Liquid chromatography mass spectrometry for the determination of salvianolic acid B, a natural compound from the herb Danshen in rat plasma and application to pharmacokinetic study

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ABSTRACT: A sensitive and specific liquid chromatographic–electrospray ionization mass spectrometric method was developed for quantification of salvianolic acid B in rat plasma with resveratrol as the internal standard. The analytes were separated on a reversed-phase column with acetonitrile (40%) and water (60%) containing 0.75% formic acid as mobile phase at a flow rate of 1 mL/min. Liquid–liquid extraction was adopted for the sample preparation, and the analytes were determined using electrospray negative ionization mass spectrometry in the selective monitoring mode. The method was validated over the concentration range 0.1–40 μg/mL using 0.1 mL of plasma with coefficients of correlation >0.999. The intra- and inter-day precisions of analysis were <10%, and accuracy ranged from 94 to 101%. This method was successfully applied to a pharmacokinetics of salvianolic acid B in rats. Copyright © 2009 John Wiley & Sons, Ltd.

Keywords: liquid chromatography–electrospray ionization mass spectrometry (LC-ESI-MS); salvianolic acid B (Sal B); pharmacokinetic

Introduction

Danshen, the dried roots of Salvia miltiorrhiza Bunge, is widely used as traditional Chinese medicine for the treatment of ischemic heart disease, hepatocirrhosis, hepatitis, heart-stroke and cerebrovascular diseases (Sugiyama et al., 2002; Sze et al., 2005). Danshen is mainly used as a decoction. The water-soluble components, which are mostly phenolic acids, are responsible for the therapeutic effects of the extract of Danshen. Sal B (Fig. 1), the major water-soluble component isolated from the Salvia miltiorrhiza Bunge, has a wide spectrum of bioactivities, including reduction of atherosclerosis (Wu et al., 1998), antioxidation (Du et al., 2000), attenuation of cyclooxygenase-2 (Chen et al., 2006) and TGF-β1 expression (Wang et al., 2005; Zhao et al., 2004), inhibition of platelet aggregation (Li et al., 2004; Tang et al., 2002), free radical scavenging effect (Wu et al., 2000), inhibition of proliferation, collagen production and protection of hepatocytes and hepatic stellate cells (Lin et al., 2006; Liu et al., 2002a). Sal B was also found capable of reversing liver fibrosis in chronic hepatitis B in a clinical study (Liu et al., 2002b).

Determination and pharmacokinetics related to Sal B have been studied in biological samples using high-performance liquid chromatography (Ma and Wang, 2007; Wu et al., 2006; Zhang et al., 2005; Chen et al., 2005). In this paper we present a selective and simple LC-ESI-MS method for determination of Sal B concentration in plasma, and its application to the pharmacokinetic study in rats. The pharmacokinetic study in rats may provide useful information for clinical trials.

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Figure 1. Chemical structure of salvianolic acid B and resveratrol.
Experimental

Chemical and Reagents
Sal B was isolated from the roots of *Salvia miltiorrhiza*, with a purity of over 98% by HPLC analysis. Sal B was dissolved in normal saline for intravenous injection in the animal experiment. Acetonitrile was of HPLC grade from Tedia (Fairfield, USA). Resveratrol (internal standard, IS, Fig. 1) was obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Formic acid and other chemicals were of analytical grade (Nanjing Chemical Reagent Co. Ltd, Jiangsu Province, China). Liquid nitrogen was from the Gas Supplier Center of China. Triple deionized water (Millipore, Bedford, MA, USA) was used for all preparations. Heparin sodium was from Shanghai Biochemical Reagent Co. Ltd. Heparin solution at 50 mg/mL in saline was used to rinse the test tubes prior to blood collection for plasma.

Instruments
A Shimadzu (Kyoto, Japan) LC-MS-2010A liquid chromatograph–mass spectrometer equipped with a SIL-HTC autosampler, two LC-10AVP pumps and an electrospray-ionization (ESI) interface was used for LC-MS analyses. Data acquisition and processing were accomplished using Shimadzu LC-MS Solution (version 3.20 with Windows XP operating system).

Animal Treatment
Male Sprague–Dawley (SD) rats (180–220 g) were purchased from Shanghai Sino-British Sippr/BKLab Animal Ltd (Shanghai, China). All rats were maintained in a clean room at 22 ± 2°C and relative humidity of 50 ± 10%, and had free access to water and standard animal diet. All experiments were carried out in accordance with the guidelines of the Committee on the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources of Jiangsu, China. The study protocol described below was approved by the Institutional Animal Care and Use Committee of China Pharmaceutical University.

Rats were given a single intravenous dose of Sal B at 10, 20 or 40 mg/kg. Sal B solutions were formulated shortly before administration and were diluted with saline. Blood samples were collected from the fossa orbitalis at 3, 10, 15, 30, 45, 60, 90, 120, 180 and 240 min post-dosing. All plasma samples were frozen and maintained at −20°C until analysis.

LC-MS Conditions
LC separation was performed using Hypersil C18 (10 μm, 200 × 4.6 mm, Dalian Elite Company, Liaoning Province, China) coupled with a SecurityGuard C18 guard column (4 × 3.0 mm, Phenomenex, Torrance, CA, USA). The mobile phase consisted of 60% water (containing a mass fraction of 0.75% formic acid) and 40% acetonitrile for an isocratic elution at 28°C. The mobile phase was delivered at a flow rate of 1.0 mL/min with post-column splitting. A quadrupole mass spectrometer equipped with an electro-spray ionization source was operated with a drying gas (N2) flow of 1.5 L/min, the detector voltage of 1.80 kV and probe voltage of 3.5 kV. The heat block temperature was 250°C and the curved desolvation line (CDL) temperature was maintained at 230°C. LC-ESI-MS was performed in negative selected-ion monitoring mode. Sal B was detected at m/z 717 and resveratrol at m/z 227. The quantification was performed using peak areas.

Preparation of Standard and Quality Control Solutions
Stock standard solutions of Sal B were prepared by weight at 1.0 mg/mL in MeOH. Standard and quality control (QC) samples (10 μg/mL) were made from separate stock solutions. Standard samples were prepared over a range of 1–400 μg/mL of Sal B. A working IS solution was prepared at 500 μg/mL. All standards were stored at 4°C.

Sample Preparation
Rat blood samples were centrifuged at 10,000g for 3 min immediately after collection and 100 μL of the plasma was promptly transferred to an Eppendorf tube. These samples were stored at −20°C until analysis. An aliquot (10 μL) of IS stock solutions was added into 0.1 mL of sample and mixed. The samples were acidified by mixing with 20 μL of hydrochloric acid (2.5 mol/L). After addition of 1 mL of ethyl acetate, the mixture was vortexed for 3 min and centrifuged at 12,000 rpm for 5 min. The organic layer (0.9 mL) was transferred to an Eppendorf tube and evaporated to dryness under a stream of air at 30°C. The residue was dissolved in 0.2 mL mobile phase. After centrifugation at 12,000 rpm for 5 min, 20 μL of the supernatant was subjected to LC-MS analysis.

Precision, Accuracy and Matrix Effect
The precision and accuracy of the method were confirmed by the five replicate determinations of plasma samples containing Sal B at three concentration levels (0.5, 2.0, and 10.0 μg/mL) in a single batch and three different batches. The determined concentrations, which were obtained from a calibration curve prepared on the same day, were used to evaluate the method precision and accuracy. The precision was evaluated by the coefficient of variation (CV), and the accuracy was expressed as the relative error (RE) according to the equation: RE (%) = 100% × (measured concentration − nominal concentration)/nominal concentration. The criteria for acceptability of the data included precision within 15% CV and accuracy within ±15% RE from the nominal values. To evaluate matrix effect, blank plasma was subjected to the sample pretreatment described above. The resulting solution was spiked with working standard solutions to prepare solutions containing Sal B at three different concentrations (0.5, 2.0, and 10.0 μg/mL). Matrix enhancement/suppression of ionization was evaluated by comparing the peak areas of spiked processed samples with corresponding neat standard solutions prepared in mobile phase.

Stability Experiments
Three concentration levels (0.5, 2.0 and 10.0 μg/mL) of spiked samples were assessed after the storage at room temperature for up to 4 h, after three freeze–thaw cycles, and at −20°C for 1 month. Sal B stability in the injection solvent was determined periodically by injecting processed samples for up to 12 h (in autosampler at 4°C) after the initial injection. Samples were considered to be stable if the bias between the concentrations determined from 0 h and under different stability conditions was within the acceptance criterion recommended by Food and Drug Administration (USFDA); http://www.fda.gov/cder/guidance/
Pharmacokinetics of salvianolic acid B in rats


The bias was calculated as follows: % bias = \( \frac{C - C_0}{C_0} \times 100 \).

Pharmacokinetic Application

Calibration curves were constructed based on LC-MS analyses of standard mixtures prior to each experiment. Pharmacokinetic calculations were performed on each individual set of data using the pharmacokinetic software WinNonlin Standard Edition Version 1.1 (Pharsight Corp., Mountain View, CA, USA) by non-compartmental method. The area under the concentration–time curve (AUC) was calculated according to the linear trapezoidal method.

Results

The LC-MS method described provided good separation of Sal B and Resveratrol from the other endogenous plasma constituents. There was no interference between the IS and Sal B. Figure 2 shows an MS chromatogram of blank rat plasma (a, b), blank rat plasma containing Sal B and Resveratrol (c, d), and a plasma sample from a rat administered Sal B and Resveratrol (e, f). The retention times were about 3.2 min for Sal B and 4.8 min for Resveratrol.

Linearity and Limits of Detection and Quantitation

Least squares regression calibration curves were constructed by plotting the peak area ratios of analyte to IS vs nominal concentration. The correlation coefficient (\( r^2 \)) was typically better than 0.99 over the concentration range of 0.1–40.0 \( \mu \)g/mL. The deviation between the nominal concentrations and measured concentrations was generally within 10% with the use of the least squares regression (\( n = 5 \)). The lower limit of quantification (LLOQ) was 0.1 \( \mu \)g/mL for Sal B in rat plasma.

Accuracy and Matrix Effect

The accuracy and matrix effect of Sal B from rat plasma are shown in Table 1. The accuracy was lower than 10%. No significant

Figure 2. Representative LC-ESI-MS negative SIM chromatograms obtained from blank rat plasma at m/z 717 (a) and at m/z 227 (b); blank rat plasma spiked with salvianolic acid B (10 \( \mu \)g/mL) and resveratrol (IS) (100 \( \mu \)g/mL) at m/z 717 (c) and m/z 227 (d); and a rat plasma sample after intravenous injection of 40 mg salvianolic acid B, monitored at m/z 717 (e) and m/z 227 (f).
Matrix effect was observed. The negligible relative matrix effect was also confirmed by the low coefficient of variation between the slopes of the five standard curves.

**Precision**

The data proved good precision of the method. As shown in Table 2, the intra- and inter-day precisions of the assay, as measured by the coefficient of variation (RSD %), were both lower than 10%. The results indicated the method was reproducible.

**Stability**

Assessment of the stability of Sal B in rat plasma at −20°C for 1 month and after three freeze–thaw cycles was performed. The results indicated that Sal B was stable in plasma under those conditions (Table 3). Processed sample stability in the autosampler for up to 12 h was evaluated. The bias between the concentrations determined from the initial injection and the re-injections was within 10%.

### Pharmacokinetics in Rats

The mean plasma concentration–time profiles of Sal B after intravenous administration are shown in Fig. 3. The pharmacokinetic parameters of three representative doses are shown in Table 4. After intravenous administration of Sal B at doses of 10, 20 and 40 mg/kg, the C₀ values for Sal B were estimated to be 11, 22 and 37 μg/mL, respectively. The AUC increased with increasing doses, and the mean AUC₀–ₚ values were 199 ± 21, 452 ± 38 and 761 ± 121 μg min/mL, respectively. The analysis of variance of CL, T½, V and MRT showed no difference among the three doses of treatments. Good linearity of the kinetics of Sal B after intravenous administration was observed in the regression analysis of the AUC–dose plot (r > 0.96), and the C₀–dose relationship was also linear (r > 0.95).

### Discussion

In this study, a liquid–liquid extraction method using acetic ether was developed for quantitative determination of Sal B in rat plasma.
The present study reported the pharmacokinetic parameters of Sal B after intravenous injection in rats. The result showed that Sal B was eliminated rapidly in rats after intravenous administration; the concentrations were about 100 times lower at 240 min than were obtained at 3 min.

In conclusion, the method of liquid chromatography mass spectrometry was validated by the fact that the calibration curve for Sal B was linear over the range 0.1–40 μg/mL with a coefficient of correlation of >0.997. The inter-day and intra-day precisions of analysis were <10%, and the assay accuracy ranged from 94.7 to 101.9%. This indicates that the LC-MS method has good reproducibility, accuracy and precision and could be applied for the quantitative assay of plasma concentration of Sal B in pharmacokinetic studies.

**Acknowledgements**

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**Table 4.** Pharmacokinetics parameters of salvinianolic acid B after intravenous injection administration to rat (*n* = 6, mean ± SD)

<table>
<thead>
<tr>
<th>Parameters</th>
<th>10</th>
<th>20</th>
<th>40</th>
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<tbody>
<tr>
<td>( C_0 (\mu g/mL) )</td>
<td>10.6 ± 1.1</td>
<td>21.9 ± 1.7</td>
<td>37.4 ± 6.4</td>
</tr>
<tr>
<td>( AUC_{0-\infty} (\mu g.min/mL) )</td>
<td>198.5 ± 21.3</td>
<td>451.8 ± 38.3</td>
<td>760.9 ± 121.0</td>
</tr>
<tr>
<td>( AUC_{\infty} (\mu g.min/mL) )</td>
<td>226.7 ± 20.4</td>
<td>494.5 ± 41.7</td>
<td>854.5 ± 120.6</td>
</tr>
<tr>
<td>( T_{1/2} ) (min)</td>
<td>154.7 ± 24.3</td>
<td>127.4 ± 15.7</td>
<td>139.0 ± 22.6</td>
</tr>
<tr>
<td>MRT (min)</td>
<td>90.1 ± 14.7</td>
<td>67.2 ± 5.3</td>
<td>82.1 ± 16.2</td>
</tr>
<tr>
<td>( V_d (L/kg) )</td>
<td>9.9 ± 2.1</td>
<td>7.4 ± 0.8</td>
<td>9.6 ± 2.5</td>
</tr>
<tr>
<td>CL (L/min/kg)</td>
<td>0.044 ± 0.004</td>
<td>0.041 ± 0.003</td>
<td>0.048 ± 0.007</td>
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