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Ion-pair reversed-phase HPLC: Assay validation of sodium tanshinone IIA sulfonate in mouse plasma

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Abstract

Sodium tanshinone IIA sulfonate (STS), a hydrophilic ionic substance, is used as a cardiovascular drug. An ion-pair reversed-phase high-performance liquid chromatography (IP-RP-HPLC) method for the determination of STS in mouse plasma was initially developed. The assay involved a rapid and simple extraction process and subsequent detection at 271 nm. The retention time for STS was 7.5 min. Based on extracted STS standard mouse plasma at 1.5,10 and 50 μg/ml, the assay precision were 2.7, 2.1 and 1.7% with a mean accuracy of 96.7, 98.5 and 99.4%, respectively. At plasma concentration of 1.5, 50 and 75 μg/ml, the mean recovery of STS were 93.1, 96.3 and 97.5%. The limit of detection (LOD) and limit of quantification (LOQ) for STS was 0.1 μg/ml and 0.5 μg/ml, respectively. Linear responses were observed over a wide concentration range (0.5–100 μg/ml) for STS in mouse plasma. STS can be detected after intravenous administration. This method was performed for the first time in pharmacokinetic studies of STS in the mouse.

Keywords: Sodium tanshinone IIA sulfonate (STS); Ion-pair; Reversed-phase HPLC; Pharmacokinetics

1. Introduction

Tanshinone IIA, which is isolated from the root of *Salvia miltiorrhiza*, is effective in the treatment of myocardial infarction and angina pectoris. The clinical use of tanshinone IIA is limited by its poor water solubility. Sodium tanshinone IIA sulfonate (STS or DS-201) is a water-soluble derivative of tanshinone IIA. STS is a cardioprotective substance and may exert a beneficial effect on the clinically important vascular endothelium [1]. In China, STS injections have been used successfully to treat patients with coronary artery disease and angina pectoris for more than 30 years. Patients administered with STS have exhibited significant improvements with respect to subjective clinical symptoms, objective clinical signs, and electrocardiogram parameters. Clinical evidence has shown that STS increases coronary blood flow and alleviates cardiac metabolic disorders [2–4]. In addition, extensive research has shown that STS has a broad range of pharmaceutical effects such as restraining adriamycin-induced lipid peroxidation, attenuating hypertrophy induced by angiotensin II in cultured neonatal rat cardiac cells, protects against ischemia-reperfusion injury through an electron transfer reaction in rat heart mitochondria by forming reactive oxygen radicals, etc. [5–10]. The molecular structure of STS is illustrated in Fig. 1.

However, an assay for STS using HPLC has not been previously reported. The lack of in vivo quantitative research for STS prompted us to develop a reliable assay for the determination of STS in plasma. STS, a hydrophilic ionic substance, would dissociate in aqueous solution and is particularly difficult to analyse using RP-HPLC since it demonstrates poor retention on traditional C_{18} columns. The purpose of this study is to establish a simple, practical, ion-pair reversed-phase HPLC method for the assay of STS, and to subsequently determine STS in mouse plasma. According to the molecular structure and physicochemical characteristics of STS, tetrabutylammonium hydroxide was added to the mobile phase as a cation agent, an ion pair composed of STS and tetrabutylammonium hydroxide was formed and the resultant ion pair was more easily retained on the C_{18} column, which resulted in a prolonged retention time for STS. Herein,

Abbreviations: STS, sodium tanshinone IIA sulfonate

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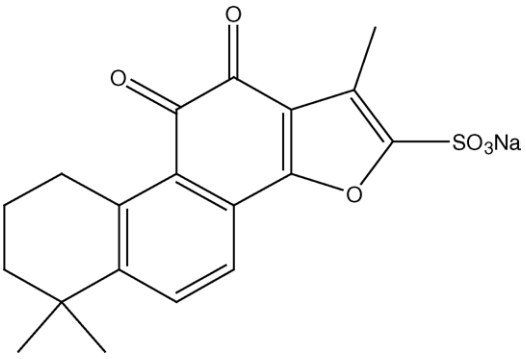

Fig. 1. Molecular structure of sodium tanshinone IIA sulfonate.

we report an assay for STS using HPLC, and the application of this method for the first time in pharmacokinetic studies of STS.

2. Materials and methods

2.1. Materials and reagents

Tanshinone IIA was isolated from the root of *S. miltiorrhiza* and STS was prepared from tanshinone IIA, according to the method of Chien et al. [11]. Its structure was identified by comparing the obtained spectrum with observations reported previously [12]. Authentic standards of STS were purchased from the National Institute for Control of Pharmaceutical and Biological Products (Beijing, China). Purity of the authentic standards was determined at 226, 253 and 271 nm respectively, using the HPLC method developed in this study. The average purity of the authentic standards was calculated as 98.6%, using the area normalization method. Compared with the authentic standards, the purity of the self-prepared STS was calibrated as 99.3%, when the same HPLC method was employed and the detection wavelength was set at 271 nm. The STS injection (5 mg/ml) was purchased from First Biochemistry Pharmaceutical Factory of Shanghai, China. Ten percent tetrabutylammonium hydroxide solution was obtained from Shanghai Chemical Reagent Co., Ltd. All chemicals and solvents were of analytical or HPLC grade. All standard solutions and mobile phases were prepared using double distilled water.

2.2. Calibration standards and quality controls (QC) samples

STS stock solution was prepared by dissolving 25 mg STS in double distilled water to a final concentration of 1000 μg/ml. The stock solution was stored at −20 °C and brought to room temperature before use. The standard stock solution was diluted with blank mouse plasma to yield the calibration standard solutions of 0.5, 5, 10, 20, 50, 75 and 100 μg/ml. The QC samples were prepared at the target concentrations of approximately 0.5, 1.5, 5, 10, 50 and 75 μg/ml in blank mouse plasma. A target amount of STS was weighed separately to prepare the stock solution for the QC samples. The QC samples were then divided into 100-μl aliquots in tightly closed microtubes and kept frozen at −20 °C until required.

2.3. Sample preparation

Seven hundred microliters of methanol was added to 300 μl of plasma sample. The obtained solution was vortexed for 10 min and centrifuged at 10,000 × *g* for 2 min to remove any protein and the resultant supernatant was used in the assay.

2.4. High-performance liquid chromatographic apparatus and chromatographic conditions

The HPLC system consisted of a variable UV detector model SPD-10Avp UV–vis (Shimadzu, Japan), a single pump model LC-10ATvp (Shimadzu, Japan) and an integrator model (N2000 work station, Zhejiang University), was used for data acquisition and processing. The mobile phase consisted of methanol/water/10% tetrabutylammonium hydroxide solution (68/30.5/1.5, v/v/v), was filtered (0.45 μm) and degassed prior to use. The mobile phase was then pumped through the system at a rate of 1.0 ml/min. Twenty microliters of sample solution was then injected onto the column. The chromatograph run required 10 min for completion. The ultraviolet (UV)-spectra of STS in the mobile phase was obtained using a UV-2450 spectrophotometer (Shimadzu, Japan) with a wavelength of 271 nm, where the UV absorption of STS reached its maximum during the mobile phase. Separation was achieved at 35 °C with a Diamonsil C_{18} column (4.6 × 150 mm with a 5 μM pore size) (Dikma). A pre-column treatment of the same constituents was employed. All chromatograms obtained were evaluated by peak area measurement. The calibration curve was plotted with peak areas against concentration. The data demonstrated a linear function for STS according to the equation:

$$Y = a + bX$$

where Y is the peak area, X is the value of sample concentration (μg/ml), a is the intercept and b is the slope.

2.5. Stability

The quality control (QC) samples were assayed under five different conditions to assess the stability of STS in mouse plasma. One set of QC samples was stored at room temperature (approximately 22–25 °C) for 24 h. The stability of the sample at room temperature was evaluated by comparing the assay results of the stored QC samples with that of the freshly thawed QC samples. Another set of QC samples was subjected to three freeze-thaw cycles and was then assayed to evaluate freeze-thaw stability of STS in mouse plasma. Long-term stability was studied by assaying samples that had been stored at −20 °C for a certain period of time. STS was considered stable under storage conditions if the assayed percent recovery was found to be 90–110% of the nominal initial concentration.

2.6. Application of the method

Kunming male mice (19–22 g) were administered STS intravenously at a dose of 50 mg/kg via the tail vein (∼0.2 ml STS injection). The mice were then sacrificed at 5, 10, 15, 30 min, 1,

2, 4, 8 and 12 h after injection. The blood samples were rapidly collected and the plasma was separated by centrifugation and stored at −20 °C until required. The STS plasma levels in the samples were determined using the above-mentioned method. The concentration data, at each time point, represented the mean ± standard deviation obtained from five mice. Pharmacokinetic parameters were calculated using a non-linear fitting program.

3. Results and discussion

3.1. Linearity, limit of detection, precision and accuracy

The linear regression equation for STS mouse plasma concentration was: $y = 11381 + 86652x$, with a correlation coefficient (r) of 0.9995 ($n = 7$). The calibration curves were found to be linear for STS in the concentration range 0.5–100 μg/ml. The limit of quantification (LOQ) in mouse plasma for STS was 0.5 μg/ml. The limit of detection value (LOD) in mouse plasma for STS was 0.1 μg/ml, calculated as the amount of the injected sample, which resulted in a signal-to-noise ration of 3.

Inter-assay precision was determined by analysing five calibration curves with quality control samples on 5 different days. The intra-assay precision was determined by analysing six replicates of quality control samples extracted on the same day. The results of inter-day and intra-day precision and accuracy for STS in mouse plasma are presented in Table 1.

3.2. Recovery

To determine STS recovery in plasma, mouse plasma samples were spiked with STS to achieve a final concentration of 1.5, 50 and 75 μg/ml. The mouse plasma samples were then extracted with methanol. Nine samples were analysed for each concentration. The recovery was calculated by comparing the peak areas of STS, extracted from mouse plasma samples, with STS solutions at the same concentrations. The analysis was performed on 3 consecutive days, each day the analysis was performed for three replicates at the concentration levels mentioned above. The mean recoveries of STS from mouse plasma at concentrations of 1.5, 50 and 75 μg/ml were 93.1, 96.3 and 97.5, respectively. The recovery results of STS at different concentrations are presented in Table 2.

Table 1
Assay precision and accuracy of STS in mouse plasma

STS nominal concentration (μg/ml)	STS measured concentration (μg/ml)	R.S.D.[a] (%)	Accuracy (%)	n
Inter-day assay				
0.5	0.46 ± 0.030	6.5	92.0	6
1.5	1.42 ± 0.06	4.2	94.7	6
10	9.73 ± 0.32	3.3	97.3	6
50	49.2 ± 1.4	2.8	98.4	6
75	72.2 ± 1.8	2.5	97.6	6
Intra-day assay				
0.5	0.46 ± 0.028	6.1	92.0	6
1.5	1.46 ± 0.04	2.7	96.7	6
10	9.85 ± 0.21	2.1	98.5	6
50	49.7 ± 0.86	1.7	99.4	6
75	73.4 ± 1.21	1.6	97.2	6

[a] R.S.D. = relative standard deviation.

Table 2
Extraction recovery of STS in mouse plasma

Time (day)	Added (μg/ml)	Found (μg/ml)	n	Recovery (%)	Mean recovery (%)
1	1.5	1.38 ± 0.13	3	92.0 ± 8.6	93.1 ± 8.0 ($n = 9$)
2	1.5	1.40 ± 0.12	3	93.3 ± 8.0	
3	1.5	1.41 ± 0.11	3	94.0 ± 7.3	
1	50	47.8 ± 0.82	3	95.6 ± 1.6	96.3 ± 1.8 ($n = 9$)
2	50	48.2 ± 0.91	3	96.4 ± 1.8	
3	50	48.5 ± 0.96	3	97.0 ± 2.0	
1	75	72.8 ± 1.29	3	97.0 ± 1.7	97.5 ± 1.7 ($n = 9$)
2	75	73.1 ± 1.26	3	97.4 ± 1.7	
3	75	73.6 ± 1.34	3	98.1 ± 1.8	

3.3. Sample stability

STS appeared stable in mouse plasma. Table 3 shows the long-term stability of STS in QC samples. Compared with theoretical STS concentrations of 0.5, 1.5, 5, 50 and 75 μg/ml, the concentrations assayed before and after the samples stored at −20 °C for 5, 15 and 30 days showed no tendency to decrease with time. These data indicate that STS in mouse plasma was stable for at least 30 days when stored frozen at −20 °C, and for 24 h when maintained at room temperature (22–25 °C). STS was also stable following three freeze-thaw cycles. STS concentrations in the QC samples after 24 h room temperature storage showed no apparent degradation: the final concentrations were 95.9–100.6% of the initial values (Table 3). STS extracted from mouse plasma samples during the mobile phase, was also stable for at least 24 h at room temperature.

3.4. Chromatography

Acceptable chromatographic results were obtained with the extracts of the plasma sample. Typical chromatograms obtained from blank plasma, blank plasma spiked with 20 μg/ml STS and a plasma sample at 15 min after injection are shown in Fig. 2. Under chromatographic conditions described previously, the retention time of STS was 7.5 min, and the corresponding peak was baseline resolved. The blank plasma chromatogram clearly demonstrated that no apparent interferences occurred.

Tanshinone IIA is a hydrophobic compound, which is simple to assay using HPLC. There have been many reports on the in vitro and in vivo determination of tanshinone IIA [13–15]. STS is an ionic substance, which is difficult to retain on a C_{18} column. It is well recognised that dissociation is the usual behavior of ionic substances in aqueous solution. Polar chromatographic column, ion suppression (the careful adjustment of the mobile phase pH to result in a non-ionized analyte) and ion-pair chromatography should be considered to assay ionic substances. We attempted to utilize the polar chromatographic column

Table 3
Stability of STS in different storage conditions in mouse plasma

STS nominal concentration (μg/ml)	Storage condition	STS measured concentration[a] (μg/ml)	R.S.D. (%)
Stored at below −20 °C			
0.50	0 days (n = 6)	0.49 ± 0.030 (100)	6.1
1.5		1.48 ± 0.062 (100)	4.2
5.0		4.95 ± 0.17 (100)	3.4
50.0		49.6 ± 1.12 (100)	2.2
75.0		74.6 ± 1.84 (100)	2.5
0.50	5 days (n = 6)	0.47 ± 0.028 (95.9)	6.0
1.5		1.51 ± 0.058 (102.0)	3.8
5.0		4.92 ± 0.19 (99.4)	3.9
50.0		49.8 ± 1.15 (100.4)	2.3
75.0		74.2 ± 1.92 (99.5)	2.6
0.50	15 days (n = 6)	0.45 ± 0.032 (91.8)	7.1
1.5		1.46 ± 0.064 (98.6)	4.4
5.0		4.89 ± 0.21 (98.8)	4.3
50.0		49.4 ± 1.08 (99.6)	2.2
75.0		73.8 ± 2.14 (98.9)	2.9
0.50	30 days (n = 6)	0.46 ± 0.030 (93.9)	6.5
1.5		1.44 ± 0.061 (97.3)	4.2
5.0		4.80 ± 0.14 (97.0)	2.9
50.0		50.8 ± 1.14 (102.4)	2.2
75.0		74.8 ± 1.71 (100.3)	2.3
After 24 h at room temperature (n = 6)			
0.50		0.47 ± 0.021 (95.9)	4.4
1.5		1.47 ± 0.045 (99.3)	3.0
5.0		4.86 ± 0.17 (98.2)	3.5
50.0		49.9 ± 1.23 (100.6)	2.5
75.0		74.1 ± 1.81 (99.3)	2.4
After three freeze-thaw cycles (n = 6)			
0.50		0.47 ± 0.025 (95.9)	5.2
1.5		1.45 ± 0.048 (98.0)	3.3
5.0		5.12 ± 0.18 (103.4)	3.5
50.0		48.8 ± 1.18 (98.4)	2.4
75.0		73.5 ± 1.68 (98.4)	2.3

[a] Value are mean ± S.D. with percentage of initial assay concentration in parentheses.

(Kromasil KR100-5NH_2), however, STS was not retained during the various mobile phases. Furthermore, when the C_8 (Kromasil) and C_{18} columns (Diamonsil) were selected, and the pH value of different eluting solutions consisting of methanol and water were adjusted to 2.5–6.0 with phosphoric acid, respectively, STS was not retained. Therefore, we attempted to assay STS using the ion-pair HPLC method. When the pH of the mobile phase composed of methanol/water (70/30, v/v), was adjusted to 7.5–8.0 by triethylamine or tetrabutylammonium hydroxide, STS could be retained and separated with an appropriate retention time and satisfactory peak shape. Following elaborate adjustment for the composition of eluting solutions, the optimal mobile phase was decided as described herein.

3.5. Application of the method

Fig. 3 shows the pharmacokinetic profile of STS in the plasma of Kunming mice following i.v. administration. The drug disappears from the plasma according to a two compartment open model with a distribution and elimination half-life of 8 min and 4.5 h, respectively. The plasma concentration of STS was higher than LOQ at 1 h post injection; however, it remained undetectable at 2 h and thereafter. The details of the pharmacokinetic study will be described in a further publication.

3.6. Robustness of method

The results regarding the robustness of this method showed that the chromatographic patterns were not significantly changed when different solvent sources and a different HPLC system (HP 1100, Agilent) were used. Identical chromatograms were achieved when different analytical columns (Kromasil C_{18} column and Diamonsil C_{18} column) were used. In addition, this method allowed variation in analytical parameters such as methanol content in the mobile phase, adjusted by ±5%, 10% tetrabutylammonium hydroxide solution content in the mobile phase adjusted to 0.5–1.7%, and column temperature adjusted to 20–40 °C.

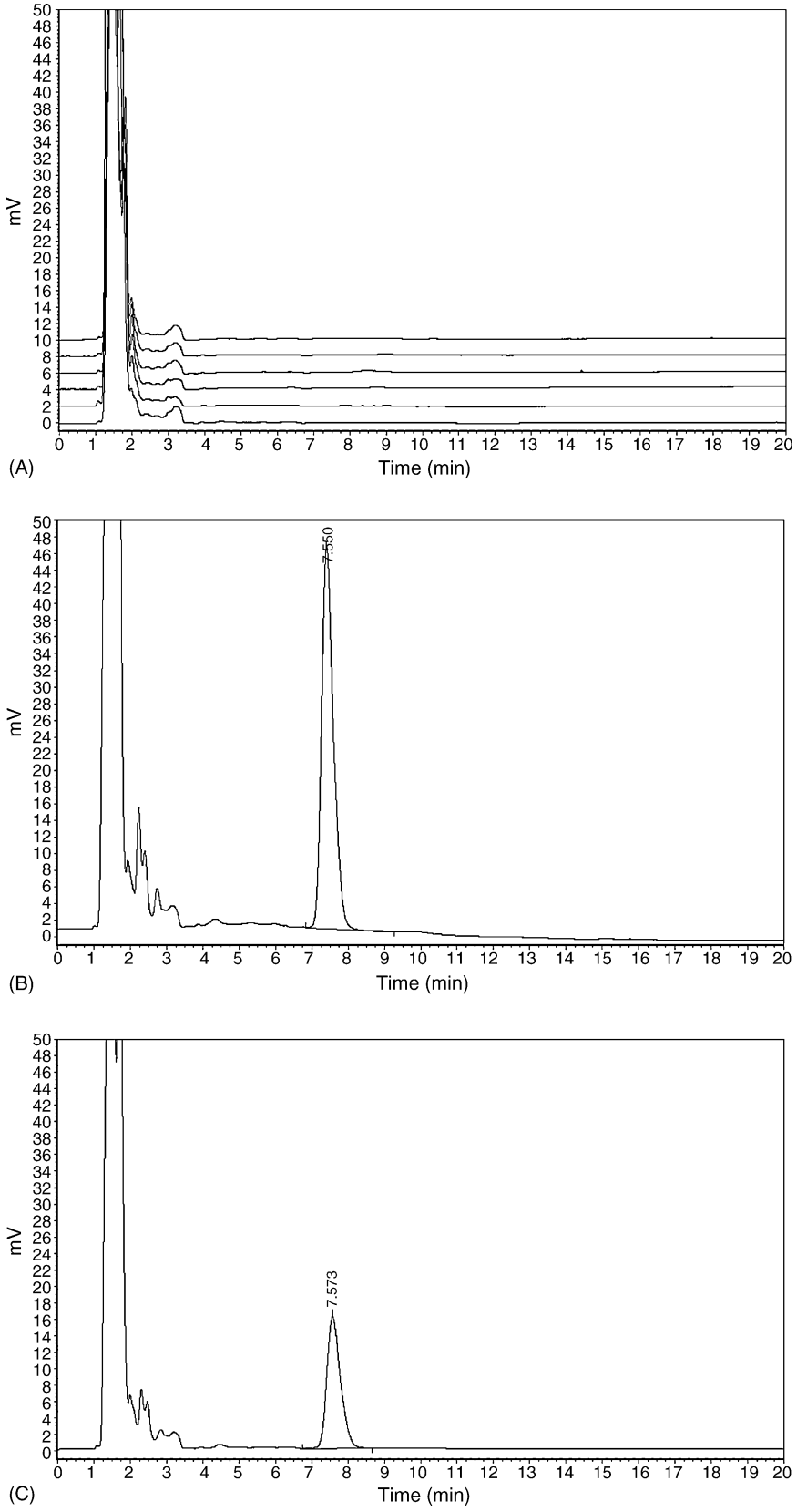

Fig. 2. Typical chromatograms obtained from HPLC analysis of six different individuals. Chromatograms of (A) extracted blank mice plasma. Typical chromatogram obtained from HPLC analysis of mouse plasma. Chromatogram of (B) extracted blank mouse plasma spiked with 10 μg/ml STS. Typical chromatogram obtained from HPLC analysis of mouse plasma. Chromatogram of (C) extracted mouse plasma sample at 15 min after the i.v. treatment with 50 mg/kg of STS.

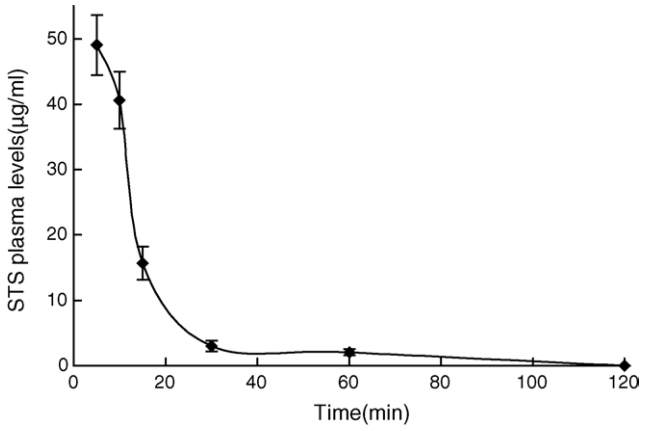

Fig. 3. STS plasma concentrations vs. time profile after an i.v. administration of 50 mg/kg of drug. Each point represented the mean of the concentration obtained from five mice. Bars are S.D.; not visible when smaller than symbols.

4. Conclusion

We have developed and validated an ion-pair RP-HPLC method with UV detection for the determination of STS. This report described the procedure to determine STS in mouse plasma. The method showed great linearity and had a high degree of selectivity, sensitivity, precision and accuracy. STS was assayed with an appropriate retention time, if the described chromatographic condition was used. It should be noted that this method is relatively practical because of the simple extraction procedure and short retention time of STS. This method has been applied successfully for the first time in pharmacokinetic studies of STS. Therefore, this method could also be used to assay STS in clinical samples and other biological fluid samples following appropriate adjustments.

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