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Simultaneous determination of tanshinone IIA and cryptotanshinone in rat plasma by liquid chromatography-electrospray ionisation-mass spectrometry

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Abstract

Tanshinone IIA (TS) and cryptotanshinone (CT) are the major active constituents contained in *Radix salvia miltiorrhiza*. This paper described a rapid, sensitive and specific assay for the simultaneous quantitative determination of TS and CT in rat plasma. After a single step of liquid–liquid extraction, plasma samples were analyzed by high performance liquid chromatography-electrospray ionization-mass spectrometry (HPLC-ESI-MS) using a reversed-phase C18 column (150 mm × 2.0 mm, 5 μm, Shim-pack VP-ODS column). The assay was linear in the concentration range of 2–200 ng/ml. The lower limits of quantification of TS and CT were 1 and 0.2 ng/ml, respectively. Recoveries of TS and CT were greater than 80%. The precisions and accuracies determined from 5 days were all within 12%. The assay was applied to a pharmacokinetic study in rats after an oral administration of total tanshinones with a dose of 150 mg/kg (containing 12% of TS and CT). Results showed that this simple and rapid method was sensitive enough to follow the plasma levels of TS and CT in rats, even though the concentration maximums of both were below 20 ng/ml after an oral administration of total tanshinones.

Keywords: Tanshinone IIA; Cryptotanshinone; Liquid chromatography–mass spectrometry; Pharmacokinetics

1. Introduction

Radix salvia miltiorrhiza, a commonly used herbal medicine in China, has a Chinese herbal name of Danshen. In virtue of its good performance and few side effects as confirmed in the long-time clinical use, Danshen was widely adopted in traditional Chinese medicinal preparations to remove blood stasis, clear away heat, relieve vexation, nourish blood, tranquilize mind, cool blood, relieve carbuncles, and treat hemorrhages, menstrual disorders and miscarriages [1,2]. Tanshinone IIA (TS) and cryptotanshinone (CT) are the main abietane-type diterpenes (tanshinones) contained in Danshen (chemical structures were shown in Fig. 1). Tanshinones have attracted particular attention from medicinal chemists and clinicians due to their diverse biological activities, such as antibacterial, antitumor, antithrombus, antiinflammatory and antiplatelet aggregation activities [3,4].

Many analysis methods including HPLC [5–7], thin layer chromatography (TLC)-fluorescence chromatography [8] and supercritical fluid extraction-capillary gas chromatography (SFE-CGC) [9] had been developed for TS determination. However, most of them were developed for the quantitative determination of TS in the plants or medical preparations, in which TS was taken as a marker component for quality control in the Chinese Pharmacopoeia [10]. Considering the poor sensitivity of these methods, they cannot be extended to bio-sample analysis. Recently, Xue et al. [11] established a reversed-phase liquid chromatography (LC) method for the quantitative determination of CT and TS in both porcine plasma and urine. Qiao et al. [12] developed a HPLC method for the determination of TS in rat plasma, and applied it to the pharmacokinetic study in rats. With a limit of detection (LOD) of 50 ng/ml, these methods obviously could not satisfy the requirements of pharmacokinetic study considering that TS was rapidly eliminated and extensively distributed leading to a plasma concentration well below the LOD. In addition, these methods involved time-consuming procedures using a relatively large volume of samples for extraction.

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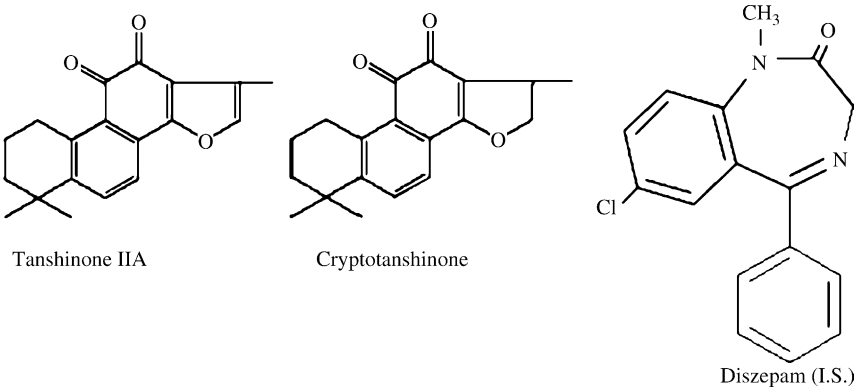

Fig. 1. Chemical structure of tanshionone IIA, cryptotanshinone and diazepam (I.S.).

A simple, rapid, sensitive and reliable method for the quantitative determination of tanshinones in biological samples was demanded for further investigations of their pharmacokinetics.

The aim of this study was to develop and validate an HPLC-ESI-MS analysis method for the simultaneous determination of TS and CT in biological samples. This method was successfully applied to the pharmacokinetic study of TS and CT in rat after an oral administration of total tanshinones with a dose of 150 mg/kg. Results showed this method was sensitive enough for the simultaneous determination of TS and CT in rat plasma up to 12-h post-dosing.

2. Experimental

2.1. Chemicals and reagents

Tanshinone IIA (mw = 294), cryptotanshinone (mw = 296), total tanshinones (containing 12% tanshinone IIA and 12% cryptotanshinone) and diazepam (internal standard (I.S.), mw = 284) were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). 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

The HPLC system consisted of a LC-10AD pump, a DGU-14AM degasser, a Shimadzu 10ATvp autosampler and a CTO-10 Avp column oven (Shimadzu, Kyoto, Japan). A Shimadzu 2010 liquid chromatograph-mass spectrometer (Shimadzu, Kyoto, Japan) equipped with an electrospray ionization (ESI) probe and a QoQ system (Q-array-Octapole-Quadrupole mass analyzer) was used in this study. A 150 mm × 2.0 mm Shim-pack VP-ODS analytical column (Shimadzu, Kyoto, Japan.) protected by a Securityguard (Phenomenex Inc.) was used. The column was maintained at 40 °C. A mobile phase composed of acetonitrile–0.05% ammonia (80:20, v/v) was used throughout the analysis at a flow rate of 0.2 ml/min. Under these conditions, CT, TS and I.S. eluted at approximately 4.5, 6.1 and 2.8 min, respectively (Fig. 2).

2.3. Mass spectrometric conditions

All measurements were carried out with the mass spectrometer operated under the positive ESI mode. Mass spectrometer conditions were optimized to obtain maximal sensitivity. The curve dissolution line (CDL) temperature was maintained at

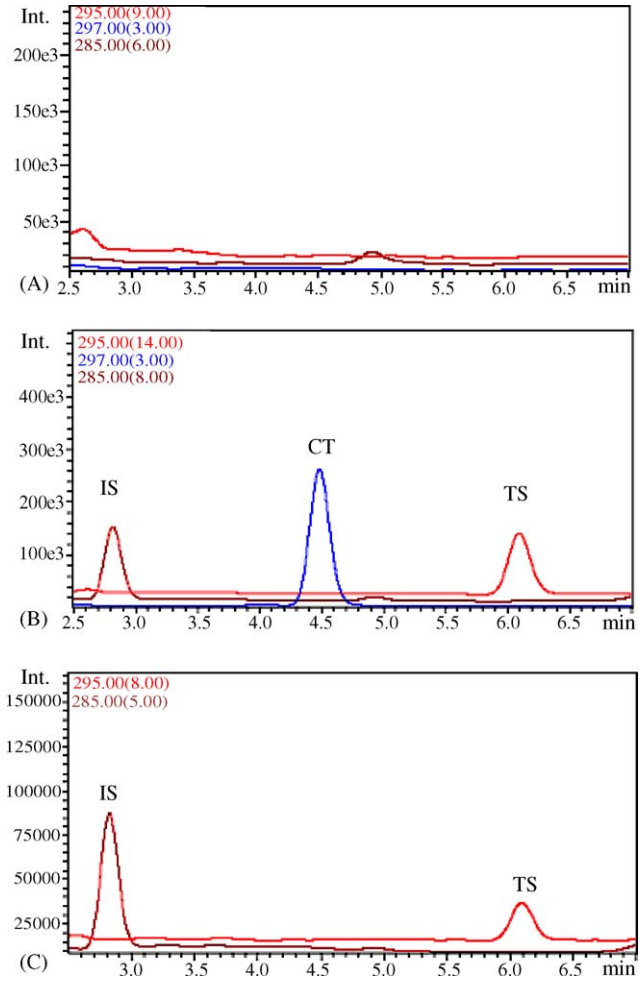

Fig. 2. Representative chromatograms obtained with the described method. (A) Drug-free plasma; (B) drug-free plasma with TS (20 ng/ml), CT (20 ng/ml) and I.S. (10 ng/ml) added; (C) plasma from a rat 90 min after intravenous administration of 20 mg/kg of TS.

250 °C and the block temperature was at 200 °C. The detector voltage 1.65 kV and probe voltage 4.5 kV were fixed as in tuning method. Mass vacuum was obtained by a Turbo molecular pump (Edwards 28, UK). Liquid nitrogen (99.995%, from Gas Supplier Center of Nanjing University, China) was used as the nebulizer gas (1.5 l/min) and curtain gas (2.0 l/min) source. Mass spectra were obtained at a dwell time of 0.2 s in SIM and 1 s in scan mode. LC/MS Solution Version 2.04 working on Windows 2000 operating system was used for data processing. All analytes were assayed by quantifying the $[M + H]^+$ ions, with TS detected at *m*/*z* 295, CT at *m*/*z* 297 and I.S. at *m*/*z* 285. The peak area ratios of analytes to I.S were calculated and the calibration curves were established by fitting these ratios to the corresponding nominal concentrations by a weighted (1/*x*) linear regression method.

2.4. Preparation of calibration samples and quality control samples

The standard stock solutions of 1 mg/ml of TS and CT were prepared in methanol. A series of standard working solutions, at concentration of 20, 50, 100, 200, 500 ng/ml, and 1 and 2 μg/ml, was obtained by further dilution of the standard stock solutions with methanol. The internal standard stock solution of 1 mg/ml was also prepared in methanol. Internal standard working solution (100 ng/ml) was prepared by diluting the stock solution with methanol. All solutions were stored at 4 °C. Appropriate amounts of working solutions were diluted with drug-free plasma to span a calibration standard range of 2–200 ng/ml (2, 5, 10, 20, 50, 100 and 200 ng/ml). Quality control (QC) samples (2, 20 and 200 ng/ml) were prepared in a similar way.

2.5. Sample preparation

Each collected blood sample was immediately centrifuged at 4000 rpm for 5 min and plasma was transferred into a clean Eppendorf tube. The plasma samples were stored at −20 °C until analysis. Aliquots (100 μl) of plasma were spiked with internal standard working solution (10 μl), vortex-mixed (Scientific industries Inc., USA) for 30 s and extracted with ethyl acetate (500 μl) using a vortex mixer for 2 min. Then the tubes were centrifuged at 10000 rpm for 5 min (Micromax RF, Thermo Electron Corporation, USA). The upper organic phase (400 μl) was transferred into the clean tubes and evaporated to dry using the Thermo Savant SPD 2010 SpeedVac System (Thermo Electron Corporation, USA). The residue was then reconstituted in 200 μl acetonitrile immediately before LC/MS analysis and 10 μl was injected.

2.6. Method validation

The method was fully validated for its specificity, linearity, lower limits of quantification (LLOQ), accuracy and precision. The assay precision was determined from inter- and intra-batch relative standard deviation (R.S.D.%), using five determinations per concentration (2, 20 and 200 ng/ml). The accuracy was determined by comparing the calculated concentration (obtained from the calibration curve) to the theoretical concentration of each sample and expressed as Bias%. Recovery experiments were performed by comparing the analytical results of extracted samples with unextracted standards at three concentrations. The lower limit of quantitation (LLOQ) was considered as the concentration that produced a signal-to-noise (S/N) ratio of 10. The stability was assessed at three concentrations (2, 20 and 200 ng/ml). The plasma samples at three concentrations for the freeze and thaw stability study were stored at −20 °C and subjected to three thaw and freeze cycles. During each cycle, triplicate 100 μl aliquots were processed and analyzed and the results were averaged. The post-processing stability of TS and CT at 4 °C, during storage in the autosampler, was performed by repeated injection every 4 h for a period of 24 h. The stability of TS and CT in plasma at ambient temperature was assessed by processing and analyzing plasma samples in triplicate after laboratory bench storage for 12 h.

2.7. Pharmacokinetic study

Sprague–Dawley rats (190–210 g) were obtained from Sino-British Sippr/BK Lab Animal Ltd. (Shanghai, China) and housed with unlimited access to food and water except for fasting 12 h before experiment. The rats were maintained on a 12 h light–dark cycle (light on from 8:00–20:00 h) at ambient temperature (22–24 °C) and ca. 60% relative humidity. After an oral administration of total tanshinones 150 mg/kg (containing 12% tanshinone IIA and 12% cryptotanshinone), 0.2 ml of blood samples via the right jugular vein were collected at 0.08, 0.25, 0.5, 1, 1.5, 2, 3, 4, 5, 6, 9 and 12 h. Blood samples were centrifuged immediately to separate plasma 100 μl, which were stored at −20 °C until analysis.

3. Results and discussion

3.1. Linearity and calibration standard range

The peak area ratios of TS and CT to I.S. in rat plasma varied linearly with concentrations over the range tested (2–200 ng/ml). Best fit for the calibration curve could be achieved by a linear equation of $y = ax + b$ with $1/x$ weighting factor (TS: $y = 0.0198x - 0.0177$; CT: $y = 0.096x - 0.088$) (where y is the peak area ratio and x the concentration). The correlation coefficients (R^2) for TS and CT were 0.998 and 0.999, respectively.

3.2. Specificity and sensitivity

LC/MS analysis of the blank plasma samples showed no endogenous peak interference with TS, CT and I.S. at their retention times. Representative chromatograms were shown in Fig. 2. At the first 2.5 min, the eluent flowed to the waste with a column switch valve, which can protect the MS system from contamination. The LLOQ was determined to be 1 and 0.2 ng/ml for TS and CT, respectively. Addition of 0.05% ammonia to the mobile phase was found to be important for acquiring the high sensitivity. It was somewhat controversial to the current under-

Table 1
Precision (R.S.D.%) and accuracy (Bias%) determined at three concentrations including 2, 20 and 200 ng/ml ($n = 5$)

Theoretical concentration	Measured concentration (mean ± S.D.)	R.S.D. (%)	Bias (%)
Tanshinone IIA (ng/ml)			
Inter-batch			
2	1.84 ± 0.09	4.64	−7.79
20	18.35 ± 0.83	4.51	−8.21
200	213.34 ± 7.38	3.46	6.67
Intra-batch			
2	2.17 ± 0.09	4.47	8.57
20	19.07 ± 0.80	4.20	−4.62
200	209.93 ± 7.96	3.80	4.73
Cryptotanshinone (ng/ml)			
Inter-batch			
2	1.82 ± 0.02	1.10	−8.98
20	17.18 ± 0.33	1.83	−11.10
200	209.66 ± 8.56	4.08	4.83
Intra-batch			
2	1.93 ± 0.02	1.04	−3.82
20	18.47 ± 0.37	2.01	−7.65
200	214.00 ± 1.63	0.76	6.99

standing that addition of the voltaic acid could enhance the sensitivity when the ion adduct $[M + H]^+$ was detected. However, in our research, when formic acid and acetate acid were added to the mobile phase, the sensitivity decreased significantly. The actual reason of the finding remained unknown though, the phenomenon indicated that for some compounds, addition of ammonia helped to form the $[M + H]^+$.

3.3. Accuracy and precision

Table 1 shows a summary of intra- and inter-batch precision and accuracy at three concentrations. The intra-batch accuracy for TS and CT samples were 95–109% and 92–107%, and the inter-batch accuracy ranged from 91 to 107% and 88 to 105%, respectively. The precisions (R.S.D.%) were all less than 5%. These results indicated that the present method had a satisfactory accuracy, precision and reproducibility.

3.4. Recovery

The recoveries of TS and CT from rat plasma were shown in Table 2. The mean recoveries were well over 80% ($n = 5$).

3.5. Stability

The stability of TS and CT during the sample storing and processing procedures was fully evaluated by analyzing triplicate replicates of QC samples at the concentrations of 2, 20 and 200 ng/ml for both analytes. The concentration variations found after three cycles of freezing and thawing were within ±8% of nominal concentrations, indicating no significant substance loss during repeated thawing and freezing. The results in detail were

Table 2
Absolute recoveries of tanshinone IIA and cryptotanshinone in rat plasma ($n = 5$)

Theoretical concentration	Measured concentration (mean ± S.D.)	Recovery (%) (mean ± S.D.)
Tanshinone IIA (ng/ml)		
2	1.73 ± 0.08	86.30 ± 3.82
20	16.96 ± 0.82	84.81 ± 4.08
200	177.18 ± 13.34	88.59 ± 6.67
Cryptotanshinone (ng/ml)		
2	1.75 ± 0.10	87.50 ± 5.04
20	17.98 ± 0.74	89.91 ± 3.69
200	182.78 ± 8.06	91.39 ± 4.03

Table 3
Freeze and thaw stability ($n = 3$)

Theoretical concentration	Measured concentration (mean ± S.D.)		
	Cycle 1	Cycle 2	Cycle 3
Tanshinone IIA (ng/ml)			
2	1.85 ± 0.07	1.89 ± 0.08	2.16 ± 0.08
20	18.40 ± 0.92	21.24 ± 0.86	18.95 ± 0.93
200	208.36 ± 10.20	208.59 ± 9.67	192.80 ± 10.66
Cryptotanshinone (ng/ml)			
2	2.11 ± 0.03	1.88 ± 0.04	1.86 ± 0.03
20	19.15 ± 0.54	20.94 ± 0.62	21.42 ± 0.76
200	208.95 ± 6.89	189.05 ± 7.56	187.46 ± 7.87

shown in Table 3. When processed samples were stored in the autosampler at 4 °C, TS and CT showed a very good stability evidenced from that the responses varied no more than ±7% at all concentrations studied within 24 h of storage. After storage at ambient temperature for 12 h, TS concentrations in plasma deviated less than ±9% from that in freshly spiked plasma. These were shown in Table 4.

3.6. Pharmacokinetic study

This simple, precise and accurate LC/MS method yielded satisfactory results for determination of TS and CT in rat plasma

Table 4
The stability of TS and CT in processed plasma at 4 °C (in the autosampler) and in plasma at ambient temperature

Theoretical concentration	Measured concentration (mean ± S.D.)	
	At 4 °C for 24 h	At ambient temperature for 12 h
Tanshinone IIA (ng/ml)		
2	2.14 ± 0.06	2.13 ± 0.07
20	21.07 ± 0.40	18.83 ± 0.72
200	190.34 ± 0.82	189.22 ± 0.85
Cryptotanshinone (ng/ml)		
2	2.12 ± 0.03	1.88 ± 0.04
20	21.03 ± 0.43	21.74 ± 0.67
200	189.66 ± 0.62	209.65 ± 0.70

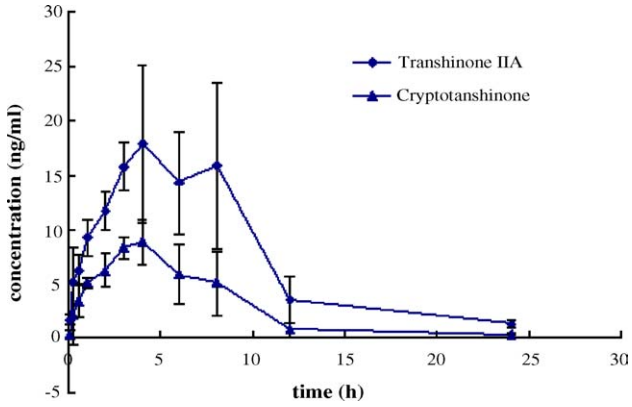

Fig. 3. Mean plasma concentration time curve in rats after oral administration of total tanshinones of 150 mg/kg.

and has been used successfully in a pharmacokinetic study in six rats following oral administration of total tanshinones. The mean plasma concentration–time profiles were illustrated in Fig. 3. The plasma concentration maximums (C_{max}) of TS and CT were below 20 ng/ml; and at 24-h post-dosing, the concentrations decreased to be about 1.38 ± 0.39 and 0.33 ± 0.06 ng/ml, respectively. All the plasma concentrations were found well below the LLOQ of the previous report method (50 ng/ml). Obviously, the previous methods based on the HPLC-UV detection could not satisfy the requirements of the pharmacokinetic study of tanshinones followed oral administration, whereas the present method based on LC/MS was sensitive enough for the pharmacokinetics research.

4. Conclusion

This paper describes a simple, rapid and specific LC/MS method for the simultaneous determination of TS and CT in rat plasma. The lower limits of quantitation of TS and CT were 1 and 0.2 ng/ml, respectively. As shown in Fig. 2, the retention times of TS, CT and I.S. were 6.1, 4.5 and 2.8 min, respectively. And the peaks were free of interference from endogenous substances in plasma. The method has been successfully applied to pharmacokinetic study in rat followed by oral administration of total tanshinones. In addition, it has now been extended to determine TS and CT concentrations in other bio-samples, such as in tissues, in urine and in bile, etc., and the results would forward to be published later.

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