Ion-Pair Reversed-Phase HPLC Method for Determination of Sodium Tanshinone IIA Sulfonate in Biological Samples and Its Pharmacokinetics and Biodistribution in Mice

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The ion-pair reversed-phase HPLC method for determination of sodium tanshinone IIA sulfonate (STS) in various biological samples was for the first time developed and validated, and was applied for pharmacokinetics and tissue distribution studies of intravenously administrated STS in mice. A linear relation was found between peak area and STS concentrations within the ranges of 0.1—5 μg/ml for plasma, 0.1—5 μg/g of tissue for kidney homogenate, 0.1—20 μg/g of tissue for liver homogenate, 0.1—1 μg/g of tissue for heart, spleen and lung homogenates, respectively. In plasma and tissues, the limit of quantification (LOQ) and the limit of detection (LOD) for STS were 100 ng/ml and 20 ng/ml. In all biological specimens, the average inter- and intra-day precision of STS were within 4.9%. The recoveries were more than 92% at all concentration levels in each type of biological specimens. STS plasma concentration–time data were best fitted with a two-compartment model, characterized by an initial rapid phase of drug concentration decrease, and a slower terminal elimination phase. The pharmacokinetics of STS was characterized with a distribution half-life ($t_{1/2\alpha}$) of 1.2±0.18 min, a terminal half-life ($t_{1/2\beta}$) of 21.6±2.4 min, a distribution volume (V) of 0.057±0.011 l/kg, a plasma clearance (CL) of 0.86±0.12 l/h/kg and an $AUC_{0-\infty}$ of 58.41±6.21 μg·h/ml. STS was widely distributed into most tissues and was obviously accumulated in liver. This results indicated that STS may be promising to treat liver disease.

Key words sodium tanshinone IIA sulfonate; HPLC; ion-pair; pharmacokinetics; biodistribution

Sodium tanshinone IIA sulfonate (STS) is a water soluble derivative of tanshinone IIa, which is the main lipophilic component contained in *Salvia miltiorrhiza* known as 'Danshen' in traditional Chinese medicine. The pharmacological activity of STS is due to tanshinone IIA, which is a cardioprotective substance and may exert a beneficial effect on the clinically important vascular endothelium.[1)] The use of tanshinone IIA is limited by its poor water solubility. STS injection was thus developed and have been used successfully in China to treat patients with coronary artery disease and angina pectoris for more than 30 years. Clinical evidence and pharmacological researches showed that STS was effective in the therapy of various cardiovascular diseases.[2—4)] Subsequent extensive research suggested that STS also has wide pharmacological effects such as acts against adriamycin-induced lipid peroxidation, attenuates hypertrophy induced by angiotensin II in cultured neonatal rat cardiac cells, protects ischemia-reperfusion injury through an electron transfer reaction in rat heart mitochondria against forming reactive oxygen radicals and blocks calcium channel.[5—9)] The chemical structure of STS is illustrated in Fig. 1.

As compared with the extensive research of pharmacological effects of STS, few studies have dealt with its pharmacokinetics and biodistribution, which is essential to understand physiological disposition of STS. Previous study has demonstrated that we developed the ion-pair reversed-phase HPLC method for determination of STS in mouse plasma.[10)] In this study, we validated the ion-pair RP-HPLC method for determination of sodium tanshinone IIA sulfonate in various biological samples, and applied the method for further pharmacokinetics and biodistribution of intravenously administrated STS in mice.

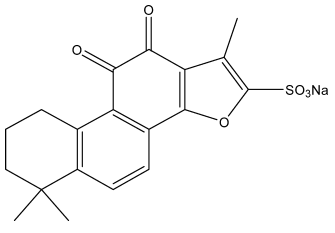

Fig. 1. Chemical Structure of Sodium Tanshinone IIA Sulfonate

Experimental

Materials Authentic standards of STS (purity >98%) was obtained from National Institute for Control of Pharmaceutical and Biological Products (Beijing, China). STS injections (5 mg/ml) were 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.

HPLC Analysis for STS The HPLC assay of Mao S. J. *et al.*[10)] was used to analyze STS in biological specimens with minor modification. The modified mobile phase consisted of methanol/water/10% tetrabutylammonium hydroxide solution (68/31.5/0.5, v/v/v) and injection volume for samples was adjusted to 100 μl.

Preparation of Stocks, Calibration Standards and Quality Control Samples STS stock solution was prepared by dissolving 25 mg STS in double distilled water to a final concentration of 1 mg/ml. The stock solution was stored at −20 °C and brought to room temperature before use. Calibration standards were prepared by adding the nominal stock solution in blank plasma and tissue homogenate. Plasma and kidney homogenate standards were prepared at concentrations of 0.1, 0.2, 0.5, 1, 2 and 5 μg/ml and μg/g of tissue, respectively. Liver homogenate standards were prepared at concentrations of 0.1, 0.5, 1, 5, 10, 15 and 20 μg/g of tissue. Heart, lung and spleen homogenate standards were prepared at concentrations of 0.1, 0.2, 0.4, 0.6, 0.8, and 1 μg/g of tissue, respectively. Six lots of quality control (QC) samples were prepared by spiking STS standard in the biological matrixes at concentrations of 0.1, 0.5, 1 and 5 μg/ml (plasma) 0.1, 0.5, 1 and 5 μg/g (kidney homogenate) 0.1, 0.5, 5 and 10 μg/g (liver homogenate) 0.1, 0.5 and

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1 μg/g (heart, lung and spleen homogenate, respectively).

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 5000 rpm for 2 min to remove any protein and resultant supernatant was used in the assay. Tissue samples were weighed and placed into a hand-held glass micro-homogenizer with 1 ml of deionized water added, and then the tissues were crushed and grinded by mechanical force to form into tissue homogenate. Three hundred microliters of obtained respective tissue homogenate were assigned, and 700 μl of methanol was added to the obtained respective tissue homogenate in order to get rid of protein, and following performance were same treatment as plasma sample.

Specificity, Linearity, Limit of Detection (LOD) and Limit of Quantification (LOQ) To evaluate assay specificity, six independent lots of mouse blank plasma and respective tissue homogenates were analyzed in order to exclude the possibility of any co-eluting endogenous interference. Plasma and tissues homogenates calibration standards at concentrations over a range of 0.1 to 20 μg/ml were prepared and assayed in duplicate on five different days to demonstrate the linearity. Calibration curves of STS were constructed using the ratio of peak area of STS and concentration of STS standard by linear regression analysis. The limit of detection was defined as the lowest concentration of STS resulting in a peak area greater or equal to three times from background noise ($S/N \geq 3$). The LOQ were investigated in plasma and tissues homogenates samples from five different days. For the determination of LOQ, the percentage deviation and %R.S.D. are to be less than 20%.

Assay Precision and Recovery To evaluate precision, six QC replicates were prepared and analyzed at respective concentration for determining intra-day and inter-day validations, along with an independent calibration curve in each type of biological matrix for quantification. The intra-day precision was determined by analyzing 6 QC replicates in each type of tissue homogenate at concentrations of 0.1, 1 and 5 μg/ml (μg/g) for plasma and kidney, 0.1, 1 and 10 μg/g for liver, and 0.1, 0.5 and 1 μg/g for heart, spleen and lung, respectively prepared within a day. The inter-day precision was determined by analyzing the QC replicates prepared on six different days. The variability was expressed as the coefficient of variation (%CV) which should be $\leq$15% at all the concentrations except LOQ whose value should not more than 20%. To determine absolute recovery of STS, the plasma and tissue samples were spiked with STS to achieve six replicates at respective concentration ranging from 0.1 to 10 μg/ml (μg/g), and were then extracted with methanol, respectively. The six replicates were analyzed in various type of biological specimens for respective concentration on three different days. The recovery was calculated by comparing analyte peak areas of the extracted samples with that of STS standard solution at the same nominal concentrations.

Pharmacokinetics and Biodistribution Study of STS Kunming male mice (19—22 g) were administered STS at a dose of 50 mg/kg *via* the tail vein. The mice were then sacrificed at 1, 5, 10, 15, 20 min, 0.5, 1, 2, 4, 8, 12 and 24 h after injection. The plasma and tissues samples were rapidly collected, and all the samples were stored at −20 °C until analysis of STS, as described above. The concentration data, at each time point, represented the

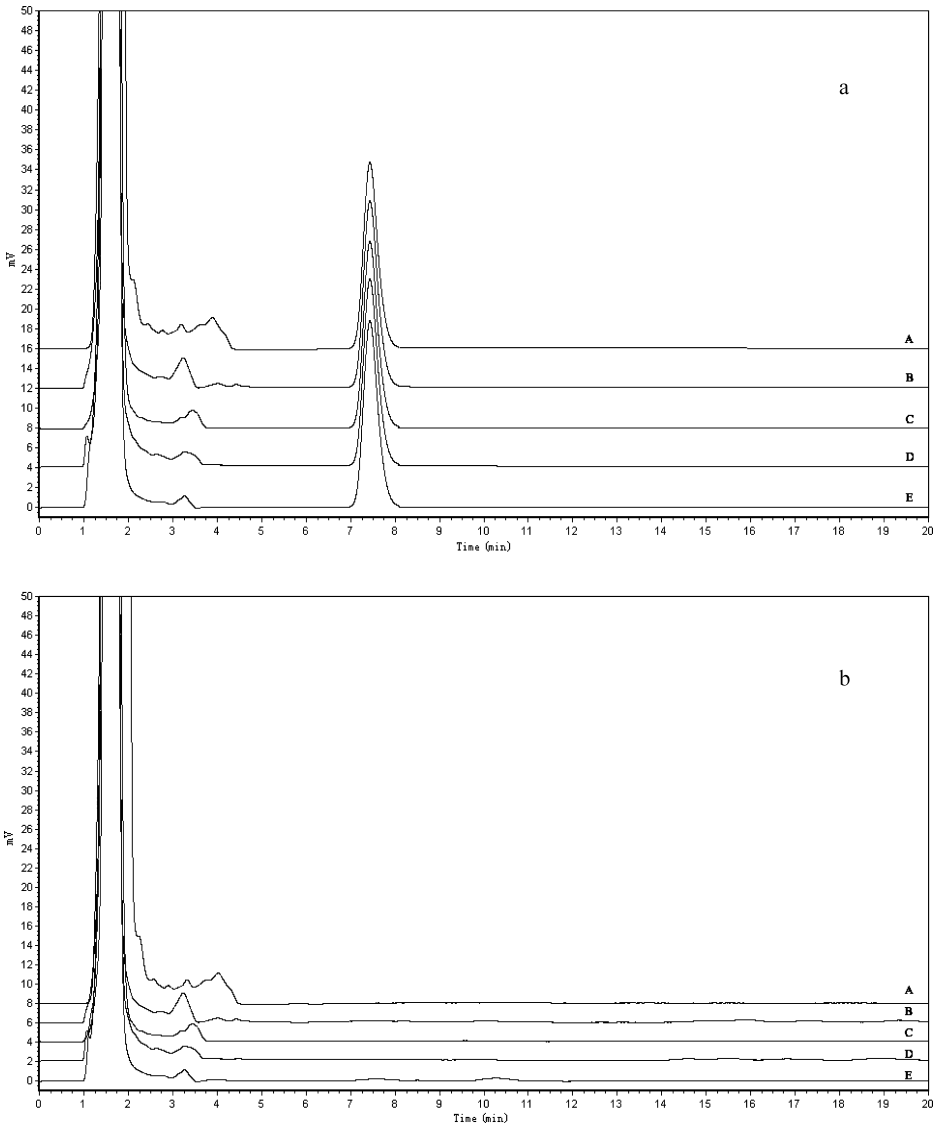

Fig. 2. (a) Representative Chromatogram of STS Spiked in Mouse Tissues and (b) Chromatogram of Blank Mouse Tissues

Peaks: A=liver, B=lung, C=kidney, D=heart, E=spleen.

Table 1. Linearity, Intra- and Inter-day Precision of STS Assay in Biological Specimens

Tissue	Linear regression equation	Correlation coefficient (r)	Intra-day precision (%) (n=18)	Inter-day precision (%) (n=18)
Plasma	Y=277273x−2493.1 (n=6)	0.9998	2.87	4.10
Heart	Y=259427x−1530.8 (n=6)	0.9989	2.63	2.53
Liver	Y=279014x−2471.6 (n=7)	0.9997	3.28	4.88
Kindey	Y=281256x−6961.5 (n=6)	0.9993	2.85	3.17
Spleen	Y=272827x+286.2 (n=6)	0.9983	2.74	2.83
Lung	Y=287151x−3588.4 (n=6)	0.9996	3.02	3.36

mean±standard deviation obtained from five mice. Pharmacokinetic analysis of STS concentration in mouse plasma samples were performed using Drug and Statistic (DAS) version 2.0 pharmacokinetic software (Shanghai University of T.C.M, China) to calculate area under the curve (*AUC*), distribution half-life ($t_{1/2\alpha}$), terminal elimination half-life ($t_{1/2\beta}$), plasma clearance (*CL*) and volume of distribution (*V*). The appropriate models fitting the plasma concentrations data were evaluated by criteria according to the goodness of fit for each model. These included the objective function, visual assessment of distribution of residuals, and Akaike's Information Criterion (AIC). The STS concentration of per tissue at various time point was calculated to express biodistribution of STS in the tissues. Data are presented as mean±S.D.

Results and Discussion

Method Validation The chromatograms of STS spiked in mouse plasma and blank mouse plasma were shown in previous reports.[10] The representative chromatograms of STS spiked in mouse tissues (A) and the blank mouse tissues (B) are shown Fig. 2.

The retention time of STS was 7.5 min. There was good baseline separation of STS and major components from mouse tissue samples. No endogenous or extraneous peaks were observed interfering with separation and quantification of STS. STS was stable during the time of analysis and no degradation products were found using the above-mentioned mobile phase. The linear regression equations for STS in mouse plasma and tissues are shown in Table 1. A linear relation was found between peak area and STS concentrations within the ranges of 0.1—5 μg/ml for plasma, 0.1—5 μg/g of tissue for kidney homogenate, 0.1—20 μg/g of tissue for liver homogenate, 0.1—1 μg/g of tissue for heart, spleen and lung homogenates, respectively. In the mouse plasma and tissues, the limit of quantification (LOQ) and the limit of detection (LOD) for STS were 100 ng/ml and 20 ng/ml.

The average precision rate for all the biological specimens are listed in Table 1. Over the range of STS tested, average inter- and intra-day precision of STS in these biological samples were within 4.9%, indicating a highly reproducible and robust assay. The average recoveries of STS after the extraction procedure are listed in Table 2. The recovery of this method was more than 92% at all concentration levels in each type of biological specimens on different days. The multi-day experiment also showed that the recovery of this method kept stable over 6 d.

It is known to all that the quality of bioanalytical methods will often be highly improved using an internal standard, which partly compensates inaccuracies in sample preparation, recovery, and chromatography. Because of the high and stable recovery of this method, we thought that there is no need to implement an internal standard.

Plasma Pharmacokinetics Figure 3 illustrates the plasma STS concentration–time profile after a intravenous dose of 50 mg/kg. STS plasma concentration–time data were best fitted with a two-compartment model, characterized by an initial rapid phase of drug concentration decrease, and a slower terminal elimination phase. At 4 h, the plasma concentrations were below 20 ng/ml. The pharmacokinetic parameters are shown in Table 3. The pharmacokinetics of STS was characterized with a distribution half-life ($t_{1/2\alpha}$) of 1.2±0.18 min, a terminal half-life ($t_{1/2\beta}$) of 21.6±2.4 min, a distribution volume (*V*) of 0.057±0.011 l/kg, a plasma clearance (*CL*) of 0.86±0.12 l/h/kg and an $AUC_{0-\infty}$ of 58.41±6.21 μg·h/ml.

Table 2. Recoveries of STS in Plasma and Tissue Samples (n=6)

Biological specimens	Added conc. (μg/ml)	Found conc. (μg/ml)	Recovery (%)
Plasma	0.1	0.095±0.0071	95.0±7.1
	1	0.96±0.062	96.0±6.2
	5	4.81±0.27	96.2±5.4
Heart	0.1	0.096±0.0082	96.0±8.2
	0.5	0.48±0.029	96.0±5.8
	1	0.97±0.047	97.0±4.7
Liver	0.1	0.094±0.0091	94.0±9.1
	1	0.94±0.052	94.0±5.2
	10	9.59±0.48	95.9±4.8
Kidney	0.1	0.093±0.0083	93.0±8.3
	1	0.97±0.057	97.0±5.7
	5	4.84±0.22	96.8±4.4
Spleen	0.1	0.092±0.009	92.0±9.0
	0.5	0.48±0.032	96.0±6.4
	1	0.97±0.053	97.0±5.3
Lung	0.1	0.093±0.0084	93.0±8.4
	0.5	0.47±0.031	94.0±6.2
	1	0.96±0.045	96.0±4.5

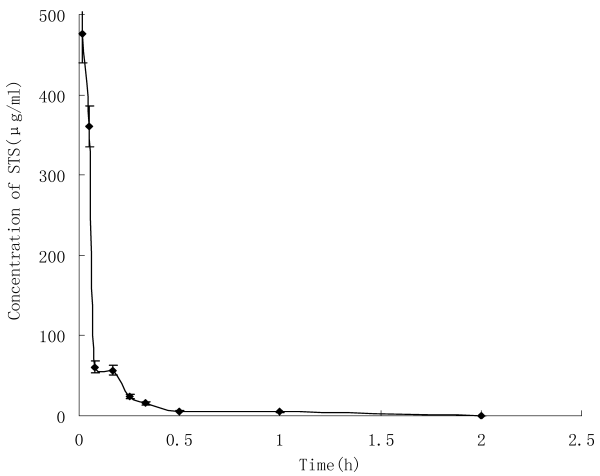

Fig. 3. Time Course of STS Levels in Mice Plasma
Each value represents the mean±S.D. of five mice per time point.

Tissue Distributions The distribution characteristics of

Table 3. Pharmacokinetic Parameters ($n=5$ Group) of STS Followed i.v. Dose of 50 mg/kg

Parameters	$t_{1/2\alpha}$ (min)	$t_{1/2\beta}$ (min)	V (l/kg)	CL (l/h/kg)	$AUC_{0-\infty}$ (μg·h/ml)
Mean±S.D.	1.2±0.18	21.6±2.4	0.057±0.011	0.86±0.12	58.41±6.21

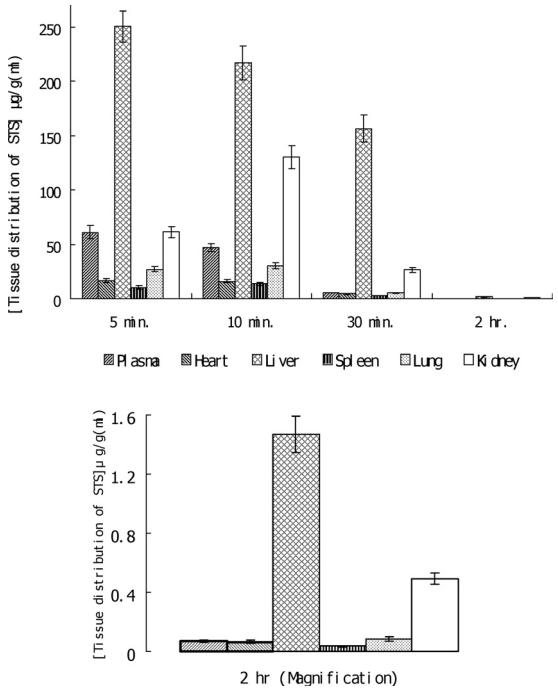

Fig. 4. STS Concentrations in Biological Specimens at 5 min, 10 min, 30 min and 2 h (Magnification) after i.v. Administration at a Dose of 50 mg/kg

Values represent the mean±S.D. of five mice at each time point.

STS to the various tissues are presented in Fig. 4. STS was widely distributed into most tissues after i.v. administration. The highest STS concentration was found in the liver. In liver, STS concentrations were maximal at 5 min after drug administration and declined progressively during 30 min, and decreased quickly with the time thereafter. STS could be determined at 12 h and 4 h after drug administration in liver and kidney, respectively. STS was hardly detected at 2 h after drug administration in the other tissues. In heart, STS concentrations were unaltered during 10 min, and declined fast after this. At 10 min after administration of STS, the highest STS concentration was found in kidney>lung>heart>spleen. This result suggested that STS was mainly cleared *via* both liver and kidney. Urine may be one of considerable excretion pathway for STS because of a relatively high STS concentrations in kidney. STS concentrations in heart, as compared with in other tissues, was not high at various time point in spite of STS as a cardiovascular drug. A dramatic concentration of STS in liver indicated that STS may be applied to treat liver disease such as chronic hepatitis, cirrhosis and hepatoma considering its extensive pharmacological activity. In fact, a few researches have demonstrated a promising potential of STS to treat liver disease.[11—13] In addition, a new chromatographic peak, which has a retention time of 2 min was determined for the first time in liver during 2 h after administration of STS injection. We presumed that the new compound may be one of STS metabolite, which may be more hydrophilic than STS. STS was metabolized not only with its original form but also with other form such as adding hydrophilic group.

Conclusion

In conclusion, we have developed and validated the ion-pair RP-HPLC method for the determination of STS in various biological samples. The method showed great linearity and had a high degree of selectivity, sensitivity, precision and recovery. We reported for the first time that pharmacokinetics and biodistribution characteristics of intravenously administrated STS in mice by using the validated HPLC method. STS was rapidly cleared from the circulation with a short half-life. STS was widely distributed into most tissues and was obviously accumulated in liver, which indicated that STS may be promising to treat liver disease. Urine may be one of considerable excretion pathway for STS.

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