Validation and Application of an Rapid HPLC–MS Method for the Determination of Salvianic Acid A in Human Plasma

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A rapid liquid chromatography–electrospray ionization-tandem mass spectrometry (LC–ESI–MS–MS) method was developed and validated for the determination of salvianic acid A in plasma of Chinese healthy subjects after oral administration of Qishenyiqi dropping pills. After liquid–liquid extraction with ethyl acetate, salvianic acid A was chromatographed on a Agilent Zorbax XDB-C18 column using a gradient mobile phase consisting of water (0.1% formic acid)–acetoniitrile (0.1% formic acid) at a flow rate of 0.45 mL/min. The detection was performed in multiple reaction monitoring mode, using the transitions of m/z 196.9→134.8 and m/z 320.9→151.9 for salvianic acid A and chloramphenicol, respectively. The method was linear over the range of 0.50–500 ng/mL using only 100 μL of plasma and the lower limit of quantification was 0.50 ng/mL. Intra-day and inter-day precisions (in terms of % RSD) were all <15% and the accuracies (in terms of % RE) were within the range of ±15%, and recoveries were between 85.0 and 115%. The validated method was successfully applied to pharmacokinetic study of Qishenyiqi dropping pills in Chinese healthy subjects. After oral administration, T\text{max} and C\text{max} values were 1.33 ± 0.52 h and 21.1 ± 3.92 ng/mL, respectively. Plasma concentrations declined with t\text{1/2} of 1.76 ± 0.33 h.

Introduction

Qishenyiqi dropping pills is a compound Chinese medicine approved by China State Food and Drug Administration (SFDA) in 2003 for treatment of cardiac dysfunction (1, 2), which is composed of four traditional Chinese medicines: Radix Salviae Miltiorrhizae, Astragalus membranaceus, Panax notoginseng and Dalbergia odorifera. Pharmacological studies have demonstrated that treatment with QSYQ attenuates pressure over-load-induced cardiac hypertrophy and myocardial fibrosis through interfering in inflammatory process (3, 4). QSYQ was also reported to attenuate rat 1/R-induced myocardial fibrosis (5). Furthermore, study by proteomic technique has shown that myocardial protective effects of QSYQ in myocardial infarction process might be closely related to the recovery of energy supply, in addition to the reduction of oxidative stress (6, 7).

There are many natural components existing in Qishenyiqi dropping pills, including salvianic acid A, protocatechualdehyde, ginsenosides Rg1, ginsenosides Rb1, astragaloside IV, flavonoids and essential oils, etc. (8). The contents of salvianic acid A, protocatechualdehyde, ginsenosides Rg1, ginsenosides Rb1, astragaloside IV in Qishenyiqi dropping pills were determined to be ~133, 32, 53, 30 and 25 μg, respectively (9). Among them, salvianic acid A (CAS number: 76822-21-4, IUPAC name: (R)-α,3,4-trihydroxy-benzene propanoic acid, chemical structure shown in Figure 1) is usually considered to be one of the main therapeutic components for cardiac dysfunction. Also, salvianic acid A is now used as one marker compound for the quality control (QC) of Qishenyiqi dropping pills. Therefore, salvianic acid A will be used as a target biomarker compound for the human pharmacokinetic evaluation of Qishenyiqi dropping pills.

For the quantification of salvianic acid A in complicated biological samples, liquid chromatography coupled to fluorescent (10), UV (11–13) or MS (14–20) methods have been reported depending on the required sensitivity, the biological matrix, and the applied pretreatment/workup procedures. HPLC-UV and fluorescent methods were general analytical methods for the determination of salvianic acid A in biological fluids, however, they both showed poor sensitivity [lower limit of quantification (LLOQ) usually above 0.1 μg/mL]. Liquid chromatography–tandem mass spectrometry (LC–MS–MS) has been used to identify and quantify salvianic acid A in biological fluids with LLOQ of ~5–50 ng/mL. However, even LLOQ of 5 ng/mL is not enough in the present study due to the above-mentioned very lower dosing level of salvianic acid A. Therefore, methodological improvements for the quantitative determination of genistein in biological fluids are still needed, especially the optimization of LLOQ and analytical throughput. Most importantly, short retention and run time are very important to improve the high-throughput efficacy in clinical studies. And, to our best knowledge, no bioanalytical methods have been reported for the quantification of salvianic acid A in human plasma after oral administration of Qishenyiqi dropping pills till now. Furthermore, no pharmacokinetic results of Qishenyiqi dropping pills in human subjects have been reported.

In the present study, a rapid LC–MS–MS method was developed and validated for the determination of salvianic acid A in plasma of Chinese healthy subjects, and the validated method has been successfully applied to an explorative pharmacokinetic study of salvianic acid A in Chinese healthy subjects following oral administration.

Experimental

Chemicals and reagents

Reference standard of sodium salvianic acid A (Purity 100%, Lot No. 110855–200809, chemical structure shown in Figure 1) was
LC–MS–MS instrumentation and analytical conditions

The HPLC system consisted of an LC-20AD pump, a DGU-20 A³ degasser, an SIL-20AC autosampler and a CTO-20A column oven (Shimadzu, Japan). HPLC separation was performed on an Agilent Zorbax XDB-C18 column (2.1 mm × 50 mm, 3.5 μm) with a gradient elution by a mobile phase consisting of water containing 0.1% formic acid (A) and acetonitrile containing 0.1% formic acid (B) with following gradient: 0.00 min 2% B, 0.50 min 2% B, 3.00 min 60% B, 3.50 min 90% B, 4.50 min 90% B, 4.51 min 2% B, 6.00 min 2% B, with the flow rate of 0.45 mL/min. The injection volume was set to be 10 μL.

The HPLC system was coupled with an API 4000 Qtrap mass spectrometer (Applied Biosystems/MDS Sciex, Concord, ON, Canada) via a Turbo IonSpray ionization interface. Following optimization of the setting parameters, the ESI source was operated in negative mode with the curtain, nebulizer and turbo-gas (all nitrogen) set at 15, 65 and 55 psi, respectively. The source temperature was 600 °C and the ion spray needle voltage was −4200 V. The mass spectrometer was operated at unit resolution for Q1 and low resolution for Q3 in the multiple reaction monitoring (MRM) mode, with a dwell time of 150 ms per MRM channel. The collision activated dissociation gas level was set at medium. Two MRM transitions (m/z 196.9 → 134.8, salvianic acid A; m/z 320.9 → 151.9, IS) were recorded and used for quantification. The optimized collision energies for the transitions of salvianic acid A and IS were set at −24 and −20 eV, respectively. The declustering potentials were set at −70 and −50 eV for salvianic acid A and IS, respectively. Data were collected and analyzed by the Analyst Data Acquisition and Processing software (Version 1.5.2, Applied Biosystems/MDS Sciex, Concord, ON, Canada).

Preparation of calibration standards and QC samples

Stock standard solution of salvianic acid A at 1.00 mg/mL was prepared in duplicate by dissolving the accurately weighed reference standard in DMSO for preparation of calibration standards and QC, respectively. Series of standard combined dilutions were prepared in methanol at 5.00, 10.0, 20.0, 50.0, 200, 500, 2000 and 5000 ng/mL for salvianic acid A. Calibration standards were prepared at 0.50, 1.00, 2.00, 5.00, 20.0, 50.0, 200 and 5000 ng/mL by adding 10 μL of standard combined dilutions to 100 μL of analyte-free plasma of healthy subjects. QC samples were similarly prepared at concentrations of 1.00, 20.0 and 400 ng/mL for low, medium and high QC, respectively. IS working solution at a concentration of 2000 ng/mL was prepared by diluting the chloroamphenicol stock solution (1.00 mg/mL) with methanol/water (1:1, v/v). All the solutions were kept refrigerated (4 °C) and were brought to room temperature before use.

Sample preparation

After thaw at room temperature for ~30 min and vortex for 30 s, aliquots of 100 μL human plasma were mixed with 10 μL of methanol (or standard or QC solution), 10 μL of IS solutions [2000 ng/mL chloroamphenicol in methanol/water (1:1, v/v)], 20 μL of 1 mol/L HCl solution and 600 μL of ethyl acetate. After vortex for 1 min and then centrifugation at 10,000 g for 10 min, aliquots of 450 μL supernatants were removed and evaporated to dryness at 40°C under a gentle stream of nitrogen.
The residues were dissolved in 100 μL of the mixture of methanol and water (1:1, v/v), and then transferred to HPLC vials. A volume of 10 μL of this solution was then injected onto the column for LC–MS–MS analysis.

Method validation

The method was validated for specificity, linearity, precision and accuracy, matrix effect, recovery and stability according to the FDA guidelines (21).

Specificity means the ability of a method to distinguish between the measured analytes and other substances, which can change according to the compound class or matrix. To investigate the specificity, one pooled batch of blank plasma from six different healthy subjects and several spiked plasma samples at LLOQ level were pretreated and analyzed in parallel. The obtained responses were compared with those of LLOQ. The peak area of coeluting interferences should be <20% of the peak area of the LLOQ.

Calibration standards were prepared and analyzed in duplicate in three consecutive days. The peak area ratios (salvianic acid A versus IS) versus the nominal concentrations of salvianic acid A were calculated to construct the calibration curves. The calibration curves were fitted via a 1/x2 weighted linear least-squares regression model.

Intra- and inter-day accuracy and precision were determined by assay of six replicates of low, medium and high QC samples on three different days and a minimum of two-thirds of the total number of QCs must not deviate by >±15% from their nominal concentration, and at least half of the QC samples at each concentration must be within 100 ± 15% of their nominal concentration. In the present study, the precision was reflected by the relative standard deviation (% RSD), and the assay accuracy was reflected by the relative percentage error from the theoretical drug concentrations (% RE).

The extraction recovery of salvianic acid A was determined at three QC levels by comparing the mean peak areas of QC samples (n = 6) with those of the blank plasma samples (n = 3) spiked with working solutions after pretreatment. The extraction recovery of IS was determined using a similar method.

To evaluate the matrix effect in the experiment, six different lots of blank plasma were pretreated and then spiked with QC solutions. Chromatographic peak areas of each analyte from the spike-after-pretreatment samples were compared with those of the solution standards at equivalent concentrations (22). In the present study, the matrix effect was evaluated at three concentration levels for each analyte. The matrix effect for IS was determined in a similar way at 200 ng/mL. Inter-subject variability at the matrix effect should be <15% (23).

In the present study, the stability of salvianic acid A was investigated by analyzing replicates (n = 3) of plasma samples at three concentration levels, which were exposed to different conditions (processed, time and temperature). The analyte was considered stable under the prescribed conditions only if 85–115% of the nominal concentrations were calculated.

Results

Method validation

Assay specificity

The validated method was high selective for the target analyte because no significant interference was observed in the blank plasma samples from six different sources. Figure 2 shows the typical chromatograms of salvianic acid A and chloramphenicol (IS) in a blank human plasma sample, and blank plasma sample spiked with salvianic acid A at the LLOQ level (0.50 ng/mL) and IS (200 ng/mL), a plasma sample collected at 2 h after oral administration of 4.5 g of Qishenyiqi dropping pills (9 pouches) to healthy subjects.

Linearity of calibration curve and LLOQ

The linear regression of the peak ratios versus concentrations was fitted over the plasma concentration range of 0.50–500 ng/mL for salvianic acid A. The typical linear regression equation of the calibration curves generated during the validation was as follows: \( y = 0.00243 \ x + 0.000205 \) (\( r = 0.9973 \)), where \( y \) represents the peak area ratio of salvianic acid A to the IS and \( x \) is the nominal concentration of salvianic acid A. The LLOQ of salvianic acid A was determined to be 0.50 ng/mL with acceptable accuracy (% RE was calculated to be 0.9%) and precision (% RSD was calculated to be 5.1%).

Precision and accuracy

Table I summarizes the intra and inter-day precision and accuracy values for the QC samples. The intra and inter-day precisions for salvianic acid A were <12.0%, while accuracy was within ±12.0%. The accuracy and precision data indicate that the method is reliable and reproducible.

Extraction recovery and matrix effect

The extraction recovery results of salvianic acid A at 1.00, 20.0 and 400 ng/mL were calculated to be 88.3, 93.5 and 94.1%,
respectively. The extraction recovery of IS at 200 ng/mL was estimated to be 90.5%. The matrix effects for salvianic acid A determined at 1.00, 20.0 and 400 ng/mL were 83.5, 81.9 and 86.4%, respectively. The matrix effect for IS determined at 200 ng/mL was 89.6%. The inter-subject variability of matrix effects for each analyte was below 11.3%. As a result, the matrix effects for salvianic acid A and IS were negligible in present conditions.

**Stability**

The stability results were presented in Table II. Salvianic acid A at the three concentrations tested had acceptable stabilities after three cycles of freeze--thaw, at room temperature for 24 h and at −20 °C for 1 month with the % RE values being within ±15%, indicating salvianic acid A remained stable during pretreatment, chromatography and sample storage processes in human plasma samples.

**Method application**

The validated method was successfully applied to an explorative pharmacokinetic study of salvianic acid A in Chinese healthy subjects after oral administration of Qishenyiqi dropping pills. Individual and mean plasma concentration–time curves of salvianic acid A in Chinese healthy subjects after oral administration of 1.5 g of Qishenyiqi dropping pills (3 pouches) (n = 6) are presented in Figure 3. Pharmacokinetic parameters of salvianic acid A in healthy subjects are shown in Table III. After oral administration of Qishenyiqi dropping pills, T_{max} and C_{max} values of salvianic acid A were found to be 1.33 ± 0.52 h and 21.1 ± 3.92 ng/mL, respectively. Plasma concentrations declined with t_{1/2} of 1.76 ± 0.33 h. MRT_{0–∞} was calculated as 3.45 ± 0.37 h. The AUC_{0–t} and AUC_{0–∞} values obtained were 68.0 ± 7.44 and 70.1 ± 7.49 ng/mL·h, respectively.

**Discussion**

**Mass spectrometry**

There are carboxyl and hydroxyl groups in the structure of salvianic acid A. Hence, it is assumed that salvianic acid A would produce stronger mass spectrometric (MS) responses in negative ionization mode, rather than in positive ionization mode. In connection with actual experiment results, negative ion detection mode was finally chosen. Furthermore, salvianic acid A exhibited higher MS responses under electronic spray ionization (ESI) source than under atmospheric pressure chemistry ionization source. The full-scan product ion mass spectra and fragmentation patterns of salvianic acid A and chloroamphenicol are shown in Figure 1. In the Q1 full-scan experiment, distinct deprotonated molecules were observed for salvianic acid A (m/z 196.9), and
no significant adductive ions were detected. Usually the most abundant fragment ion was selected in the MRM transitions. For salvianic acid A, the most abundant ion was observed at \( m/z \) 178.8 (a dehydrated ion). However, the background noise of the transition \( (m/z \) 196.9–178.8) was relative higher than that of the transition \( (m/z \) 196.9–134.8). Therefore, the MRM transition of \( m/z \) 196.9–134.8 was finally chosen as it provided a better signal-to-noise ratio (S/N), reproducibility and response than other transitions.

**Chromatography**

During the optimization of chromatographic conditions, salvianic acid A might be extensively retained on several kinds of columns due to its physical–chemical properties. To achieve symmetric peak shapes and shorten chromatographic running time compromising the potential matrix effect mostly arising from all kinds of coexisting components, the mobile phase consisting of acetonitrile with 0.1% formic acid and water with 0.1% formic acid was used on a Zorbax XDB C18 column. It is well known that addition of acid modifier in the mobile phase might perhaps improve the peak shapes of acidic analytes, but these acid modifiers would also induce ion suppression at the same time. Therefore, the effect of formic acid and acetic acid as acidic modifier on the ion suppression had been compared during the development of present method. No significant difference was obtained. Considering the different boiling point of formic acid (100.7°C) and acetic acid (117.9°C), formic acid was finally chosen as pH modifier in the mobile phase. By applying a first slowly ascending gradient and a second relative sharp ascending gradient, salvianic acid A and IS were eluted between early eluting hydrophilic components and later eluting hydrophobic components, while maintaining a relative short analysis time of 6.0 min. All these hydrophilic and hydrophobic components were considered to be potentially response-suppressing matrix components. As shown in Figure 2, the retention times for salvianic acid A and IS were 2.39 and 3.40 min, respectively. The chromatograms show baseline separation of salvianic acid A and the internal standard without any interference from endogenous plasma components and other coexisting components in Qishenyiqi dropping pills. The total run time of one sample using present method is only 6.0 min, and >100 samples could be analyzed in 1 day.

**Sample preparation**

Considering the complexities of Qishenyiqi dropping pills and human plasma matrix, protein precipitation (PPT) was first excluded to be an applicable sample preparation method, because PPT is well known as a non-selective purification method, which may introduce high amounts of endogenous components and can cause signal suppression or enhancement (i.e., matrix effect), especially with an ESI ionization source. Liquid–liquid extraction (LLE) and solid-phase extraction (SPE) techniques were further investigated to be utilized in the sample preparation of human plasma. However, as thousands of plasma samples would be analyzed during the clinical trials, relative economic LLE technique had become our first and preferred consideration for its economic and efficiency. In the present experiment, different LLE conditions were evaluated including different pH modifiers and extraction solvents. Three organic extraction solvents (diethyl ether, dichloromethane and ethyl acetate) were

**Figure 3.** Individual (A) and mean (B) plasma concentration–time curves of salvianic acid A in Chinese healthy subjects after oral administration of 1.5 g of Qishenyiqi dropping pills (3 pouches) \( (n = 6) \).

**Table III**

| Pharmacokinetic Parameters of Salvianic Acid A in Healthy Subjects Determined After Oral Administration of Qishenyiqi Dropping Pills \( (n = 6) \) |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Parameter       | Unit            | Subject 1       | Subject 2       | Subject 3       | Subject 4       | Subject 5       | Subject 6       | Mean            | SD |
| AUC\(_0\rightarrow t\) | ng/mL*hr       | 70.1            | 73.5            | 77.0            | 56.0            | 63.9            | 67.8            | 68.0            | 7.44 |
| AUC\(_0\rightarrow \infty\) | ng/mL*hr       | 70.8            | 76.8            | 79.3            | 59.2            | 64.7            | 69.6            | 70.1            | 7.49 |
| MRT\(_0\rightarrow t\) | hr             | 2.71            | 3.30            | 3.37            | 3.11            | 3.08            | 3.45            | 3.17            | 0.27 |
| MRT\(_0\rightarrow \infty\) | hr             | 2.80            | 3.72            | 3.64            | 3.65            | 3.19            | 3.68            | 3.45            | 0.37 |
| \( t_{1/2}\) | hr             | 1.43            | 2.09            | 1.70            | 2.21            | 1.45            | 1.69            | 1.76            | 0.33 |
| \( t_{max}\) | hr             | 1.00            | 1.00            | 2.00            | 1.00            | 1.00            | 2.00            | 1.33            | 0.52 |
| \( C_{max}\) | ng/mL          | 27.9            | 21.6            | 22.0            | 16.4            | 18.8            | 19.6            | 21.1            | 3.92 |

SD, standard deviation.
evaluated. Among them, the last one (ethyl acetate) yielded the best clean-up of the plasma samples and highest and most stable recovery values. It was found that addition of 1 mol/L HCl solution could significantly increase the extraction of salvianic acid A.

Selection of internal standard
An appropriate internal standard is usually required in LC–ESI–MS-MS analysis in order to eliminate the effects from matrix and the pretreatment efficiency. Usually stable isotope-labeled internal standards are the optimal choice, however, an isotopically labeled internal standard for salvianic acid A was not readily available and could not be obtained. Other naturally compounds, which may be present in Qishenyiqi dropping pills, should not be used as internal standard. In this study, chloroamphenicol, a chemical synthetic compound, was selected as the internal standard (IS); its chromatographic behavior and extraction efficiency were similar to that of salvianic acid A, and in addition, there were no interferences from the analytes and endogenous substances.

Pharmacokinetic
After oral administration of Qishenyiqi dropping pills, the plasma concentrations of salvianic acid A were very low, with $C_{\text{max}}$ of $21.1 \pm 3.92$ ng/mL. As a compound formulation of TCM, there are many natural components existing in Qishenyiqi dropping pills. We had tried our best to quantify other components, including protocatechualedehyde, ginsenosides Rg1, ginsenosides Rb1, astragaloside IV. However, the concentrations of these compounds were almost near or below their respective LLOQ (LLOQs ~ 1–10 ng/mL, data not shown). The contents of salvianic acid A, protocatechualedehyde, ginsenosides Rg1, ginsenosides Rb1, astragaloside IV in Qishenyiqi dropping pills were determined to be ~ 133, 32, 53, 30 and 25 µg, respectively. As a result, salvianic acid A is now used as a target biomarker compound for the human pharmacokinetic evaluation of Qishenyiqi dropping pills. And we will further struggle to develop more sensitive bioanalytical methods to quantify other component in Qishenyiqi dropping pills in order to clarify its pharmacological effects in human.

Conclusion
The optimized method was validated to guarantee the reliable determination of salvianic acid A in the plasma of Chinese healthy subjects. The LLOQ of the method is 0.50 ng/mL for salvianic acid A using 100 µL of human plasma sample. Relative simple and economic LLE procedure and the running time of no more than 6 min allowed sample throughput of 120–150 samples per day and thus made the method easily applied to clinical trials.

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