Journal of Pharmaceutical and Biomedical Analysis 53 (2010) 698–704

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

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

Short communication

Simultaneous determination of danshensu, rosmarinic acid, cryptotanshinone, tanshinone IIA, tanshinone I and dihydrotanshinone I by liquid chromatographic–mass spectrometry and the application to pharmacokinetics in rats

Yun Liu [1], Xiaorong Li [1], Yuhang Li, Lijuan Wang, Ming Xue *

Department of Pharmacology, School of Chemical Biology and Pharmaceutical Sciences, Capital Medical University, Youanmen, Beijing 100069, PR China

ARTICLE INFO

Article history:
Received 14 October 2009
Received in revised form 27 March 2010
Accepted 31 March 2010
Available online 8 April 2010

Keywords:
Phenolic acids
Tanshinones
Quantification
Pharmacokinetics
LC–ESI–MS

ABSTRACT

A sensitive and specific liquid chromatography–tandem mass spectrometry method (LC–MS) was developed and validated for the separation and simultaneous determination of danshensu, rosmarinic acid and tanshinone compounds including cryptotanshinone, tanshinone I, dihydrotanshinone I and tanshinone IIA in rat plasma. Chromatographic separation of the analytes was successfully achieved on a C_{18} column using a mobile phase composed of acetonitrile–water containing 0.5% glacial acetic acid. This method demonstrated good linearity and did not have endogenous material interfering with the active compounds and I.S. peaks. The limit of quantification of danshensu, rosmarinic acid, cryptotanshinone, dihydrotanshinone I, tanshinone I and tanshinone IIA were 5, 0.75, 0.1, 0.1, 1 and 0.5 ng/mL. The average extraction recoveries of these analytes from rat plasma were all over 60%. The precisions determined from five days were all within 10%. This method has been successfully applied in the simultaneous quantification and the pharmacokinetic studies of these six compounds in animals which were orally administered with danshen preparations.

1. Introduction

Salvia Miltiorrhiza Bunge (SMB), which is a well-known traditional Chinese medicine (TCM) named 'Danshen' in China, has been widely adopted in many TCM prescriptions for treating various diseases including coronary heart diseases such as angina pectoris, myocardial infarction and atherosclerosis as well as liver diseases such as hepatitis and liver fibrosis in clinical practice. Moreover, SMB had anti-inflammatory, antibacterial and antineoplastic actions [1–6]. In terms of its chemical structure, the major bioactive constituents in roots of SMB can be classified into two main active groups: the hydrophilic phenolic acid compounds such as danshensu and rosmarinic acid; and the lipophilic tanshinone compounds (named abietane-type diterpenes or danshen diterpenoid quinones) such as cryptotanshinone, tanshinone IIA, dihydrotanshinone I and tanshinone I [7,8]. The in vivo simultaneous quantification analysis of these two kinds of active components from the roots of SMB has become more and more important due to the increasingly extensive application of danshen preparations in worldwide. The in vitro and in vivo quantitative methods of HPLC equipped with UV or MS in detecting either the water-soluble phenolic acids or the lipophilic tanshinone components have been reported [9–16], However, until now, there are very few reports on the in vivo simultaneous detection of the two active components with HPLC–UV methods and the quantitative analyses of these components are very limited [17,18]. Due to the significant differences on the chemical structures and the pharmacological activities between the two groups of the active components, it is very necessary to develop a more efficient method for in vivo simultaneous detection of the two groups of the active components which are as the main active biomarkers in TCM compound preparations containing danshen extracts. The development of this method is very useful in monitoring the quality controls of the manufacturing process as well as the pharmacokinetics, in vivo drug–drug interaction and the therapeutic concentration of danshen preparations. In this study, a time-segment program in selected multiple reaction monitoring (MRM) mode was developed for the simultaneous liquid chromatography–tandem mass spectrometry (LC–MS) analysis of danshensu, rosmarinic acid, cryptotanshinone, tanshinone IIA, dihydrotanshinone I and tanshinone I from danshen extracts in biological samples. Using electrospray LC–MS, positive ion mode provided good sensitivity for detecting tanshinones while negative ion mode was better suited for detecting phenolic components. MS

* Corresponding author. Tel.: +86 1083911520; fax: +86 1083911520.
E-mail address: xuem@ccmu.edu.cn (M. Xue).
[1] Co-first author.

0731-7085/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2010.03.041

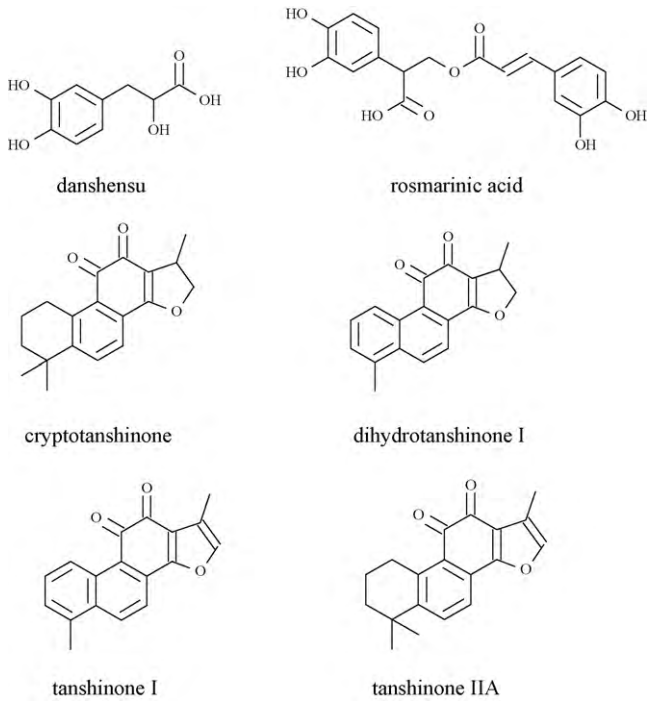

Fig. 1. Chemical structures of danshensu, rosmarinic acid, cryptotanshinone, dihydrotanshinone I, tanshinone I and tanshinone IIA.

parameters under the positive and negative electrospray ionization conditions were optimized for achieving good sensitivity for both phenolic acids and tanshinones in one single analytical run and this method was successfully applied to the in vivo simultaneous quantitative analysis, the pharmacokinetic studies and drug interaction from danshen preparations.

2. Experimental

2.1. Chemicals and reagents

Danshensu (DS), rosmarinic acid (RA), cryptotanshinone (CT), dihydrotanshinone I (DT), tanshinone I (TI), tanshinone IIA (TS) and fenofibrate (the internal standard, I.S.) were purchased from the National Institute for Control of Pharmaceuticals and Biological Products (Beijing, China). The chemical structures of these compounds were shown in Fig. 1. The raw materials of tanshinones and phenolic acid extracts were purchased from Xi'an Honson Biotechnology Co. Ltd. (Xi'an, Shanxi, China). HPLC grade methanol was purchased from Fisher Scientific (Fair Lawn, NJ, USA). HPLC grade acetonitrile, ethyl acetate and glacial acetic acid were purchased from Dikma Reagent Company (Beijing, China). Water was triply distilled and filtered through a 0.45 μm filter prior to the use as HPLC mobile phase. The other chemicals, reagents and solvents used were all of chromatographic or analytical grade.

2.2. Instrument and chromatographic-mass conditions

The LC–ESI–MS system consisted of a HPLC system (Series 1100, Agilent technology, Palo Alto, CA, USA) including a HP G1312A binary pump, a G1379A vacuum degasser and G1313A autosampler and coupled to Finngan LCQ Deca XP ion-trap spectrometer equipped with electrospray source (Thermo Finnigan, San jose CA,USA). LC separation was performed on a Capcell Pak MG C_{18} column (100 mm × 2 mm, i.d., 5 μm; Shiseido, Japan) coupled with a C_{18} guard column (10 mm × 2 mm, i.d., 5 μm; Shiseido, Japan) of the same packing material. All chromatography was performed at 25 °C. The LC–ESI–MS system was controlled by Xcalibur® (version 1.3) software.

The mobile phase was composed of acetonitrile–water containing 0.5% (v/v) glacial acetic acid (pH 2.5). Gradient elution (from 8:92 to 78:22, v/v) was started with 8% acetonitrile for 5 min, and increased acetonitrile to 78% with 10 min, and then run for 13 min at a flow rate of 0.2 mL/min. The sample injection volume was 10 μL and the run time of samples was 28 min. The effluent was on-line transferred to ESI–MS system without splitting.

The compound-dependent parameters were optimized for the target compounds to achieve the highest instrument response. MS parameters under the positive and negative electrospray ionization conditions were optimized for achieving good sensitivity for both phenolic compounds and tanshinones in one single analytical run. ESI was operated at the capillary temperature of 350 °C and multiplier voltage of −940 V. The operating conditions were optimized by direct infusion of a mixture of all analytes. Nitrogen was used as sheath gas and aux/sweep gas in the ion trap and the flow rate was optimized as follows: sheath gas flow rate, 30 psi for first 18 min, 20 psi from 18 min to 28 min; aux/sweep gas flow rate, 5 psi for first 18 min, 0 psi from 18 min to 28 min capillary voltage of −35 V for first 18 min, 12 V from 18 min to 28 min and kV.

During the LC–ESI–MS/MS analysis, a time-segment program was developed to switch the ionization mode from negative ion to positive ion mode at the retention time of 28 min. Negative ion mode was used to monitor phenolic acid compounds within 18 min and positive ion mode was used to analyze tanshinone compounds from 18 min to 28 min. Both positive and negative ion modes were performed with selected multiple reaction monitoring (MRM) for the quantitative analysis. The MRM quantitative ions were then selected from the MS/MS data. The optimal MRM transitions of the analytes for quantification were determined as follows: DS m/z 197 → 179, RA m/z 359 → 161, CT m/z 297 → 279, DT m/z 279 → 261, TI m/z 277 → 249, TS m/z 295 → 277, and fenofibrate m/z 361 → 233.

2.3. Animals and materials

Male Sprague–Dawley rats (250 ± 20 g) were obtained from Animal Center of Capital Medical University (ACCMU, Beijing, China). Pooled drug-free plasma was obtained from the healthy rats, Plasma controls were aliquoted and stored at −80 °C and then thawed at room temperature for use in calibration curves and quality control (QC) samples. Animal studies were carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Health Ministry of China. Protocols of animal experiments had been approved by Animal Center of Capital Medical University.

2.4. Calibration samples and quality control samples preparation

The mixture of stock standard solutions containing danshensu (5 μg/mL), rosmarinic acid (0.75 μg/mL), dihydrotanshinone I (0.2 μg/mL), cryptotanshinone (0.2 μg/mL), tanshinone I (0.5 μg/mL) and tanshinone IIA (0.1 μg/mL) was prepared in methanol. The stock solution was further diluted with methanol to form a series of working standard solutions. The internal standard stock solution (fenofibrate) of 40 μg/mL was also prepared in methanol and then diluted with methanol to working solution at the concentration of 1 μg/mL. Calibration samples in plasma were prepared by spiking appropriate amount of standard mixture and I.S. with rat blank plasma at a volume ratio of 200 μL:100 μL: 40 μL to form a concentration series of 2500, 500, 100, 50, 10 and 5 ng/mL for DS; 375, 75, 37.5, 7.5, 3.75 and 0.75 ng/mL for RA; 100, 50, 10, 5, 1, 0.5 and 0.1 ng/mL for CT and DT; 250, 50, 10, 5 and 1 ng/mL for TI; 50, 10, 5, 1, 0.5 and 0.1 ng/mL for TS, and 20 ng/mL for I.S.

Quality control (QC) samples were prepared using the pooled plasma for low, intermediate and high concentration (10, 100, 2500 ng/mL for DS; 3.75, 37.5, 375 ng/mL for RA; 1, 10, 250 ng/mL for TI and 0.5, 5, 50 ng/mL for DT, CT and TS) to evaluate the precision, accuracy, stability and recovery of the assay method. The spiked samples were treated following the sample preparation procedure as indicated in Section 2.5. All the solutions were stored at 4 °C prior to use.

2.5. Extraction procedure

Plasma samples (200 μL) were spiked with 100 μL each of working solution containing danshensu, rosmarinic acid, cryptotanshinone, tanshinone I, dihydrotanshinone I, tanshinone IIA and 100 μL of I.S. working solution. 100 μL of 12% hydrochloric acid (v/v) was then added to adjust the pH to 2.0 in the combined samples. The samples were mixed by vortexing for 1 min, and 1 mL of ethyl acetate was added to each tube, followed by another vortex for 1 min. The samples were then centrifuged at 3500 rpm for 10 min and 1 mL of the supernatant was transferred to a clean test tube. The residue was resolved in 0.9 mL of ethyl acetate and extracted again with the same method. Then the two supernatants were combined and evaporated to dryness under a flow of nitrogen gas at 30 °C. The residue was reconstituted in 100 μL of methanol and a 10 μL aliquot was injected into the LC–MS/MS system for analysis.

2.6. Method validation

The method was validated for the selectivity, linearity, limits of detection (LOD), limits of quantification (LOQ), accuracy, precision, recovery and stability. To evaluate the selectivity, five independent samples of rat blank plasma were analyzed by comparing with the plasma-spiked analytes for excluding endogenous material interference. Quantification was based on the I.S. method of plotting peak areas ratios of analyte/I.S. versus the concentration of the samples with a weighting factor 1. The concentrations of each analyte were determined using the equations of linear regression obtained from the calibration curves. The limit of detection was defined as the final concentration that produced a signal-to-noise (S/N) ratio of 3 and the limit of quantification was 10 times the S/N ratio. The precision and accuracy of method were assessed by performing replicate analyses of QC samples spiked with low, middle and high concentration against calibration standards. Five replicates of QC samples at each concentration were evaluated on the same day for intra-day precision, while repeated analysis at each concentration of QC samples five times per day over five consecutive days for inter-day precision and accuracy. Standard deviations and relative standard deviations (R.S.D. %) was calculated from the QC values and used to estimate the inter- and intra-day precision. The extraction recovery was determined by calculating the ratio of the amount of the extracted compounds from drug-free plasma spiked with low, medium and high concentrations of DS, RA, CT, TI, DT and TS to the amount of these compounds added at the same concentrations to methanol. Five replicates at each concentration were determined. The means, the standard deviations and the relative standard deviations were calculated. The stability of the sample was assessed by measuring the analysis data of QC samples under ambient, frozen and freeze–thaw storage conditions with fresh prepared QC samples.

2.7. Animal pharmacokinetic study and data analysis

Male Sprague–Dawley rats were kept in an environmentally controlled breeding room for five days. The rats were housed with unlimited access to food and water except for fasting 12 h before experiment, with water available *ad libitum*. The raw materials of tanshinones and phenolic components was mixed and dissolved in normal saline. Blood samples were collected from orbital vein of the rats before and after receiving a single oral dose of the mixture above (each dose containing DS 10.25 mg/kg, RA 6.39 mg/kg, CT 9.82 mg/kg, DT 3.58 mg/kg, TI 3.90 mg/kg and TS 5.79 mg/kg). Approximately 0.4 mL of blood was collected in heparinized tubes before drug administration and post-dosing at 5, 10, 20, 30, 45, 60, 75, 90, 120, 240, 360, 480 and 720 min, and then 200 μL of plasma was separated by centrifuged at 3000 rpm for 15 min immediately and stored at −80 °C until further analysis. The plasma samples were extracted as described in Section 2.5.

The LC–ESI–MS/MS procedure was successfully applied to simultaneously investigate the plasma concentration–time profiles of DS, RA, CT, TI, DT and TS in animals. The pharmacokinetic model and parameters were calculated by the practical pharmacokinetic program-version 87 (3P87), edited by the Committee of the Mathematics Pharmacology, the Chinese Society of Pharmacology (Beijing, China). The compartment model was established by the methods of the survival square sum (SUM), the Akaike's information criterion (AIC) and the fitted degree (r^2).

3. Results and discussion

3.1. Method development

Liquid–liquid extraction and organic precipitation were tested for sample preparation in our expedriments. Organic precipitation with acetonitrile did not provide the satisfactory recovery of phenolic acid components. A liquid–liquid extraction method was then developed and proved to be simple and suitable for simultaneous detection of both the tanshinones and the phenolic acid components from danshen extracts in rat plasma. Different extraction solvents, pH condition and extraction time were also evaluated. Better recovery, particularly for the phenolic componets, was achieved by adding 12% hydrochloric acid in the extraction solution to adjust pH to 2.0 and conducting the extraction twice with ethyl acetate. It is difficult to simultaneous separation of danshen components due to the polarity and unstability of danshensu and rosmarinic acid. Prolonged retention time is more beneficial in analysis of these plasma samples in order to avoid co-elution with early eluting endogenous compounds that produce ion suppression. The Capcell Pak MG C_{18} column were evaluated and proved to be more suitable for simultaneously separating these two kinds of compounds especially for phenolic acid components. The ion intensities of danshensu and rosmarinic acid are inverse ratio to pH value, and the concentration of 0.5% glacial acetic acid aqueous solution (v/v) was proved to be a better response condition. Gradient elution changed linearly from acetonitrile–0.5% acetic acid aqueous solution (8:92 to 78:22, v/v) at a flow rate of 0.2 mL/min. As the I.S., fenofibrate had a more suitable retention time in the chromatographic separation, as well as more efficiency for the ESI ionization of the analytes.

Electrospray ionization (ESI) was adopted to quantify the analytes in rat plasma due to its lower levels of background noise. Negative ion mode was found to provide better sensitivity for detecting the phenolic components in danshen, while positive ion mode was better for the detection of tanshinones. Negative ion mode was applied for the first time-segment running to the chromatographic retention time at 19 min because the phenolic compounds were eluted first, and then the ion mode was switched to positive mode for the analysis of tanshinone compounds. The capillary temperature, vaporizer temperature and flow rate were optimized to obtain protonated molecules of the analytes. The fragment energy was optimized to achieve maximum response of the compound fragment ion peaks. Selected multiple reaction ion

Table 1
Precision and accuracy for the LC–MS analysis of danshensu, rosmarinic acid, dihydrotanshinone I, cryptotanshinone, tanshinone I and tanshinone IIA in rat plasma.

Analyte	Theoretical concentration (ng/mL)	*n*	Measured concentration (ng/mL) (mean ± S.D.)	Precision (R.S.D. %)	Accuracy percent error (%)
Danshensu	Intra-day				
	10	5	10.42 ± 1.04	9.83	3.82
	100	5	92.65 ± 2.18	2.43	−7.27
	2500	5	2367 ± 43.4	1.81	−5.28
	Inter-day				
	10	15	9.68 ± 0.65	6.74	−3.12
	100	15	92.91 ± 1.63	1.77	−7.13
	2500	15	2331 ± 99.32	4.32	−6.76
Rosmarinic acid	Intra-day				
	3.75	5	3.65 ± 0.07	2.03	−2.22
	37.5	5	38.03 ± 1.18	3.24	1.20
	375	5	387.44 ± 2.09	0.54	3.12
	Inter-day				
	3.75	15	3.58 ± 0.08	3.15	3.46
	37.5	15	37.79 ± 2.10	5.50	0.82
	375	15	370.65 ± 14.81	4.04	1.38
Dihydrotanshinone I	Intra-day				
	0.5	5	0.48 ± 0.002	0.41	−7.71
	5	5	5.12 ± 0.35	7.60	1.46
	50	5	51.60 ± 1.25	2.48	3.28
	Inter-day				
	0.5	15	0.48 ± 0.02	4.1	−7.9
	5	15	4.69 ± 0.18	3.2	−5.3
	50	15	51.23 ± 1.61	3.04	2.48
Cryptotanshinone	Intra-day				
	0.5	5	0.52 ± 0.02	5.03	−3.20
	5	5	5.12 ± 0.16	3.39	1.57
	50	5	48.9 ± 3.21	6.68	−2.52
	Inter-day				
	0.5	15	0.51 ± 0.03	4.60	9.04
	5	15	5.02 ± 0.28	6.72	−0.88
	50	15	54.60 ± 1.91	3.48	9.17
Tanshinone I	Intra-day				
	1	5	0.93 ± 0.03	2.79	−6.66
	10	5	9. 81 ± 0.56	5.64	−2.23
	250	5	260.54 ± 14.0	5.40	3.92
	Inter-day				
	1	15	1.02 ± 0.03	3.39	−5.30
	10	15	9.42 ± 0.36	4.35	−6.3
	250	15	253.1 ± 8.9	3.53	1.3
Tanshinone IIA	Intra-day				
	0.5	5	0.48 ± 0.03	5.65	−5.6
	5	5	4.81 ± 0.17	3.10	−5.01
	50	5	50.62 ± 2.44	4.67	1.28
	Inter-day				
	0.5	15	0.47 ± 0.03	6.90	−7.49
	5	15	5.24 ± 0.26	6.03	3.61
	50	15	50.68 ± 1.24	2.32	1.4

monitoring (MRM) was used for the simultaneous quantification of danshen components and the quantitative ions were chosen because all of the product ions were the most abundant ion. The optimal chromatographic conditions were determined as described in Section 2.2.

3.2. Selectivity

The results for selectivity are shown in Fig. 2. The LC–ESI–MS/MS method described was selective and specific. The analysis from the rat plasma samples showed that there were no endogenous substance peaks and drug metabolite peaks interfered with the analytes and I.S. at the retention times. The retention times for DS, RA, CT, DT, TI, TS and I.S. were approximately 4.35, 16.05, 22.60, 21.17, 22.85, 25.08 and 25.26 min, respectively. Fig. 2(A) and (B) showed a blank rat plasma and a blank plasma spiked with the analytes and I.S., the graph (C) showed a plasma sample at 1.5 h after an oral dose of danshen extracts, indicating that there were no endogenous substances and metabolites interfered, Moreover, as shown in Fig. 2, phenolic acids and tanshinone components were well separated, which confirmed the selectivity of the current method.

3.3. Linearity and sensitivity

To evaluate the linearity of the LC–ESI–MS method, the calibration curves of plasma were determined in triplicate on three separate days. Calibration graphs were constructed using a linear regression of test compound/I.S. peak area ratio (y) to nominal plasma concentration of the test compound (x, ng/mL). The equations for the calibration curves for these six active compounds were: $y = 2.96 \times 10^{-4}x + 0.0898$ in the ranges of 5–2500 ng/mL (DS), $y = 3.01 \times 10^{-3}x + 0.187$ in the ranges of 0.75–375 ng/mL (RA), $y = 0.181x + 0.363$ in the ranges of 0.1–100 ng/mL (DT), $y = 1.47 \times 10^{-3}x + 0.657$ in the ranges of 0.1–100 ng/mL (CT), $y = 9.41 \times 10^{-3}x + 0.0248$ in the ranges of 1–250 ng/mL (TI), $y = 4.76 \times 10^{-2}x + 0.0523$ in the ranges of 0.5–500 ng/mL (TS),

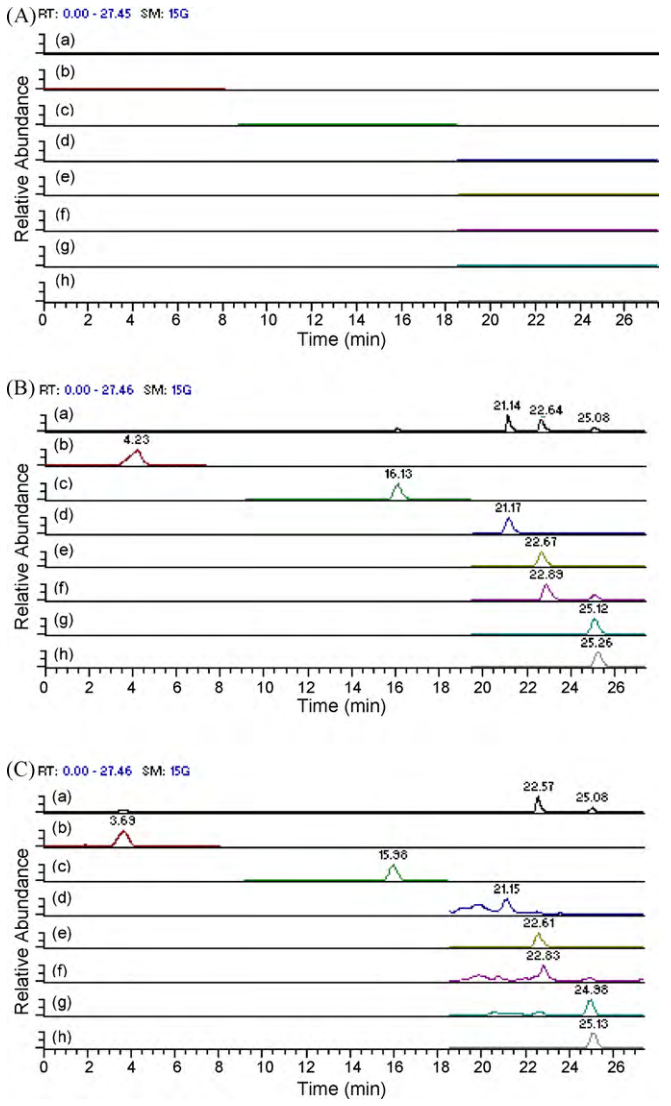

Fig. 2. LC–MS chromatogram of blank rat plasma (A); blank rat plasma spiked with danshensu, rosmarinic acid, cryptotanshinone, tanshinone I, dihydrotanshinone I and tanshinone IIA and I.S. (B); rat plasma sample at 1.5 h after an oral dose of danshen extracts to a rat (C). Total ion current (a); SRM of m/z 197 → 179 for danshensu (b); SRM of m/z 359 → 161 for rosmarinic acid (c); SRM of m/z 297 → 279 for cryptotanshinone (d); SRM of m/z 279 → 261 for dihydrotanshinone I (e); SRM of m/z 277 → 249 for tanshinone I (f); SRM of m/z 295 → 277 for tanshinone IIA (g); SRM of m/z 361 → 233 for fenofibrate (h).

respectively. The coefficient of correlation (r^2) for these six calibration curves were 0.9954–0.9996 in rat plasma. The assay data proved to be linear and acceptable and the range of concentrations was found to be suitable for pharmacokinetic analysis. Sensitivity was evaluated by determining the LOD and the LOQ, which are defined as the lowest concentration that can be reliably and reproducibly measured at least five replicates. The LOD had to have precision of ≤10% and a signal/noise ratio ≥3. The LOQ had to have precision of ≤10% and a signal/noise ratio ≥10. The LOD and the LOQ were 2.5 and 5 ng/mL (DS), 0.38 and 0.75 ng/mL (RA), 0.05 and 0.1 ng/mL (DT and CT), 0.5 and 1 ng/mL (TI), 0.1 and 0.5 ng/mL (TS), respectively.

3.4. Precision and accuracy

The precision and accuracy of the method were assessed in rat plasma by performing replicate analyses of spiked samples against calibration standards at low, medium and high concentrations. The procedure was repeated on the same day and between five different days on the same spiked standard series. The inter- and intra-day precisions were expressed as the relative standard deviation (R.S.D.%). As shown in Table 1, the precisions (R.S.D.%) and accuracy percent error were all within 10%. The data indicated that the precision and the accuracy of the method are acceptable.

Table 2
Extraction recoveries of danshensu, rosmarinic acid, dihydrotanshinone I, cryptotanshinone, tanshinone I, tanshinone IIA in rat plasma.

Analyte	Added (ng/mL)	Recovery (mean ± S.D.)	R.S.D. (%)
Danshensu	10	57.28 ± 3.39	5.92
	100	67.11 ± 3.36	5.14
	2500	70.58 ± 6.32	9.01
Rosmarinic acid	3.75	76.32 ± 4.04	5.24
	37.5	81.81 ± 3.82	4.73
	375	87.65 ± 2.51	2.89
Dihydrotanshinone I	1	83.66 ± 2.69	3.26
	10	84.02 ± 1.75	2.18
	250	91.16 ± 3.25	3.59
Cryptotanshinone	0.5	76.42 ± 4.15	5.46
	5	83.26 ± 2.17	2.65
	50	78.56 ± 5.31	6.72
Tanshinone I	1	81.26 ± 1.88	2.32
	10	73.83 ± 2.41	3.21
	250	77.48 ± 1.85	2.40
Tanshinone IIA	0.5	82.12 ± 3.46	4.15
	5	88.84 ± 5.01	5.57
	50	88.52 ± 1.45	1.70

3.5. Recovery

The extraction recovery was determined for five replicates of rat plasma spiked with low, medium and high concentrations of DS, RA, CT, TI, DT and TS, respectively. The results are summarized in Table 2. The mean recoveries of the samples were more than 60%. The data indicated that the recoveries of the analytes and I.S. from the rat plasma were concentration-independent in the concentration range evaluated and the recoveries were acceptable for the pharmacokinetic analysis.

3.6. Stability

The stability of stock and standard solution kept at 20 °C and frozen (−20 °C) plasma samples, as well as frozen plasma extracts, was checked. Plasma QC samples were: (1) allowed to stand at ambient temperature for at least 24 h before extraction; (2) allowed to stand at ambient temperature for at least 24 h after extraction; and (3) subjected to three freeze–thaw cycles for at least five days. Analysis of these samples consistently produced values that were nearly identical to those from freshly prepared QC samples, confirming the overall stabilities of DS, RA, DT, CT, TI and TS in plasma under frozen storage, assay processing and freeze–thaw conditions.

3.7. Pharmacokinetics of the six active compounds from danshen extracts in rats

This LC–MS/MS method demonstrated satisfactory effects for the in vivo separation and simultaneous determination of danshensu, rosmarinic acid, cryptotanshinone, tanshinone I, dihydrotanshinone I and tanshinone IIA in rat plasma and was successfully utilized for studying the pharmacokinetics of these components from danshen extracts following a single oral administration to rats. The plasma concentration–time profiles for the six main active components (DS, RA, CT, TI, DT and TS) from danshen

Table 3
The main pharmacokinetic parameters describing disposition danshensu, rosmarinic acid, cryptotanshinone, dihydrotanshinone I, tanshinone I and tanshinone IIA in rats after a single oral dose of danshen extracts (mean ± S.D.).

Parameter	Danshensu	Rosmarinic acid	Cryptotanshinone	Dihydrotanshinone I	Tanshinone I	Tanshinone IIA
$t_{1/2\ Ka}$(h)	0.51 ± 0.13	0.35 ± 0.09	0.37 ± 0.21	0.28 ± 0.13	0.17 ± 0.11	0.24 ± 0.04
$t_{1/2\alpha}$(h)	0.89 ± 0.16	0.54 ± 0.11	0.69 ± 0.18	0.54 ± 0.14	0.94 ± 0.36	0.40 ± 0.04
$t_{1/2\beta}$(h)	3.04 ± 1.28	3.68 ± 0.41	2.81 ± 0.39	3.65 ± 1.74	4.72 ± 1.90	3.70 ± 0.65
K_{21}(1/h)	0.33 ± 0.19	0.43 ± 0.31	0.42 ± 0.11	0.54 ± 0.24	0.32 ± 0.11	0.68 ± 0.08
K_{10}(1/h)	0.66 ± 0.07	0.92 ± 0.07	0.62 ± 0.086	0.56 ± 0.09	0.50 ± 0.08	0.52 ± 0.09
K_{12}(1/h)	0.10 ± 0.08	0.38 ± 0.20	0.26 ± 0.12	0.46 ± 0.18	0.26 ± 0.34	0.81 ± 0.09
$V_{(c)}$ (L/kg)	0.07 ± 0.02	0.18 ± 0.07	0.13 ± 0.02	0.33 ± 0.17	0.06 ± 0.010	0.24 ± 0.02
AUC (ng h/mL)	220.67 ± 52.54	89.12 ± 12.10	123.40 ± 8.04	34.82 ± 22.9	169.64 ± 32.22	81.05 ± 12.08
$Cl_{(s)}$ (L (kg/h))	0.05 ± 0.01	0.12 ± 0.04	0.08 ± 0.01	0.18 ± 0.10	0.03 ± 0.002	0.14 ± 0.01
T_{max} (h)	1.11 ± 0.18	0.74 ± 0.12	0.86 ± 0.35	0.74 ± 0.14	0.60 ± 0.21	0.61 ± 0.05
C_{max} (ng/mL)	71.98 ± 22.86	37.19 ± 13.85	42.85 ± 13.58	11.29 ± 11.49	54.64 ± 17.72	22.24 ± 3.42

extracts to rats are shown in Fig. 3 and the main pharmacokinetic parameters of the six active compounds in rats are presented in Table 3. The plasma drug concentration–time data of DS, RA, CT, TI, DT and TS after a single oral administration of danshen preparation to rats were all best fitted to a two-compartment open model. There were significant differences in the absorption, distribution and elimination of the main pharmacokinetic parameters among DS, RA, CT, TI, DT and TS. The results indicated that the absorptions, distribution and elimination of all the six active compounds in rats were relatively moderate. It was found that CT could be further biotransformed to TS in animals [19] and the concentration of TS in rat plasma after the administration of danshen extracts was an accumulation of the parent drug TS and the metabolite of CT. Therefore, the pharmacokinetics of TS from danshen extracts was complex and need to be investigated and described further.

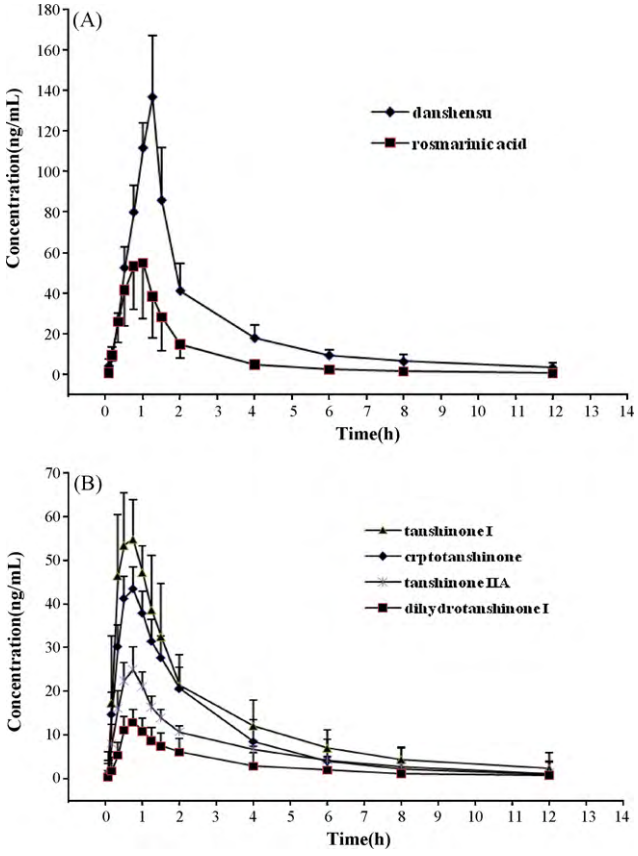

Fig. 3. Pharmacokinetics profiles of: (A) danshensu and rosmarinic acid; (B) cryptotanshinone, tanshinone I, dihydrotanshinone I and tanshinone IIA in plasma following administration of a single oral dose of danshen extracts to rats.

In the current report, the in vivo simultaneously quantitative analysis and the pharmacokinetic studies of two water-soluble phenolic acid compounds and four lipophilic diterpenes compounds are investigated. The hydrophilic phenolic acids and lipophilic diterpenes compounds are two kinds of important and representative constituents in danshen. Up to now, there are some studies investigating either the main lipophilic diterpenes components or the hydrophilic phenolic compounds, while the simultaneously quantitative and pharmacokinetics studies of the two kinds of the compounds were rare. Therefore, our data in this report make significant contribution to the fully understanding of the comprehensive pharmacokinetics profiles of danshen preparations.

4. Conclusion

A LC–ESI–MS/MS method has been developed for the simultaneous determination of danshensu, rosmarinic acid, cryptotanshinone, tanshinone I, dihydrotanshinone I and tanshinone IIA from danshen preparations in rat plasma. The performance criteria for specificity, precision, accuracy, recovery, sensitivity, linearity and stability have been assessed and were within the FDA recommended guidelines. The results indicated that this method could be successfully used for simultaneous determination of the six main active components from danshen extracts. It was successfully applied to the pharmacokinetic studies of these active compounds after oral administration to rats.

Acknowledgements

This work is supported by grant from the National Foundation of Natural Sciences of China (no. 30472057), Beijing Natural Science Foundation Program (no. 7052007) and Funding Project for Academic Human Resources Development in Institutions of High Learning Under the Jurisdicrion of Beijing Municipality (PHR201007111).

References

[1] W.Z. Chen, Acta Pharmacol. Sin. 19 (1984) 876–880.
[2] H. Wang, X.M. Gao, B.L. Zhang, J. Ethnopharmacol. 99 (2005) 93–98.
[3] A.M. Elsharkawy, F. Oakley, D.A. Mann, Apoptosis 10 (2005) 927–939.
[4] Y.G. Gao, Y.M. Song, Y.Y. Yang, J.X. Tang, W.F. Liu, Acta Pharmacol. Sin. 14 (1979) 75–81.
[5] R. Lin, W.R. Wang, J.T. Liu, G.D. Yang, C.J. Han, J. Ethnopharmacol. 108 (2006) 217–222.
[6] J.K. Jiang, C.G. Ruan, Z.L. Gu, W.X. Zh, C.Y. Guo, Acta Pharmacol. Sin. 19 (1998) 47–50.
[7] Y.G. Li, L. Song, M. Liu, Z.B. Hu, Z.T. Wang, J. Chromatogr. A 1216 (2009) 1941–1953.
[8] H.M. Chang, K.Y. Chui, F.W. Tan, Y. Yang, Z.P. Zhong, C.M. Lee, H.L. Sham, H.N. Wong, J. Med. Chem. 34 (1991) 1675–1692.
[9] M. Gu, G.F. Zhang, Z.G. Su, F. Ouyang, J. Chromatogr. A 1041 (2004) 239–243.

[10] Z.C. Zhang, M. Xu, S.F. Sun, X. Qiao, B.R. Wang, J. Han, D.A. Guo, J. Chromatogr. B 871 (2008) 7–14.
[11] H.P. Hao, G.J. Wang, P. Li, J. Pharm. Biomed. Anal. 40 (2006) 382–388.
[12] J.L. Wu, L.P. Yee, Z.H. Jiang, Z.W. Cai, Talanta 73 (2007) 656–661.
[13] A.H. Liu, H. Guo, M. Ye, Y.H. Lin, J.H. Sun, M. Xu, D.A. Guo, J. Chromatogr. A 1161 (2007) 170–182.
[14] Z.H. Shi, J.T. He, T.T. Yao, W.B. Chang, M.P. Zhao, J. Pharm. Biomed. Anal. 37 (2005) 481–486.
[15] E.J. Park, H.Y. Ji, N.J. Kim, W.Y. Song, Y.H. Kim, Y.-C. Kim, D.H. Sohn, H.S. Lee, Biomed. Chromatogr. 22 (2008) 548–555.
[16] X.C. Li, Y. Chen, Y.B. Cai, G.Y. Liu, J.Y. Jia, Y.P. Wang, J. Chromatogr. B 820 (2005) 41–47.
[17] X.L. Li, X.R. Li, L.J. Wang, Y.H. Li, Y.X. Xu, M. Xue, J. Pharm. Biomed. Anal. 44 (2007) 1106–1112.
[18] X.L. Wang, Z.R. Zhang, H.L. Fu, J. Liu, Q. Chen, Y. Nie, L. Deng, T. Gong, Biomed. Chromatogr. 21 (2007) 1180–1185.
[19] M. Xue, Y. Cui, H.Q. Wang, Y.J. Luo, B. Zhang, J. Pharm. Biomed. Anal. 21 (1999) 207–212.