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Simultaneous quantification of cryptotanshinone and its active metabolite tanshinone IIA in plasma by liquid chromatography/tandem mass spectrometry (LC–MS/MS)

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Abstract

A rapid and sensitive method for the simultaneous determination of cryptotanshinone and its active metabolite tanshinone IIA in rat plasma was developed and well validated, using high-performance liquid chromatographic separation with tandem mass spectrometric detection. This method entailed a single step of liquid–liquid extraction with ethyl acetate from a small volume of plasmas. The analytes and internal standard diazepam were baseline separated on a Shim-pack VP-ODS analytical column. Detection was performed on a triple quadrupole tandem mass spectrometer equipped with electrospray ionization source operated under selected reaction monitoring (SRM) mode. The method was linear in the concentration range of 1–100 ng/ml for both tanshinone IIA and cryptotanshinone. The intra- and inter-day precisions (R.S.D.%) were within 10.2% for both analytes. Deviation of the assay accuracies was within ±12.0% for both analytes. Both analytes were proved to be stable during all sample storing, preparation and analytic procedures. The method was successfully applied to a pharmacokinetic study after an oral administration of cryptotanshinone to rats with a dose of 20 mg/kg. With the lower limits of quantification at 1.0 ng/ml for tanshinone IIA and 0.2 ng/ml for cryptotanshinone, this method was proved to be sensitive enough and reproducible for the pharmacokinetics study of both tanshinones.

Keywords: Tanshinone IIA; Cryptotanshinone; Simultaneous quantification; LC–MS/MS; Pharmacokinetics

1. Introduction

Tanshinone IIA and cryptotanshinone are the derivatives of phenanthrene–quinone isolated from *Salvia miltiorrhiza* (Danshen, in Chinese), a widely used Chinese herbal medicine. They have attracted much attention from researchers in China and worldwide due to their powerful and wide pharmacological activities. In particular, tanshinone IIA had been previously observed to posses various kinds of pharmacological activities including antioxidant [1], prevention of angina pectoris and myocardial infarction [2], antineoplastic [3], anticancer [4,5], and anti-inflammation. Cryptotanshinone possesses the most powerful antibacterial activity among the tanshinones contained in *S. miltiorrhiza.* *C*ryptotanshinone had been found to be rapidly metabolized into tanshinone IIA to a great extent when administered to porcine, thus the similar effects as tanshinone IIA in vivo can be expected.

Considering its prominent pharmacological activities and high content in Danshen, tanshinone IIA was selected to be the marker compound for quality control of Danshen and any other medicinal preparations containing Danshen. The prerequisite for determining the content of tanshinone IIA or other tanshinones is to develop and validate the suitable separation and detection methods. In the previous decade, lots of methods including high performance liquid chromatography separation coupled with ultraviolet detection (HPLC-UV) [6], non-aqueous micellar electrokinetic chromatography [7], nonaqueous capillary electrophoresis [8], and supercritical fluid extraction (SFE) coupled with capillary gas chromatography (CGC), had been developed for the quantitative detec-

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tion of tanshionone IIA alone or simultaneous detection of multiple tanshinones in the plants or medicinal preparations. All of these methods can no doubt satisfy the need of quantitative determining the tanshinones contained in the plants or medical preparations.

However, few methods had been developed and validated for quantitative determining the tanshinones in biological samples such as plasma, urine or tissue homogenates. Up to now, only two methods, both of which based on HPLC-UV detection, have been developed for the quantitative determination of tanshionone IIA [9], and simultaneous determination of cryptotanshinone and tanshionone IIA [10], respectively. Although both methods were precisely and accurately enough for quantitative determining the tanshinones in biological samples, the sensitivity with a lower limit of detection (LLOD) of 50 ng/ml could not satisfy the requirements of the pharmacokinetic research. Both cryptotanshinone and tanshionone IIA underwent a rapid and extensive distribution due to their extreme hydrophobicity, and also a rapid elimination with a half life shorter than 180 min, resulting in a plasma concentration lower than 50 ng/ml 3 h after an intravenous injection of cryptotanshinone 500 mg [10]. Obviously, more sensitive analytic method is demanded for the pharmacokinetics research of tanshinones.

We report here a much more sensitive method based on the LC separation coupled with tandem mass detection for the simultaneous quantitative determination of tanshinone IIA and cryptotanshinone in rat plasma. This method has been fully validated for its specificity, accuracy, precision and sensitivity, and has been successfully applied to the pharmacokinetics study of cryptotanshinone and its active metabolite tanshinone IIA followed a single oral dose of cryptotanshinone (20 mg/kg).

2. Experimental

2.1. Chemicals and reagents

Tanshinone IIA (MW = 294), cryptoshinone (MW = 296) and diazepam (internal standard, IS, MW = 284) were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). The purity of all chemicals was above 99% and their chemical structures were shown in Fig. 1. HPLC grade acetonitrile was obtained from Fisher Scientific (Toronto, Canada). Deionized water was purified using a Milli-Q system (Millipore, Milford, MA, USA). Methanol, ethyl acetate and other chemicals and solvents used were of analytical grade.

2.2. Instrument and analytical conditions

LC experiments were conducted using a Finnigan Surveyor^{TM} HPLC system (Thermo Electron, San Jose, CA, USA). Separations of analytes were achieved using a 150 mm × 2.0 mm Shim-pack VP-ODS analytical column

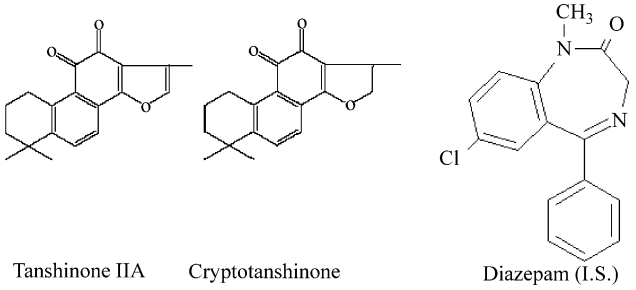

Fig. 1. Chemical structure of tanshinone IIA, cryptotanshinone and diazepam (IS).

(Shimadzu, Kyoto, Japan) protected by a Security guard (Phenomenex, Inc.). The column and autosampler tray temperature were set at 40 and 4 °C, respectively. A mobile phase composed of acetonitrile–0.05% ammonia (80:20, v/v) at a flow rate of 0.2 ml/min was used to obtain the baseline separation of all analytes. The mass detection was conducted using a Finnigan TSQ Quantum Discovery max system (Thermo Electron, San Jose, CA, USA) equipped with an electrospray ionization source. Data acquisition was performed with Xcalibur 1.2 software (Thermo Finnigan, USA). Peak integration and calibration were performed using LCQuan software (Thermo Finnigan, USA). Mass spectrometer was operated in positive ion mode. In order to find the most abundant ion, tanshinone IIA, cryptotanshinone and the internal standard diazepam were separately scanned under the Q1 MS fullscan mode to determine the parent ion, and under the Q1/Q3 (MS/MS) product ion scan mode to locate the most abundant product ion. The scan results were shown in Fig. 2. Quantification was thus performed using selected reaction monitoring (SRM) of the transitions of m/z 295 → 277 for tanshinone IIA, m/z 297 → 251 for cryptotanshinone, and m/z 285 → 193 for the diazepam (IS), respectively, with a scan time of 0.2 s per transition. The tuning parameters were optimized for tanshinone IIA, cryptotanshionone and diazepam by direct infusing a solution containing 1 μg/ml of each analytes at a flow rate of 20 μl/min into the mobile phase (0.2 ml/min) using a flow injection system. The optimized MS parameters obtained were shown in Tables 1 and 2.

2.3. Preparation of standard and quality control samples

Primary standard stock solutions of tanshinone IIA and cryptotanshinone were prepared separately by dissolving

Table 1
Optimized parameters of mass spectrometry

Parameters	Value
Spray voltage	5.0 kV
Sheath/auxiliary gas	Nitrogen
Sheath gas pressure	49 (arbitrary units)
Auxiliary gas pressure	10 (arbitrary units)
Ion transfer capillary temperature	398 °C
CID conditions	Argon at 1.0 mTorr

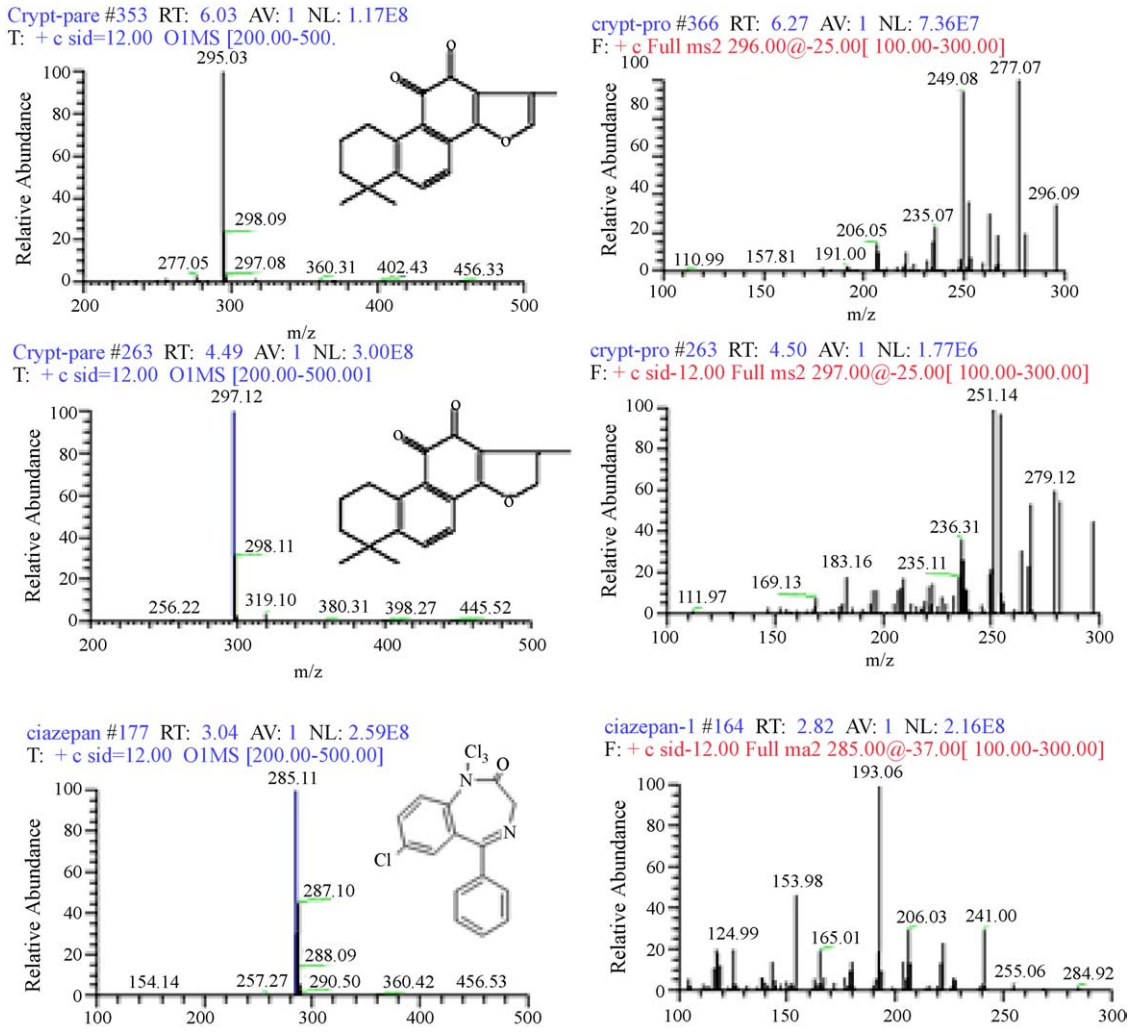

Fig. 2. Mass spectrum of tanshinone IIA, cryptotanshinone and IS obtained from full scan. Showing the protonated ions for both analytes and IS; and the corresponding abundant products ions.

10 mg, accurately weighed, in 10 ml methanol, and stored in a glass vial at 4 °C. A series of standard working solutions of their mixtures at appropriate concentrations were obtained by mixing and further diluting of the standard stock solutions with methanol. The standard stock solution of the internal standard with a concentration of 1 mg/ml was prepared in methanol same as that for the analytes. Internal standard working solution (100 ng/ml) was prepared by diluting internal standard stock solution with the methanol. To prepare the calibration samples, 10 μl of the working solutions were diluted each day with 100 μl blank rat plasma to span a calibration standard range of 1–100 ng/ml. The final concentration of the internal standard was 10 ng/ml in all samples. Quality control (QC) samples (1, 10, 100 ng/ml for tanshinone IIA and 0.2, 1, 10, 100 ng/ml for cryptotanshinone) were prepared in a similar way.

Table 2
Collision energy for the analytes and internal standard

Analytes	SRM transitions	Collision energy	Scan time (s)
Tanshinone IIA	295 → 277	24	0.2
Cryptotanshinone	297 → 251	21	0.2
Diazepam	285 → 193	37	0.2

2.4. Sample preparation

Prior to extraction, frozen plasma samples were thawed at ambient temperature. A 100 μl aliquot of plasma was added to a disposable Eppendorf tube followed by spiking with the standard working solutions and into which 10 μl of the internal standard working solution was added. This mixture was then vortexed for 30 s using a vortex mixer (Scientific industries, Inc., USA). A single step of liquid–liquid extraction was adopted to extract the analytes from plasmas. To each tube, 400 μl of ethyl acetate was added followed by vortexing vigorously for 2 min on an automated multi-tube shaker. The well vortexed solutions were then centrifuged at 2000 × *g* for

10 min and 300 μl of the upper organic layer was transferred to a new disposable Eppendorf tube and evaporated to dry in the Thermo Savant SPD 2010 SpeedVac System (Thermo Electron Corporation, USA) set at 40 °C. The residues were then reconstituted in 200 μl acetonitrile followed by centrifugation at 10,000 × *g* for 10 min before LC–MS/MS analysis. The supernatant was transferred to a polypropylene autosampler vial and a volume of 10 μl was injected.

2.5. Method validation

The method was fully validated for its specificity, linearity, lower limits of quantification (LLOQ), accuracy and precision. To evaluate assay specificity, six independent lots of rat blank plasma were analyzed for excluding any endogenous co-eluting interference by comparing them with the assay of plasma spiked with analytes. Plasma calibration curves over a linear range from 1 to 100 ng/ml at seven concentrations were prepared and assayed in duplicate on three different days to demonstrate the linearity of this method. The lines of best fit for calibration standards were determined using linear least-squares regression analysis based on the peak area ratios of the analytes to IS.

The lower limits of quantification (LLOQ), defined as the lowest concentration at which both precision and accuracy were less than or equal to 20%, were evaluated by analyzing the six replicates of plasma samples spiked with analytes at a final concentration of 1.0 ng/ml for tanshinone IIA and 0.2 ng/ml for cryptotanshinone at which the signal to noise ratio (S/N) was preliminary found to be larger than 10.

To evaluate accuracy and precision, three concentration levels of QC samples (1, 10, and 100 ng/ml) were prepared and analyzed on the same day, along with an independent standard curve for quantification. Six QC replicates were prepared at each concentration for intra- and inter-day validations, respectively. The accuracy was expressed as the percent of the determined concentration to the nominal concentration. The precision was evaluated as the relative standard deviation of the mean expressed as percent (R.S.D.) for each sample.

For determining the absolute recovery and matrix effect, triplicates of extracted and unextracted QC samples and a set of post-extraction spiked QC samples were analyzed in the same assay run. The recovery was determined by measuring an extracted sample against a post-extraction spiked sample and expressed as the ratio of the peak responses. The matrix effect was measured by comparing the peak response of the post-extracted spiked sample with that of the unextracted sample (pure sample prepared in methanol).

The stability of tanshinone IIA and cryptotanshinone during the sample storing and processing procedures was fully evaluated by analyzing triplicate replicates of QC samples at the concentration of 1, 10 and 100 ng/ml for both analytes. The obtained results were compared with the nominal concentration of the analytes added to justify whether the analytes were stable or not after different treatments. A compound was considered unstable if the calculated concentration was less than the nominal concentration by more than 15%.

2.6. Application to pharmacokinetics study of tanshinone IIA

Sprague-Dawley rats (190–210 g) were obtained from Sino-British Sippr/BK Lab Animal Ltd. (Shanghai, China) and housed with free access to food and water. The animals were maintained on a 12 h light–dark cycle (light on from 8:00 to 20:00 h) at ambient temperature (22–24 °C) and ca. 60% relative humidity. The rats were fasted for 12 h before the pharmacokinetics study. The rats were orally administered with the cryptotanshinone dispersion (20 mg/kg, which was the dose frequently used in the pharmacological study). About 0.2 ml of blood samples via the right jugular vein were collected into a preheparinized tube at pre-dosing, and at 0.08, 0.25, 0.5, 1, 2, 3, 4, 6, 8, 12 and 24 h post-dosing. Blood samples were centrifuged immediately to separate plasma 100 μl, which were stored at −20 °C until analysis.

3. Results and discussion

3.1. Chromatography and mass spectrometry conditions

Under the current chromatographic conditions, all analytes were rapidly eluted within 6.0 min. Although the analytic time in a single run could be shortened to 5 min when the content of the organic phase, acetonitrile, was further enhanced to 85%, the sensitivities of both tanshinone IIA and cryptotanshinone were significantly decreased. Addition of 0.05% ammonia to the mobile phase was found to be an important factor for acquiring the high sensitivity. Similar phenomenon was found in the LC–MS system. It was somewhat controversial to the current understanding in which it was believed that addition of the voltaic acid such as formic acid and acetate acid could enhance the sensitivity when the ion adduct $[M + H^+]$ was detected. However, reverse result had been obtained in the present research. Both formic acid and acetate acid addition caused a significant sensitivity decrease. The underlying causes remained unexplained though, the present finding indicated that for some compounds, addition of ammonia helped to form the protonated molecule ion $[M + H^+]$ resulting an increased sensitivity.

The mass spectrums of tanshionone IIA, cryptotanshinone, and the internal standard diazepam exhibited a protonated molecular ion at *m*/*z* 295, 297 and 285, respectively. The high collision energy gave the most abundant product ion at *m*/*z* 277, 251 and 193 for tanshinone IIA, cryptotanshinone and diazepam, respectively. Therefore, the precursor to product transition was assigned in the selected reaction monitor mode as follows: *m*/*z* 295 → 277 for tanshinone IIA, *m*/*z* 297 → 251 for cryptotanshinone, and *m*/*z* 285 → 193 for diazepam. In order to obtain the highest sensitivity, the mass spectrometry parameters were optimized by the auto-tuning.

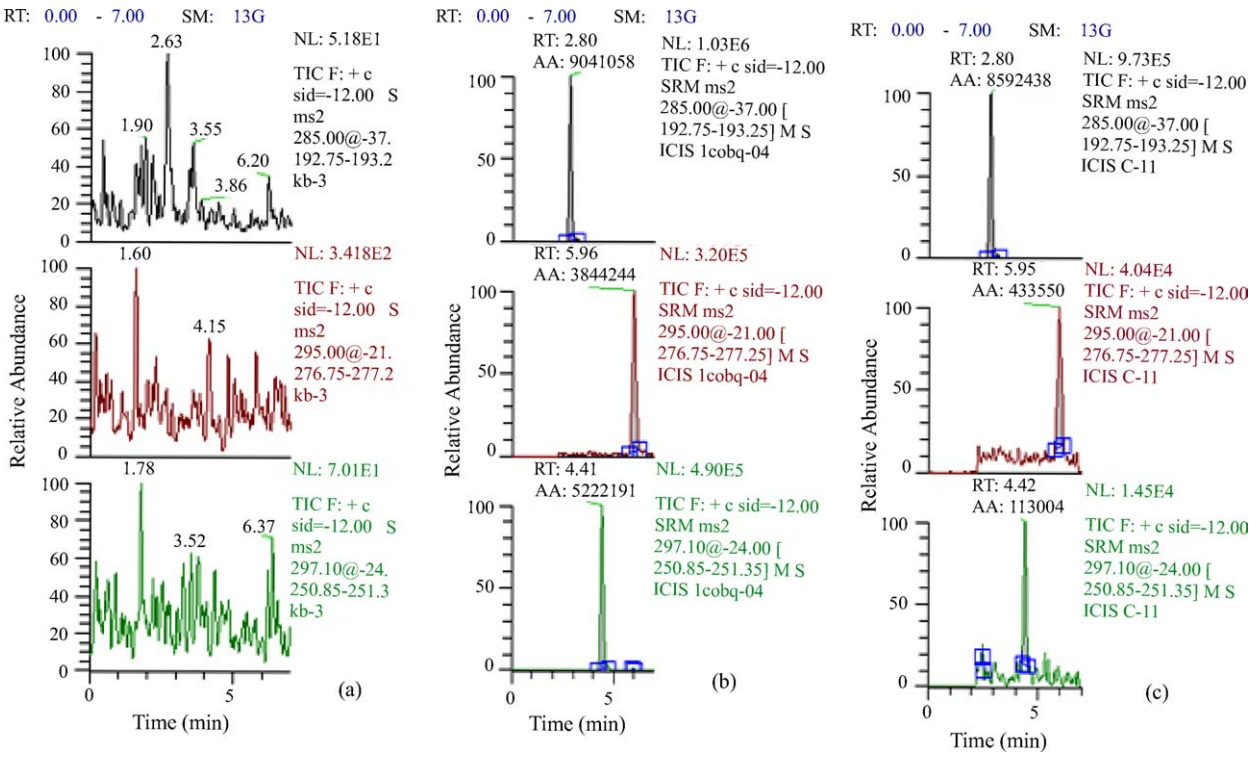

Fig. 3. Representative SRM chromatograms of tanshinone IIA, cryptotanshinone and the internal standard diazepam. (a) A blank rat plasma; (b) rat plasma spiked with both analytes and IS at a concentration of 10 ng/ml; (c) a plasma sample 24 h after oral-dosing of 20 mg/kg cryptotanshinone.

Obtained optimal parameters as shown in Tables 1 and 2 were saved in a tuning method which was used in all the following analysis.

3.2. Method validation

3.2.1. Selectivity

Under the current optimized HPLC and MS/MS conditions, tanshinone IIA, cryptotanshinone and the internal standard were baseline separated chromatographically with the retention times of 5.9 ± 0.06, 4.4 ± 0.05 and 2.8 ± 0.05 min, respectively. Blank rat plasmas from six lots were allowed to run a long time up to 30 min to observe any possible late-eluting interfering peaks. Due to high selectivity of the selected reaction mode, no any interference had been observed up to 30 min; therefore, regeneration of the column using a gradient elution step or prolongation of the analytical time was not necessary. Fig. 3 shows the representative chromatograms of the blank rat plasma, plasma sample spiked with tanshinone IIA, cryptotanshinone and internal standard, and the plasma sample obtained at 24 h post-dosing of cryptotanshinone, supporting the high selectivity of this method.

3.2.2. Linearity of calibration curves and lower limits of quantification

The calibration curves were prepared over a linear range from 1 to 100 ng/ml of both analytes at seven concentrations, 1, 2, 5, 10, 20, 50 and 100 ng/ml. Good linearity with a correlation coefficient r^2 exceeding 0.995 was observed for both analytes. The representative regression equation was $y = 0.032x - 0.0175$ for tanshinone IIA, and $y = 0.1316x - 0.3275$ for cryptotanshinone, respectively where y indicates the ratios of analytes to internal standard and x indicates the plasma concentrations. The slopes of the regression equations were consistent for the calibration curves prepared in three separate days. Although the calibration curves were prepared and validated in a relatively narrow concentration range in this method, it can be easily extended to explore a much broader range of concentrations. Actually, good linearity and acceptable accuracy and precision had been obtained for a broad range of concentrations from 1 to 1000 ng/ml observation when the weighed ($1/x^2$) linear regression or segment regression strategy (1–100 and 10–1000 ng/ml) was used to create the regression equations.

The lower limits of quantification under the optimized conditions were judged as 1.0 ng/ml for tanshinone IIA, and 0.2 ng/ml for cryptotanshinone from the fact that the signal to noise ratios were well larger than 10 and the relative standard deviation (R.S.D.%) of six replicates were 8.5 and 11.2, respectively, well less than 20%.

3.2.3. Recovery and matrix effect

The extraction recoveries of tanshinone IIA and cryptotanshinone from spiked rat plasma were determined at the concentrations of 1, 10, 100 ng/ml in triplicates. A single step of liquid–liquid extraction with ethyl acetate was proved

Table 3
Recoveries of tanshinone IIA and cryptotanshinone at three different concentrations

Concentration (ng/ml)	Recovery (%, mean ± S.D., $n = 3$)	
	Tanshinone IIA	Cryptotanshinone
1	86.3 ± 5.6	84.8 ± 3.5
10	90.5 ± 6.9	89.2 ± 6.6
100	88.2 ± 7.8	91.5 ± 7.3

to be simple, rapid and successful with an average recovery ratio over 85% for both analytes at all tested concentrations. The results in detail were shown in Table 3. The extraction recovery of the internal standard was determined to be 85.6% at the spiked concentrations (10 ng/ml). The possibility of a matrix effect caused by ionization competition occurred between the analytes and the endogenous co-eluents was evaluated at three concentrations as mentioned above in triplicates. Results from comparing the peak responses of the post-extraction spiked samples with that of the pure standards prepared in methanol suggested negligible matrix effect occurred in this method. For all the tested concentrations, the ratios of the peak response were within the acceptable ranges (95.6–105.9%).

3.2.4. Accuracy and precision

QC samples at three concentrations were analyzed in six replicates for determining the accuracy and precision of this method. The results were shown in Table 4. The intra-batch accuracy for tanshinone IIA and cryptotanshinone ranged from 105.4 to 109.0% at the tested concentrations with the precision (R.S.D.) between 4.3 and 8.5%. The inter-batch accuracy for tanshinone IIA and cryptotanshinone ranged from 104.7 to 112.0% at three different concentrations with the precision (R.S.D.) between 4.5 and 10.2%. These results indicated that the present method has a satisfactory accuracy, precision and reproducibility.

3.2.5. Stability

For both tanshinone IIA and cryptotanshinone, no significant degradation (the losses were within 8%) had been found during all of the sample storage, preparation and analytic periods. Results of the stability evaluation were shown in detail in Table 5.

3.2.6. Pharmacokinetic study

The well-validated method was successfully applied to determine the plasma concentrations of cryptotanshinone and its main active metabolite tanshinone IIA in rats following a single oral administration of cryptotanshinone (20 mg/kg). The SRM chromatograms of plasma obtained from pre- and post-dosed rats showed that no significant interfering peak was detected at the retention times of cryptotanshinone, tanshinone IIA and the internal standard, indicating the method was specific enough for their pharmacokinetics study. The time–plasma concentration profile of cryptotanshinone and tanshinone IIA was depicted in Fig. 4. The plasma concentration maximum (C_{max}) of cryptotanshinone was only 25.1 ± 6.5 ng/ml (six rats) and decreased to be about 1.1 ng/ml at 24 h post-dosing. The main active metabolite tanshinone IIA was detected at the first sample point, 5 min post-dosing, with the average concentration of 3.2 ng/ml (57% of corresponding cryptotanshinone), indicating that the

Table 4
Accuracy and precision for the analysis of tanshinone IIA and cryptotanshinone

Analyte	Statistical variable	Intra-day (ng/ml)			Inter-day (ng/ml)		
		1	10	100	1	10	100
Tanshinone IIA	Mean (ng/ml)	1.09	10.75	105.35	1.12	10.96	108.74
	RSD (%)	8.5	5.6	6.7	10.2	4.5	7.5
	Accuracy (%)	109.0	107.5	105.4	112.0	109.6	108.0
Cryptotanshinone	Mean (ng/ml)	1.07	10.63	107.68	1.08	10.47	110.56
	RSD (%)	5.6	4.3	6.9	7.8	6.3	9.7
	Accuracy (%)	107.0	106.3	107.7	108.0	104.7	110.6

Table 5
Stability test of tanshinone IIA and cryptotanshinone during the storing and preparation procedures

Analytes	Nominal concentration (ng/ml)	Observed at time zero	Remaining (%)		
			Freeze–thaw (three cycles)	Plasma samples at room temperature for 24 h	Autosampler tray at 4 °C for 24 h
Tanshinone IIA	1	1.08	95.6	99.7	101.5
	10	10.56	98.5	94.6	97.2
	100	108.35	96.5	92.1	95.8
Cryptotanshinone	1	1.08	96.4	97.9	92.9
	10	11.2	92.6	98.3	95.1
	100	106.5	97.5	95.7	103.5

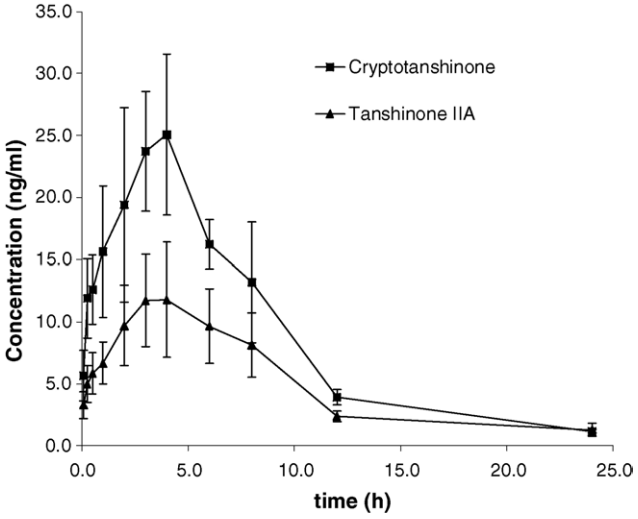

Fig. 4. Mean plasma concentration–time curves of cryptotanshinone and tanshinone IIA after a single oral administration of cryptotanshinone to rat with a dose of 20 mg/kg (mean ± S.D., $n = 6$).

metabolite formation of tanshinone IIA was rapid. Tanshinone IIA reach its maximal concentration, 11.8 ng/ml at 4 h post-dosing and decreased to be about 1.2 ng/ml at 24 h post-dosing. Obviously, the previously reported method based on HPLC-UV cannot satisfy the requirements of the pharmacokinetics study on cryptotanshinone followed oral administration, whereas the present method based on LC–MS/MS with a LLOQ of 0.2 and 1.0 ng/ml was sensitive enough for the pharmacokinetics research of cryptotanshinone and its main active metabolite tanshinone IIA.

4. Conclusion

A LC–MS/MS assay for the simultaneous quantitation of tanshinone IIA and cryptotanshinone plasma concentration was developed and fully validated. The new assay was proved to be rapid, sensitive, specific, accurate and reproducible. In addition, the assay required very small volume of plasma, which allows serial sampling in small laboratory animals. The new method was successfully applied to the characterization of pharmacokinetics of cryptotanshinone and its main active metabolite tanshionone IIA in rats after oral administration. In addition, this method has now been extended to determine cryptotanshinone and tanshinone IIA in other biological samples including cell and tissue homogenates, urine and feces.

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