat after single-dose (2.0 mg/kg) to twelve rats including oral and intravenous administration. The mean plasma concentration-time profiles of raddeanin A were showed in Fig. 4. The major pharmacokinetic parameters of raddeanin A were calculated by a non-compartmental model and were presented in Table 4. A lower absolute bioavailability (0.295%) and systemic absorption for raddeanin A was observed in our study due to poor liposolubility which was similar to most of saponins reported in the literatures [10,11].

4. Conclusions

A simple, rapid and sensitive LC–MS/MS method for the quantification of raddeanin A in rat plasma was successfully developed and validated. This method showed excellent sensitivity, linearity, precision and accuracy, and was successfully applied to pharmacokinetic study of raddeanin A in rat after oral and intravenous administration. This study could provide a scientific basis for the further study of raddeanin A.

Acknowledgement

The study was supported by a grant from the “333 High Level Talent Cultivation Project” of Jiangsu Province (2012).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jchromb.2012.09.038.

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