Determination of Tanshinone I in Rat Plasma by High-Performance Liquid Chromatography and its Application to Pharmacokinetic Studies

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Abstract

This paper describes a rapid and sensitive high-performance liquid chromatographic (HPLC) method for the determination of the concentration of tanshinone I in rat plasma, and applies the method to pharmacokinetic study. The plasma is deproteinized with acetonitrile containing an internal standard (estradiolbenzoate). The HPLC assay is carried out using a Cosmosil C18 column. The mobile phase is acetonitrile, 0.05 mol/L^{-1} ammonium acetate buffer with 1% acetic acid (66:34, v/v). The flow rate is 1.0 mL/min. The detection wavelength is set at 263 nm. The assay accuracy is better than 92%, and the precision of tanshinone I at low to high concentrations is better than 9% and 11% for intra-day and inter-day assays, respectively. The recovery of the method exceeds 88.3% for tanshinone I. The assay shows good linearity (r = 0.9998) over a relatively wide concentration range from 0.05 to 10.0 µg/mL. The method is used to determine the concentration-time profiles of tanshinone I in plasma following an intravenous injection of tanshinone I solution, and the pharmacokinetic parameters of tanshinone I are calculated for the first time by the Drug and Statistics 1.0 program. This assay is successfully applied to the determination of tanshinone I in rat plasma, and the developed method is applied to pharmacokinetic studies for the first time.

Introduction

Danshen (Chinese name), the dried root of *Salvia mitiorrhiza* Bunge, is a well-known traditional Chinese medicinal herb which has attracted particular attention from medicinal chemists for several thousand years. Because of its special therapeutic effects, Danshen has been widely adopted to improve blood microcirculation, relieve vexation, treat hemorrhages, reduce blood pressure, etc. (1–3). Tanshinones are mainly hydrophobic active components which have been isolated from Danshen. It is reported that tanshinones can dilate coronary arteries, increase coronary flow, modulate mutagenic activity, and protect the myocardium against ischaemia. They also have some activity as a broad-spectrum bactericide. Among tanshinone constituents in Danshen, tanshinone I (TS I), tanshinone IIA (TS IIA), and cryptotanshinone (CT) (structures shown in Figure 1) are present in the greatest amounts (4). They are abietane-type diterpenes which exhibit diverse biological activities, such as antibacterial, anti-inflammatory, and anti-platelet aggregation activities (5–7).

Up to now, pharmacokinetic studies have been mainly focused on TS IIA and CT, and there is a lack of information concerning the other bioactive components, such as TS I. Several methods for the determination of TS IIA and CT in biological samples have been described in the literature. They include high-performance liquid chromatography (HPLC) (8), liquid chromatography–tandem mass spectrometry (LC–MS–MS) (9), and ion-pair reversed-phase HPLC (10). As the metabolite of CT, TS IIA has been studied widely and sodium tanshinone IIA sulfonate injections have been used successfully to treat patients in clinics. The preliminary experiments concerning TS I were mainly aimed at determining TS I in plasma (11,12); however, there is no literature about the pharmacokinetic investigation of TS I because it has not been not easy to obtain its concentration in biological fluids. In order to further study the actions of the compounds in Danshen more completely, it is important to investigate the pharmacokinetics of TS I in rat plasma.

The aim of this study was to develop a rapid and sensitive HPLC method for determining TS I in rat plasma after intravenous injection, and the developed method was applied to pharmacokinetic studies for the first time.

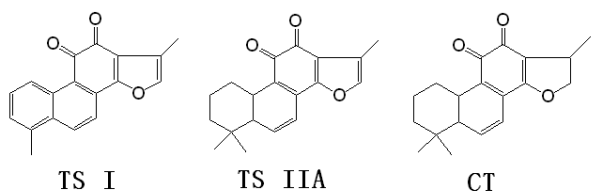

Figure 1. Structures of TS I, TS IIA, and CT.

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Materials and Methods

Chemicals and materials

TS I (analytical grade) and estradiolbenzoate were commercially available from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). TS I used in animal experiments was isolated from the extraction of *Salvia emiltriorrhizae* and has a high purity identified as more than 95% by HPLC analysis. Acetonitrile and methanol were HPLC grade, obtained from Shandong Yuwang Co. Ltd. (Shandong, China). Acetic acid, ammonium acetate, and ethanol were of analytical grade, obtained from Laiyang Chemical Plant (Shandong, China). Water was daily obtained from a Milli-Q water purification system (Millipore Corp., Bedford, MA).

Apparatus and chromatographic conditions

The HPLC was carried out using a Shimadzu LC-20A HPLC system (Kyoto, Japan) which consisted of a binary gradient pump (model LC-20A), a SPD-M20A diode-array detector, a SiL-20A auto sampler, a DGU-20A3 degasser, and a CTO-20A Column oven. The apparatus was interfaced to a Dell PC-compatible computer using LC solution software.

The separation was performed on a Cosmosil C_{18} column, 4.6 × 150 mm, 5 µm (Kyoto, Japan). The mobile phase consisted of acetonitrile and 0.05 mol/L^{-1} ammonium acetate buffers with 1% acetic acid in a volume ratio of 66:34 was filtered through a 0.45 µm hydrophilic membrane filter prior to use. The mobile phase was delivered at a flow rate of 1.0 mL/min. Detection was performed at a wavelength of 263 nm at 40ºC. The sample injection volume was 20 µL.

Animals and blood sampling

Male Wistar-strain rats (220–250 g) were obtained from the Laboratory Animal Center of Lanzhou University and fasted for 12 h with free access to water, prior to the experiments. A polyethylene tube (0.28 mm i.d., 0.61 mm o.d.) was inserted into the right femoral artery of the rat while the animal was under anesthesia with ether. The supernatant solution of TS I in ethanol–PEG400–water (1:4:5, v/v/v) was then intravenously injected into the rat at a dose of 3 mg/kg. Blood samples (0.25 mL) were collected at 0, 2, 5, 8, 13, 18, 25, 45, 60, and 90 min after intravenous injection. After each sampling, loss of blood volume was supplemented with an equal volume of saline containing 100 IU/mL heparin. Water was supplied to each rat 1 h after dosing.

Preparation of plasma samples

Each collected blood sample was immediately transferred to a heparinized microcentrifuge tube and centrifuged in a 1.5 mL microcentrifuge at 10800 rpm for 5 min. And the plasma was separated and stored at –20ºC until processing. The plasma (0.1 mL) was then vortex-mixed with 0.2 mL of acetonitrile containing estradiolbenzoate (10 µg/mL) as internal standard (IS) for 30 s. After 10 min, the mixture was centrifuged for 5 min to separate precipitated proteins. Then the supernant solution was filtered through a 0.45 µm membrane filter, and 20 µL solution of filter was directly injected into the HPLC. The same sample processing was applied to the recovery and to the precision in plasma.

Calibration curve

Calibration curves in the concentration range of 0.05–10.0 µg/mL for TS I were constructed by plotting the peak-area ratio of each analyte/IS versus TS I concentration in rat plasma. In order to avoid undue bias to the low concentrations of the standard curve by the high concentrations, the calibration curve was split into two ranges: 0.05–1.0 and 1.0–10.0 µg/mL, respectively. Least squares linear regression analysis was used to determine the slope, intercept, and correlation coefficient.

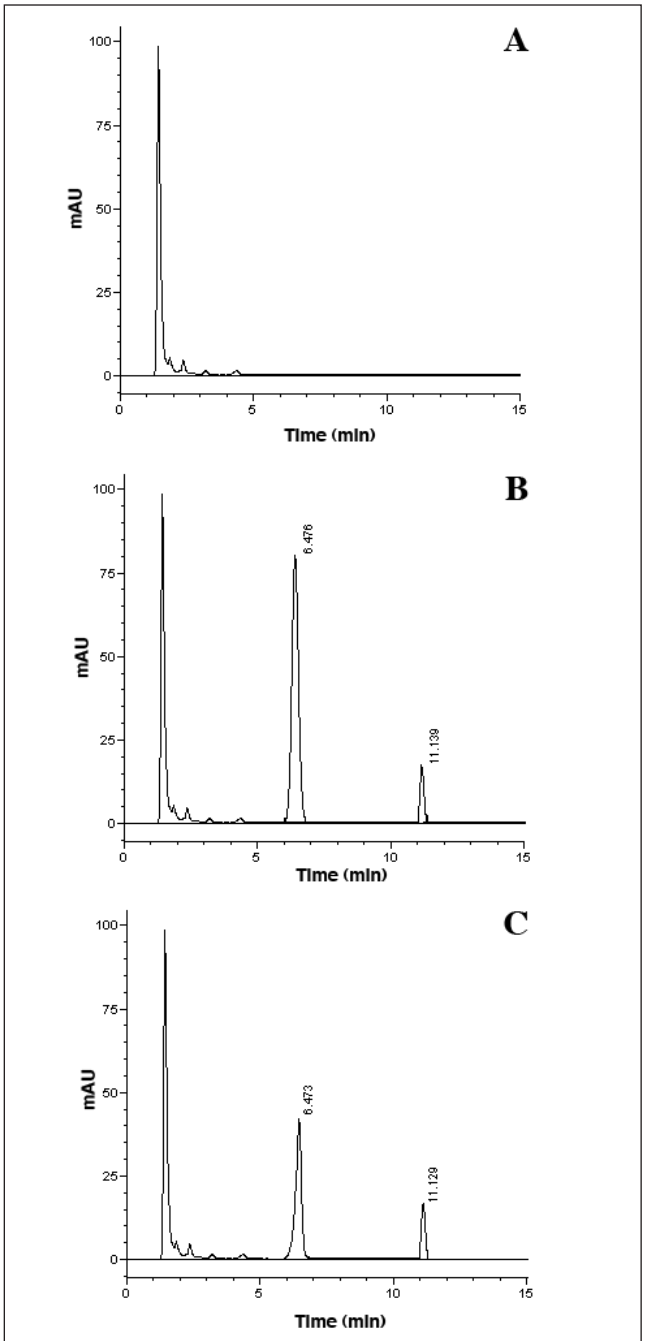

Figure 2. Chromatograms of TS I in rat blank plasma (A). Chromatograms of TS I in rat plasma spiked with TS I at a concentration of 0.5 µg/mL and estradiolbenzoate (IS) (B). Chromatograms of TS I in rat plasma sample obtained 10 min after intravenous injection of TS I supernatant solution at a dose of 3 mg/kg. Retention times of TS I and IS were approximately 6.47 and 11.12 min, respectively (C).

The concentration of TS I in plasma was determined from the peak-area ratios by using the equations of linear regression obtained from the calibration curves.

Recovery

For the recovery study, three concentrations of TS I solution were prepared in acetonitrile and plasma. Plasma samples were spiked with TS I at concentrations of 0.1, 1.0, and 10.0 µg/mL. The recovery was determined as the ratio of peak-area (analyte/IS) of plasma to that of acetonitrile.

Precision, accuracy, and limit of quantitation

Plasma samples were spiked with TS I at concentrations of 0.05, 0.10, 0.5, 1.0, 5.0, and 10.0 µg/mL. Samples were processed in replicates (n = 5) and subjected to HPLC analysis. The precision was calculated as the relative standard deviation of measurements (RSD). To determine intra-day variance, the assays were carried out on the same samples at different times during day. The inter-day RSD was determined by assaying the spiked samples over five consecutive days. To determine the limit of quantitation (LOQ), dilutions of 5, 10, and 20 ng/mL TS I in plasma were prepared using a solution of 0.1 µg/mL TS I in plasma for calibration.

Stability

The plasma samples at concentrations of 0.05, 1.0, and 10.0 µg/mL were assayed under three different conditions to assess the stability of TS I for three days in the plasma. The stability was studied by assaying plasma samples that had been stored at room temperature, at 2ºC and at –20ºC for a certain period of time, respectively. TS I was considered stable under storage conditions if the assayed percent recovery was found to be 90–110% of the nominal initial concentration.

Pharmacokinetic analysis

The developed method was used to determine the concentration of TS I in rat plasma after intravenous injection of TS I aqueous solution. The results were analyzed using Drug and Statistics 1.0 program (DAS 1.0 program) (Medical College of Wannan, China). The pharmacokinetic parameters of $t_{1/2}$, mean residence time (MRT), area under the plasma concentration-time curve (AUC), clearance (CL), and apparent volume of distribution (V_d) were calculated based on the moment theory. All data were expressed as the mean ± SD.

Results and Discussion

HPLC chromatograms

Under the conditions described previously, the HPLC chromatograms of blank plasma, plasma spiked with TS I at a concentration of 0.5 µg/mL, and the plasma obtained after 10 min intravenous injection of TS I aqueous solution at a dose of 3 mg/kg are shown in Figure 2. It can be seen that endogenous plasma components do not interfere with the elution of TS I and IS. The retention times of TS I and IS were approximately 6.47 and 11.12 min, respectively. The peaks were sharp and symmetrical with good baseline resolution and minimal tailings.

For the separation of TS I, IS, and endogenous plasma components by reversed-phase HPLC, the following experimental conditions, choice of mobile phase, mobile phase flow rate have been investigated. A mixture of standard TS I and IS, dissolved in acetonitrile, was injected into a Cosmosil C_{18} column at flow rate of 1.0 mL/min. Several mobile phases were investigated using an isocratic system in all cases. The initial mobile phase was acetonitrile–water–acetic acid (74:26:1, v/v/v) (13), which gave poor separation of TS I and endogenous plasma components, and TS I and IS could not be baseline resolved. To improve the baseline stability, water in the mobile phase was replaced by 0.05 mol/L^{-1} ammonium acetate buffer throughout the HPLC analysis. After adding acetic acid to the buffer, the peaks of TS I and IS became shaper. The optimum mobile phase was therefore acetonitrile–0.05 mol/L^{-1} ammonium acetate buffer with 1% acetic acid (66:34, v/v).

Calibration curves

TS I was dissolved in acetonitrile and diluted to give a series of standard solutions (0.05, 0.1, 0.5, 1.0, 5.0, and 10.0 µg/mL) for the calibration curves of the drug in rat plasma. The linear regression analysis results of the standard calibration plot for rat plasma were $Y = 0.8584X - 0.0367$ for the low concentration range (0.1–1.0 µg/mL) and $Y = 0.9424X - 0.4675$ for the high concentration range (1.0–10.0 µg/mL) with the same r^2 values of 0.9998, where Y and X represented the peak area ratio and TS I concentration, respectively.

Recovery

The recovery of the assay was assessed by comparing the peak-area ratios (analyte/IS) obtained from spiked plasma samples of different TS I concentrations with the peak-area ratios for the samples containing the equivalent amounts of analyte and IS directly dissolved in acetonitrile. The recoveries of TS I from rat plasma are shown in Table I, and the data showed that the recoveries from this method exceeded 88.3%. The data meet the requirement of the standards of biosample analysis (14).

Accuracy, reproducibility, and limit of quantitation

Accuracy, reproducibility, and precision data are summarized in Tables II and III, where the values are expressed as mean detected concentration and the RSD of measurements. The reproducibility of the method was assessed by examining both intra- and inter-day variance. The assay precision of TS I at low to high concentrations was better than 9% and 11% for intra-day and inter-day assays, respectively. Assay accuracy was better than 92% (Tables II and III). These validations demonstrate the reliability of the developed method. The limit of quantitation, defined as the lowest concentration on the calibration curve at which both accuracy and precision should be within 20% (15), was deemed to be 10 ng/mL, which was lower than that of TS IIA reported by Li et al. (11).

Stability

The stability of TS I was fully evaluated while the sample was stored in three different conditions. TS I appeared stable in rat

plasma. Table IV shows the long-term stability of TS I in rat plasma. The results indicated that the TS I concentrations in the samples after 72 h under room temperature storage showed more apparent degradation than after storing at 2ºC. Compared with the three different TS I of 0.1, 1.0, and 10.0 µg/mL, the concentrations assayed before and after the samples stored at –20ºC for 72 h showed no apparent tendency to decrease with the assayed percent recovery above 92%. TS I stored at –20ºC showed a very good stability, as evidenced by a RSD no more than ± 6%. This result was different from the stored condition which have been reported (12).

Table I. Recovery of the TS I Assay*

Spiked conc. (µg/mL)	Peak-area ratio		Recovery (%)	RSD (%)
	Untreated	Treated		
0.1	0.16 ± 0.024	0.142 ± 0.006	91.61 ± 0.25	0.273
1.0	3.05 ± 0.033	3.06 ± 0.018	88.35 ± 0.54	0.611
10	18.47 ± 0.012	18.29 ± 0.007	90.4 ± 0.58	0.642

* Each value represents the mean ± S.D. (n = 3).

Table II. Validation of the Inter-Day Assay*

Spiked conc. (µg/mL)	Measured conc. (µg/mL)	Accuracy (%)	RSD (%)
0.05	0.046 ± 0.005	92.00	10.87
0.10	0.112 ± 0.009	112.00	8.04
0.5	0.536 ± 0.057	107.20	10.63
1.0	0.964 ± 0.106	96.40	11.00
5.0	4.912 ± 0.177	98.24	3.60
10.0	9.821 ± 0.143	98.21	1.46

* Each value represents the mean ± S.D. (n = 5).

Table III. Validation of the Intra-Day Assay*

Spiked conc. (µg/mL)	Measured conc. (µg/mL)	Accuracy (%)	RSD (%)
0.05	0.049 ± 0.003	98.00	6.12
0.1	0.095 ± 0.006	95.00	6.32
0.5	0.556 ± 0.021	112.00	3.78
1.0	0.922 ± 0.079	92.20	8.57
5.0	4.714 ± 0.322	94.28	6.83
10.0	9.133 ± 0.094	91.33	1.03

* Each value represents the mean ± S.D. (n = 5).

Application

In order to estimate the pharmacokinetic parameters of TS I, the plasma concentration of TS I was analyzed in rat plasma following injection of TS I supernatant solution at a dose of 3 mg/kg. The plasma concentration of TS I was determined at 0, 2, 5, 8, 13, 18, 25, 45, 60, and 90 min after intravenous injection. Figure 3 shows the mean ± SD plasma concentration-time profile of TS I after intravenous injection at a dose of 3 mg/kg. The average concentration-time profiles of TS I after intravenous injection at a dose of 3 mg/kg in rats were well described with a two-compartment model. The pharmacokinetic parameters of TS I are listed in Table IV. After intravenous injection of TS I supernatant solution, TS I was eliminated rapidly with a short value of $t_{1/2\beta}$. The plasma concentration was low, with a mean maximum plasma concentration (C_{max}) value of 0.274 µg/mL. According to the reported literature, TS IIA could be distributed rapidly while the plasma concentration was at a low level after injection (11). Therefore, our study further demonstrated that the tanshinone compounds were absorbed badly, eliminated rapidly, and could be easily metabolized as another compound (10). And because of the low systemic bioavailability of diterpenoid tanshinones by oral administration, the limitation of this research was not determining the concentration of TS I in rat plasma after oral administration at a dose of 30 mg/kg. Although TS I has the low value of $t_{1/2\beta}$ and C_{max}, it was found to exhibit some pharmacological effects (16,17). Every traditional Chinese medicine can be considered as a complex system which plays curative roles depending on the interaction of various kinds of active compounds contained in it. When combined, the effects and bioavailability of one constituent may sometimes be influenced by others. This may reflect the advantage of multiple constituent characteristics possessed by most traditional Chinese medicines or by many other natural medicines. It is therefore reasonable to suggest that the cooperation with other tanshinones is

Table IV. Stability of TS I in Different Storage Conditions in Rat Plasma*

TS I concentration (µg/mL)	Storage condition	TS I measured concentration (µg/mL)	RSD (%)
Stored at –20ºC	72 h (n = 5)		
0.1		0.094 ± 0.003 (95.2)	3.19
1.0		0.935 ± 0.052 (94.9)	5.56
10.0		9.812 ± 0.161 (99.7)	1.64
Stored at 2ºC	72 h (n = 5)		
0.1		0.087 ± 0.005 (92.9)	5.75
1.0		0.838 ± 0.044 (91.8)	5.25
10.0		8.210 ± 0.326 (87.6)	3.62
Stored at room temperature	72 h (n = 5)		
0.1		0.068 ± 0.007 (77.3)	10.29
1.0		0.501 ± 0.058 (64.6)	11.58
10.0		4.783 ± 0.214 (58.9)	4.47

* Each value represents the mean ± S.D. (n = 5).

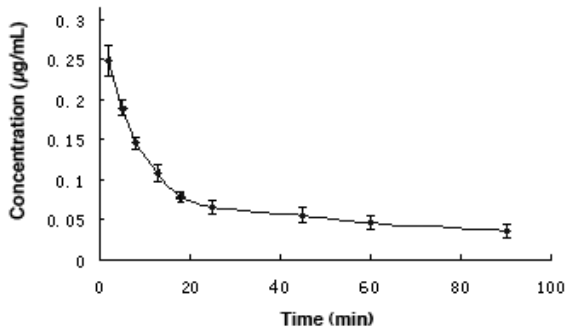

Figure 3. Plasma concentration profiles of TS I after injection at a dose of 3 mg/kg. Data are mean S.D. of five experiments.

Table V. Pharmacokinetic Parameters of TS I in Rat Plasma (n = 5) After Intravenous Injection of TS I Solution (at a dose of 3 mg/mL)

Parameters	Estimate (mean ± S.D.)
AUC_{0-1} (mg/L • min)	2.10 ± 0.34
$AUC_{0-\infty}$ (mg/L • min)	17.16 ± 2.15
$t_{1/\alpha}$ (min)	1.92 ± 0.16
$t_{1/\beta}$ (min)	56.1 ± 6.83
C_{max} (mg/L)	0.273 ± 0.017
T_{max} (min)	2.53 ± 0.27
V d (L/kg)	148.150 ± 21.68
CL (L/min/kg)	0.299 ± 0.091
MRT (min)	22.74 ± 3.77

probably responsible for the absorption and bioavailability of TS I after oral administration in rats. Therefore, further studies are needed to understand whether the other tanshinones' interaction could play an important role in the absorption and/or elimination of TS I. The next study on the investigation of pharmacokinetic studies in rats after oral administration of the extract of Danshen is in progress.

Conclusion

In conclusion, the HPLC method we have developed is simple, sensitive, and specific, and can be used for the analysis of large numbers of plasma samples. The assay was validated to meet the requirements of pharmacokinetic studies, and the results of validation showed that this method is sensitive, accurate, and reproducible. This method has been applied successfully for the first time in pharmacokinetic studies of TS I. Therefore, this method could be used to assay TS I in clinical samples and other biological fluid samples following appropriate adjustments.

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