ORIGINAL ARTICLE

Pharmacokinetic study of salvianolic acid D after oral and intravenous administration in rats

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Abstract A sensitive, specific and rapid LC-MS method was developed and validated for the determination of salvianolic acid D (SalD) in rat plasma. This method used a single quadrupole mass spectrometer with an electrospray ionization (ESI) source. A single ion monitoring scanning (SIM) mode was employed. It showed good linearity over the concentration range from 3.3 to 666.7 ng/mL for the determination of SalD. The R.S.D.% of intra-day and inter-day precision values were no more than 7.69%, and the accuracy was within 91%−104% at all quality control levels. This LC-MS method was applied to the pharmacokinetic study of SalD in rats. A two-compartmental model analysis was employed. The plasma concentrations at 2 min (C_{2min}) were 5756.06 ± 719.61, 11,073.01 ± 1783.46 and 21,077.58 ± 5581.97 μg/L for 0.25, 0.5 and 1 mg/kg intravenous injection, respectively. The peak plasma concentration (C_{max}) was 333.08 ± 61.21 μg/L for 4 mg/kg oral administration. The area under curve (AUC_{0-\infty}) was 14,384.379 ± 8443.184, 22,813.369 ± 11,860.823, 46,406.122 ± 27,592.645 and

Abbreviations: AUC, the area under curve; CI, confidence interval; CL, clearance; C_{max}, peak plasma concentration; ECE-1, endothelin converting enzyme 1; ESI, electrospray ionization; IS, internal standard; LLOQ, lower limit of quantification; QC, quality control; R.E., relative error; R.S.D., relative standard deviation; SalB, salvianolic acid B; SalD, salvianolic acid D; SIM, single ion monitoring; TCM, traditional Chinese medicine; ULOQ, upper limit of quantification

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1. Introduction

Traditional Chinese medicine (TCM) has been used in clinical practice for thousands of years and plays an indispensable role in prevention and treatment of diseases, especially the complicated and chronic ones. Pharmacokinetic studies on the effective components of TCM are important to illustrate their distribution and clearance from the body. *Salvia miltiorrhiza*, one of the oldest and most versatile Chinese herb drug, is of great importance for the treatment of cardiovascular and cerebrovascular diseases. It had been listed in the Pharmacopoeia of the People's Republic of China due to its notable pharmacological activities.

Salvianolic acid D (SalD) is one of the major antioxidants from *S. miltiorrhiza*. Although the cardiac anti-ischemic effects of salvianolic acid B (SalB) have been widely reported, an experiment based on neural network analysis indicated that SalD was more potent than SalB against myocardial ischemia induced by pituitrin. Moreover, water-soluble extracts of *S. miltiorrhiza* (especially SalA, SalB and SalD) exhibited potent inhibitory effects against HIV-1 integrase and this activity mainly resulted from the acid component of SalD. SalD was also a promising compound against endothelin converting enzyme 1 (ECE-1), supporting its use in cardiovascular and cerebrovascular diseases. *SalD* is also the major component of some TCM preparations including "Danshen" injection, "QI-SHEN-YI-QI" dropping pills, "SHUANGDAN" granules, and "Fufang Danshen" tablets. These preparations have been used for the treatment of cardiovascular diseases in China for several decades and lots of pharmacological effects have been reported. It is of great need to study the pharmacokinetics of SalD. Pharmacokinetic parameters, such as maximum plasma concentration, time to maximum plasma concentration, area under the concentration-time curve, volumes of distribution and clearance, are quite useful for assessing the margins of safety of the drug in clinical use. To the best of our knowledge, several methods have been reported for the determination of SalD including HPLC-UV and LC-MS methods. However, no reports have addressed the pharmacokinetic profile of SalD yet. In the present study, we developed a sensitive and reliable HPLC–ESI-MS for the quantification of SalD in rat plasma after oral and intravenous administration. Furthermore, the pharmacokinetic patterns and bioavailability were also investigated.

2. Materials and methods

2.1. Chemicals and reagents

SalD (98% of purity) (Fig. 1) was provided by Institute of Materia Medica (Beijing, China). Naringin (internal standard (IS), 99% of purity) (Fig. 1) was purchased from the National Institutes for Food and Drug Control (Beijing, China). Methanol and acetonitrile (LC-MS grade, J.T. Baker) were purchased from Avantor Performance Materials (USA). Formic acid was obtained from TEDIA (Fairfield, USA). Ultrapure water was prepared by a Milli-Q Reagent water system (Millipore, USA).

2.2. Chromatography and mass spectrometry conditions

Chromatography was carried out on an Agilent 1200 HPLC system (Agilent, CA, USA). The analyte and IS were chromatographed by injection of 20 μL sample onto a Zorbax SB-C18 column (3.5 μm, 2.1 mm × 100 mm, Agilent, USA). The mobile phase consisted of solvent A (water with 0.05% formic acid) and solvent B (water with 0.05% formic acid). A gradient elution program was applied as follow: at the initiation time, the solvent A was set at 5% (v/v) then gradually increased to 30% at 12 min. Then the solvent A was decreased to 5% suddenly, and the system was then equilibrated for 5 min before next injection. The flow rate was 0.3 mL/min with an operating temperature at 35 °C.

An Agilent 6110 quadrupole mass spectrometer with electrospray ionization (ESI) was operated in negative ion mode. The capillary voltage, nebulizer pressure, drying gas flow rate and drying gas temperature were 3000 V, 35 psig, 10 L/min and 350 °C, respectively. The quantification was performed using single ion monitoring (SIM) method with m/z 417 for SalD and m/z 579 for IS. The fragmentation voltage of SalD was set to 100 V and IS was set to 190 V. The gain and dwell time were 1.5 and 144 ms for both SalD and IS.

2.3. Preparation of calibration standards and quality control samples

Stock solutions of SalD and IS were prepared by dissolving the reference standards in acetonitrile at a precise concentration of 1000 μg/mL. All stock standard solutions were stored at –20 °C. The stock solution of IS was further diluted with acetonitrile to 1 μg/mL as working solution. The SalD stock solution was diluted and added into blank plasma to obtain calibration curves with final concentration levels of 3.3, 6.7, 16.6, 33.3, 83.3, 166.7, 333.3, 500.0 and 666.7 ng/mL. The quality control (QC) samples were similarly prepared at concentrations of 6.7, 166.7 and 500.0 ng/mL.
2.4. Plasma sample preparation

For the preparation of 150 μL plasma sample, 15 μL IS solution (1 μg/mL), 10 μL HCl (3 mmol/L), 10 μL vitamin C (10 mg/mL) and 1 mL ethyl acetate were added to it in an Eppendorf tube. After vortex-mixing for 5 min, the sample was centrifuged at 13,400 rpm for 10 min. After carefully separation of the sample, another 1 mL ethyl acetate was added to the same tube for the second extraction. All the supernatant was carefully transferred to a new tube and evaporated under a nitrogen atmosphere at 40 °C. The residue was reconstituted in 75 μL of acetonitrile-H2O (20:80, v/v). After centrifugation at 13,400 rpm for 10 min, 20 μL of aliquot was injected into the HPLC-MS system for measurement.

2.5. Method validation

The validation process was carried out according to Guidance for Industry-Bioanalytical Method Validation (U.S. Food and Drug Administration, 2013). The validation aspects accessed consisted of specificity, carryover, sensitivity, linearity, accuracy, precision, recovery, matrix effect, stability, dilution effect and incurred sample reanalysis.

2.5.1. Specificity

Selectivity was investigated by comparison of the chromatograms of six different blank plasmas obtained from six rats with the corresponding spiked samples and plasma samples after single intravenous administration of SalD.

2.5.2. Carryover

Carryover was evaluated by three injections of an upper limit of quantification (ULOQ) sample, immediately followed by three injections of a blank plasma sample. It was considered acceptable if the mean peak area of SalD or IS was no more than 20% for SalD and 5% for IS, compared to the mean area in the lower limit of quantification (LLOQ) sample.

2.5.3. Linearity of calibration curves and LLOQ

Linearity was investigated over the concentration range of 3.3–666.7 ng/mL. Six calibration curves of SalD were performed with nine concentrations. The calibration curves were constructed by plotting the peak area ratio (Y) of SalD to IS against the nominal concentration (X). The correlation coefficients were calculated by weighted least-square regression (1/X^2). The LLOQ samples were prepared with six different batches of blank plasma at the lowest point of calibration curve with an acceptable accuracy within ±20% and the precision below 20%.

2.5.4. Accuracy and precision

Five replicates of QC samples at three concentration levels (6.7, 166.7 and 500.0 ng/mL) on the same day were analyzed to determine the intra-day precision and accuracy, and the inter-day precision and accuracy were estimated by determining the QC samples over five consecutive days. Relative standard deviation (R.S.D.%) and relative error (R.E.%) were used to express the precision and accuracy, respectively. The intra-day and inter-day precision should not exceed 20% for the low QC samples and 15% for the other QC samples and accuracy should be within ±20% for the low QC samples and ±15% for the other QC samples.

2.5.5. Extraction recovery and matrix effect

Extraction recovery of SalD was determined by comparing the peak area of blank plasma spiked with analyte before and after extraction at three QC levels (6.7, 166.7 and 500.0 ng/mL) (n=5). Matrix effects were measured by comparing the peak areas of the analytes dissolved in the pretreated blank plasma with that of neat standard solution containing equivalent amounts of the analytes (n=5).

2.5.6. Stability

The stability of this method was investigated under the different circumstances including freeze-thaw cycles (three cycles of freezing at −20 °C and thawing at 20 °C), at room temperature (20 °C for 12 h), post-preparation procedure (in the autosampler for 12 h) and long-term sample storage (−20 °C for 30 days). All the stability experiments were conducted at low, medium and high QC samples (n=5) and the obtained results were compared with the nominal concentration.

2.5.7. Dilution effect

The dilution effect was investigated to ensure that samples could be diluted with blank rat plasma without affecting the final concentration. SalD spiked rat plasma samples prepared at 20,000 ng/mL were diluted with blank rat plasma with the dilution factors of 40 in five replicates and analyzed for three successive days. Samples were considered stable if assay values were within the acceptable limits of accuracy (±15% R.E.) and precision (15% R.S.D.).

2.5.8. Incurred sample reanalysis

Six rats were used for the incurred sample reanalysis after 1 mg/kg intravenous administration of SalD. Blood samples (1 mL) from these rats were collected into heparinized tubes by retro-orbital puncture and then centrifuged at 5000 rpm for 10 min. 150 μL of the total plasma was used for the original analysis, another 150 μL was used for repeat analysis. The variability difference (%) was calculated using the original and repeat results as described by the following formula: variability (%) = 2 × (repeat–original)/(repeat+original) × 100. For the acceptance criterion, at least two thirds of the original results and repeat results should be within ±20%.

2.6. Application to pharmacokinetic study

For the pharmacokinetic study, 18 rats were divided into six per group with half males and half females and given SalD at 0.25, 0.5 and 1 mg/kg via the tail vein. Another six rats were orally given 4 mg/kg SalD by gastric gavage. Blood samples (0.5 mL) were collected into heparinized tubes by retro-orbital puncture at 0 (pre-dose), 2, 5, 10, 20, 30 min, 1, 2, 3, 4, 6, 8 and 12 h after intravenous administration and at 5, 10, 20, 30 and 45 min, 1, 2, 3, 4, 6, 8 and 12 h after oral administration. Plasma was separated by centrifugation at 5000 rpm for 10 min and stored at −20 °C until analysis. The pharmacokinetic parameters were calculated using the DAS 3.0 pharmacokinetic program (Chinese Pharmacology Society). A two-compartmental model analysis was employed. Absolute bioavailability was determined from the ratio of AUC\(_{0\rightarrow t}\) for the low, medium and high concentration QC samples. All working solutions were stored at −20 °C.
values obtained from p.o. administration versus that obtained from i.v. administration and expressed as \( F(\%) = \frac{Dose_{i,v} \times AUC_{p.o.}}{Dose_{p.o.} \times AUC_{i,v}} \times 100 \).

3. Results

3.1. Validation

3.1.1. Specificity

Fig. 2 shows full MS spectra of SalD and IS. The specificity of the method was demonstrated by comparing SIM chromatograms of a drug-free plasma sample, a spiked plasma sample, and a plasma sample from rats at 1 h after intravenous administration. As shown in Fig. 3, no significant peaks interfering with analytes were observed in the drug-free rat plasma. The retention times for SalD and IS were approximately 12.7 and 13.0 min, respectively. These observations indicate that no endogenous substances significantly influenced the ionization of these analytes.

3.1.2. Carryover

No peak was observed at the retention times of SalD or IS in the chromatogram of a blank sample analyzed after the injection of ULOQ sample, indicating the absence of carryover.

3.1.3. Linearity and LLOQ

The validation concentration range was from 3.3 to 666.7 ng/mL (\( Y = 0.0065X + 0.0051, R^2 = 0.9995, n = 6 \)). The lowest SalD plasma level on the calibration curves 3.3 ng/mL was recognized as LLOQ. The accuracy and precision data for LLOQ determination were –9.43% and 12.83% which were both within the acceptable criteria.

3.1.4. Precision and accuracy

The intra-day, inter-day precision and accuracy of SalD are summarized in Table 1. The accuracies for all tested concentrations were within ±9% of nominal and both the within- and between-run precisions were acceptable. All the results of the tested samples were within the acceptable criteria (R.S.D. %: 7.38%; R.E. %: –5.78%).

3.1.5. Extraction recovery and matrix effect

The average extraction recoveries at three QC levels were 90.20% ± 11.57%, 84.04% ± 2.69%, and 88.70% ± 2.92% for low, middle, and high QC samples, respectively. The data indicated that extraction recovery was consistent and reproducible. The matrix effect values were 95.12% ± 7.73%, 94.07% ± 2.19% and 87.25% ± 5.01% for SalD at the three QC concentration levels, respectively (Table 2).

3.1.6. Stability

Several stability experiments were performed and the results are summarized in Table 3. No significant changes of SalD concentrations were measured after three freeze-thaw cycles (storage at –20 °C for 30 days, storage for 12 h at room temperature, and 12 h in the autosampler after preparation). These results indicate that the compound was stable during storage and sample preparation.

3.1.7. Dilution effect

The dilution effect was confirmed for QC samples that exceeded the ULOQ. SalD-spiked rat plasma samples prepared at 20,000 ng/mL were diluted with blank rat plasma at a dilution factor of 40 in five replicates for three successive days. The results showed that the diluted samples were within the acceptance range (R.S.D. %: 7.38%; R.E. %: –5.78%).

3.1.8. Incurred sample reanalysis

For the incurred-sample reanalysis, 3 time points from 6 rats were used. Variability of SalD content in all reanalyzed samples was from –15.86% to 13.56%, suggesting good reproducibility of this validated method.

3.2. Pharmacokinetic study of SalD

The described method was applied to quantify SalD in rat plasma following a p.o. 4 mg/kg dose and i.v. 0.25, 0.5, 1 mg/kg doses. The concentration-time profiles are shown in Fig. 4. The main pharmacokinetic parameters of SalD after i.v. and p.o. administration were calculated by a two-compartmental model analysis (Table 4). The AUC was calculated by the linear trapezoidal method. The main pharmacokinetic parameters, including area under concentration-time curve (AUC_{0–t} and AUC_{0–∞}), distribution half-life (\( t_{1/2d} \)), elimination half-life (\( t_{1/2e} \)), apparent central volume of distribution (\( V_{c} \)), clearance (CL), elimination rate constant (\( K_{e} \)), transfer rate constants (\( K_{10} \) and \( K_{12} \)), first-order absorption rate constant (\( K_{a} \)), and absorption half-life (\( t_{1/2K_{a}} \)) were determined according to standard equations. Following the 4 mg/kg p.o. administration, the \( C_{max} \) was 333.08 ± 61.21 ng/mL, and the \( T_{max} \) was 1.133 ± 0.689 h. SalD showed low bioavailability in rats with the absolute bioavailability 4.159% ± 0.517%.

Dose proportionality was assessed using the power model in a manner analogous to the method of Smith et al. (\( P = a \times Dose^{0.9} \rightarrow lnP = \beta_0 + \beta_1 \lnDose \), in which \( P \) is the pharmacokinetic
variable, $\alpha$ is the typical value of the variable, and $\beta_1$ is the effect of dose as a covariate). An ideal proportional model corresponds to $\beta_1 = 1$ (e.g., $P = \alpha$ × Dose), indicating exact dose proportionality. Deviations of $\beta_1$ from 1 correspond to deviations from ideal dose proportionality. Dose proportionality is claimed when the estimate of the 90% confidence interval (CI) for $\beta_1$ falls between the equivalent limits ($L$, $U$) proposed by Smith et al.\(^{25}\), where $L$ and $U$ can be derived according to equations $L = 1 + \ln \theta_L/r$ and $U = 1 + \ln \theta_H/r$, respectively. In these equations, $\theta_L$ is the lower limit of the range, $\theta_H$ is upper limit of the range, and $r$ is the ratio between the highest and lowest tested doses. The ranges $0.8 \theta_L/C_0$ to $1.25 \theta_H/C_0$ and $0.7 \theta_L/C_0$ to $1.43 \theta_H/C_0$ were used as the criteria for dose independence of AUC and $C_{2\text{min}}$ over the entire range of doses administered in this study, respectively. As a result, the corresponding 90% CI for $C_{2\text{min}}$ fell into the acceptance interval, and the corresponding 90% CI for AUC0–t and AUC0–1 was slightly outside the acceptance interval (Fig. 5 and Table 5). $\beta_1$ was 0.90, 0.86 and 0.92 for AUC0–t, AUC0–1 and $C_{2\text{min}}$, respectively. These results indicated that SalD showed relatively good dose proportionality for $C_{2\text{min}}$, AUC0–t, and AUC0–1, but more studies are needed for detailed information.

### Table 1: Intra-day and inter-day accuracy and precision in rat plasma ($n=5$).

| Item       | Nominal conc. (ng/mL) | Observed conc. (mean ± SD, ng/mL) | Accuracy R.E. (%) | Precision R.S.D. (%) |
|------------|-----------------------|----------------------------------|-------------------|----------------------|
| Intra-day  | 6.7                   | $6.89 \pm 0.53$                 | 3.23              | 7.69                 |
|            | 166.7                 | $157.99 \pm 5.24$               | −5.21             | 3.32                 |
|            | 500.0                 | $455.20 \pm 16.57$              | −8.96             | 3.64                 |
| Inter-day  | 6.7                   | $6.47 \pm 0.33$                 | −2.97             | 5.08                 |
|            | 166.7                 | $161.31 \pm 3.64$               | −3.22             | 2.26                 |
|            | 500.0                 | $474.28 \pm 24.34$              | −5.14             | 5.13                 |

### Figure 3
Typical chromatograms of SalD and IS (A) blank rat plasma; (B) blank rat plasma spiked with SalD and IS; and (C) rat plasma sample at 1 h after intravenous injection of SalD spiked with IS.

### 4. Discussion and conclusions

Up to now, several papers reporting analytical methods for SalD determination have used HPLC\(^{13,26}\). However, the HPLC method requires a relative long running time with a low sensitivity. SalD was also detected by LC-MS methods, but these methods were mainly developed for determination of the chemical fingerprint of TCM drug preparations, which also takes a relatively long running time and has no method validation\(^{13,23,26}\). No validated method has been reported for use with the plasma of rat, dog and human yet. Our single quadrupole mass spectrometry (LC-MS) has a high sensitivity, a good resolution, a moderate price and is quite suitable for the pharmacokinetic study of SalD.

Our method was validated by the fact that the calibration curve for SalD was linear over the range 3.3–666.7 ng/mL with a coefficient of correlation $> 0.999$. The inter-day and intra-day precision of analysis was < 8%, and the assay accuracy ranged from 91.04% to 103.23%. The present optimized method was validated to guarantee reliable determination results for SalD and was successfully applied to the pharmacokinetic study after both p.o. and i.v. administration in rat.
A low $C_{\text{max}}$ of 333.08 $\pm$ 76.12 $\mu$g/L and very small AUC0/$C_{\text{0}}$ value of 820.1740 $\pm$ 4711.961 $\mu$g/L/$C_{\text{0}}$h were found after 4 mg/kg oral administration in rats. The bioavailability of SalD was calculated to be about 4.159% $\pm$ 0.517%. Since the structure of SalD is similar to the structures of SalA and SalB, these phenolic compounds may have similar pharmacokinetic patterns. Although there was no previous pharmacokinetic study on SalD, we can get some guidance from the studies of SalA and SalB pharmacokinetics. The bioavailability of SalA was reported and calculated to be 1.42% in beagle dogs27, and the bioavailability of SalB was calculated to be about 2.3% in freely moving rats28. These results indicated that salvianolic acids (including SalA, SalB, SalC and SalD, etc.) may all have extremely low bioavailability. It was reported that more than 60% of dosed SalB remained in the gastrointestinal tract even 4 h after oral administration, indicating the poor absorption of SalB in rat small intestine29. The low bioavailability of SalD may also be due to its poor absorption in rat small intestine. For the further study of SalD, an intravenous injection method is preferred. In our study, SalD showed relatively good dose proportionality by i.v. administration of 0.25/$C_{\text{0}}$1 mg/kg.

Since SalD is a polyphenol compound with highly potent antioxidation activity, a high dose of SalD could affect the body's redox and metabolic systems, which could affect the elimination of SalD and lead to a poor dose proportionality. The dose proportionality of SalB in beagle dogs has been reported30. A linear relationship of SalB concentrations was observed between AUC and doses after i.v. 3, 6 and 12 mg/kg, but the clearance and volume of distribution values obtained at 12 mg/kg were lower than those at the lower dose, suggesting that a saturated distribution and metabolism might occur at the high dose of SalB. This profile of SalB will be an important clue for dose proportionality investigation of SalD.

### Table 2 Recovery and matrix effect in rat plasma (n=5).

| Analyte | Nominal conc. (ng/mL) | Recovery | Matrix effect |
|---------|-----------------------|----------|--------------|
|         |                       | Mean $\pm$ SD (%) | R.S.D. (%) | Mean $\pm$ SD (%) | R.S.D. (%) |
| SalD    | 6.7                   | 90.20 $\pm$ 11.57 | 12.83 | 95.12 $\pm$ 7.73 | 8.13 |
|         | 166.7                | 84.04 $\pm$ 2.69  | 3.20  | 94.07 $\pm$ 2.19 | 2.32 |
| IS      | 500.0                | 88.70 $\pm$ 2.92  | 3.29  | 87.25 $\pm$ 5.01 | 5.74 |
|         | 100.0                | 95.22 $\pm$ 3.94  | 4.14  | 97.89 $\pm$ 3.59 | 3.67 |

### Table 3 Stability of SalD in rat plasma (n=5).

| Condition                        | Nominal conc. (ng/mL) | Observed conc. (mean $\pm$ SD, ng/mL) | R.E. (%) | R.S.D. (%) |
|----------------------------------|-----------------------|---------------------------------------|----------|------------|
| 12 h in the autosampler           | 6.7                   | 6.55 $\pm$ 0.66                        | -1.75    | 10.00      |
|                                  | 166.7                 | 164.78 $\pm$ 16.79                     | -1.13    | 10.19      |
|                                  | 500.0                 | 483.72 $\pm$ 40.12                     | -3.26    | 8.29       |
| Three freeze-thaw cycles         | 6.7                   | 6.72 $\pm$ 0.62                        | 0.81     | 9.22       |
|                                  | 166.7                 | 161.76 $\pm$ 13.42                     | -2.95    | 8.30       |
|                                  | 500.0                 | 493.76 $\pm$ 46.13                     | -1.25    | 9.34       |
| 12 h at room temperature         | 6.7                   | 6.40 $\pm$ 0.48                        | -4.06    | 7.49       |
|                                  | 166.7                 | 156.33 $\pm$ 7.35                      | -6.21    | 4.70       |
|                                  | 500.0                 | 467.88 $\pm$ 39.91                     | -6.42    | 8.53       |
| $-20^\circ C$ for 30 days        | 6.7                   | 6.88 $\pm$ 0.70                        | 3.21     | 10.21      |
|                                  | 166.7                 | 160.56 $\pm$ 16.40                     | -3.67    | 10.22      |
|                                  | 500.0                 | 483.16 $\pm$ 31.40                     | -3.37    | 6.50       |

**Figure 4** Mean plasma concentration-time curves of SalD. (A) Mean plasma concentration-time curves of rats (i.v., n=6); (B) semi-logarithmic axis mean plasma concentration-time curves of rats (i.v., n=6); (C) mean plasma concentration-time curves of rats with 4 mg/kg dose (p.o., n=6).
Table 4  Compartmental pharmacokinetic parameters of SalD in rat plasma (n = 6).

| Parameter | Unit | i.v. | p.o. |
|-----------|------|------|------|
| t1/2α | h | 0.063 ± 0.049 | 0.067 ± 0.072 | 0.079 ± 0.068 | 1.464 ± 1.459 |
| t1/2β | h | 1.514 ± 1.252 | 0.969 ± 0.547 | 1.405 ± 0.934 | 17.306 ± 43.768 |
| V1 (i.v.) or V1/F (p.o.) | L/kg | 0.028 ± 0.016 | 0.023 ± 0.025 | 0.027 ± 0.023 |
| CL (i.v.) or CL/F (p.o.) | L/h/kg | 0.025 ± 0.020 | 0.031 ± 0.024 | 0.030 ± 0.022 |
| AUC(0–t) | µg/L·h | 14,384.379 ± 8443.184 | 22,813.369 ± 11,860.823 | 46,406.122 ± 27,592.645 |
| AUC(0–∞) | µg/L·h | 14,790.764 ± 8572.595 | 23,269.485 ± 11,926.715 | 47,441.218 ± 27,763.107 |
| R AUC(0–∞) | % | 96.883 ± 2.058 | 97.417 ± 1.821 | 96.950 ± 2.552 |
| K10 | 1/h | 6.951 ± 15.027 | 25.241 ± 40.132 | 24.793 ± 37.427 |
| K12 | 1/h | 30.763 ± 55.694 | 58.500 ± 61.077 | 43.829 ± 61.132 |
| K21 | 1/h | 3.502 ± 2.035 | 2.205 ± 1.013 | 1.945 ± 0.735 |
| F | % | – | – | – |
| t1/2K0 | h | – | – | – |

Figure 5  Relationship between lnPK and lnDose. The dashed lines are the 90% confidence intervals. (A) AUC0–t; (B) AUC0–∞; and (C) Cmin.

Table 5  Assessment of dose proportionality of SalD based on power model.

| Parameter | Acceptance range | b1 | 90% Confidence interval |
|-----------|------------------|----|------------------------|
| AUC0–t | 0.84 – 1.16 | 0.90 | 0.74 – 1.05 |
| AUC0–∞ | 0.84 – 1.16 | 0.86 | 0.69 – 1.04 |
| Cmin | 0.74 – 1.26 | 0.92 | 0.77 – 1.06 |

Because SalD is the major component of more than 20 commercial drug preparations being sold in China, the bioavailability and dose proportionality studies of SalD could benefit the clinical use of these preparations. With the results, the concentration of SalD in patients could be easily estimated and the analysis method in human plasma could be further developed based on this method.

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References

1. Li MH, Li QQ, Zhang CH, Zhang N, Cui ZH, Huang LQ, et al. An ethnopharmacological investigation of medicinal Salvia plants (Lamiaceae) in China. Acta Pharm Sin B 2013;3:273–80.
2. Chinese Pharmacopoeia Commission. Pharmacopoeia of the People’s Republic of China. 2010 ed. Beijing: China Medical Science Press; 2010.
3. Ai CB, Li LN. Salvianolic acids D and E: two new depsides from Salvia miltiorrhiza. Planta Med 1992;58:197–9.
4. Pan CS, Lou LX, Hou YQ, Singh G, Chen M, Zhang DM, et al. Salvianolic acid B and Tanshinone IIA attenuate myocardial ischemia injury in mice by NO production through multiple pathways. Ther Adv Cardiovasc Dis 2011;5:99–111.
5. Xue L, Wu Z, Ji XP, Gao XQ, Guo YH. Effect and mechanism of salvianolic acid B on the myocardial ischemia-perfusion injury in rats. Asian Pac J Trop Med 2014;7:280–8.
6. He HB, Shi MQ, Zeng XW, Yang J, Li YK, Wu LM, et al. Cardioprotective effect of salvianolic acid B on large myocardial infarction mediated by reversing upregulation of leptin, endothelin pathways, and abnormal expression of SERCA2a, phospholamban in rats. J Ethnopharmacol 2008;118:35–45.
7. Yin YQ, Zhu JF, Shen ZB, Tang CP, Yang CY, Sun Y. Neural network analysis on spectra-effects correlation of anti-ischemic active ingredients in Xiangdan injection. Chin Tradit Herb Drugs 2009;40:1284–7.
8. Queffeux C, Bailly F, Mbemba G, Moussadet JF, Hayes S, Debyser Z, et al. Synthesis and antiviral properties of some polyphenols related to Salvia genus. Bioorg Med Chem Lett 2008;18:4736–40.
9. Yu S, Zhao G. Development of polyphenols as HIV-1 integrase inhibitors: a summary and perspective. Curr Med Chem 2012;19:5536–61.
20. Li C, Wang Y, Qiu Q, Shi T, Wu Y, Han J, et al. Qishenyiqi protects
19. Shang H, Zhang J, Yao C, Liu B, Gao X, Ren M, et al. Qi-shen-yi-qi
18. Zhang L, Wang Y, Yu LY, Liu L, Qu HB, Wang Y, et al. QI-SHEN-
16. Lv YH, Zhang X, Liang X, Liu XR, Dai WX, Yan SK, et al.
11. Chen SJ, Cui MC, Chen HJ. Virtual screening of active components
12. Wang X, Xiang YH, Ren ZZ, Zhang YL, Qiao YJ. Rational questing
13. Zhang JL, Cui M, He Y, Yu HL, Guo DA. Chemical fingerprint and
15. He Q, Hu XI. Cheng YY. Analysis of ‘SHUANGDAN’ granules by
14. Li YF, Qu HB, Cheng YY. Identi
17. Han JY, Horie Y, Miura S, Akiba Y, Guo J, Li D, et al. Compound
10. Wang XH, Morris-Natschke SL, Lee KH. New developments in the
16. Li C, Wang Y, Qiu Q, Shi T, Wu Y, Han J, et al. Qishenyiqi protects
19. Shang H, Zhang J, Yao C, Liu B, Gao X, Ren M, et al. Qi-shen-yi-qi
18. Zhang L, Wang Y, Yu LY, Liu L, Qu HB, Wang Y, et al. QI-SHEN-
16. Lv YH, Zhang X, Liang X, Liu XR, Dai WX, Yan SK, et al.
11. Chen SJ, Cui MC, Chen HJ. Virtual screening of active components
12. Wang X, Xiang YH, Ren ZZ, Zhang YL, Qiao YJ. Rational questing
13. Zhang JL, Cui M, He Y, Yu HL, Guo DA. Chemical fingerprint and
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14. Li YF, Qu HB, Cheng YY. Identi
17. Han JY, Horie Y, Miura S, Akiba Y, Guo J, Li D, et al. Compound
10. Wang XH, Morris-Natschke SL, Lee KH. New developments in the
16. Lv YH, Zhang X, Liang X, Liu XR, Dai WX, Yan SK, et al.
11. Chen SJ, Cui MC, Chen HJ. Virtual screening of active components
12. Wang X, Xiang YH, Ren ZZ, Zhang YL, Qiao YJ. Rational questing
13. Zhang JL, Cui M, He Y, Yu HL, Guo DA. Chemical fingerprint and
15. He Q, Hu XI. Cheng YY. Analysis of ‘SHUANGDAN’ granules by
14. Li YF, Qu HB, Cheng YY. Identi