A systematic study on pharmacokinetics and tissue distribution of danshensu (one of the major active components from *Salvia miltiorrhiza*) is conducted using a rapid and sensitive high-performance liquid chromatographic (HPLC) method. Before HPLC analysis, biological samples are pretreated with a liquid–liquid extraction. Separation of danshensu and internal standard is achieved on an Agilent Zorbax C18 column with a mobile phase made up of acetonitrile and 0.05% trifluoroacetic acid at a flow rate of 0.8 mL/min. The calibration curves in plasma and tissues are linear in the given concentration ranges, with $r^2$ no less than 0.99. The intra-day and inter-day relative standard deviations in the measurement of quality control samples are less than 15%, and the accuracies are in the range of 86–115%. The recoveries of danshensu in plasma and tissues are among 80% to 118%. Meanwhile, the multi-peaks in pharmacokinetic profiles are observed. The method is successfully applied to the pharmacokinetics and tissue distribution study of danshensu after a single oral administration of 50.0 mg/kg sodium danshensu to rats.

**Introduction**

The dried root of *Salvia miltiorrhiza* Bunge, called Danshen in Chinese, is widely used in traditional Chinese medicine for the treatment of coronary heart disease, cerebrovascular disease, bone loss, hepatocirrhosis, and chronic renal failure (1). It is commonly considered that the active constituents in Danshen can be divided into two groups: phenolic acids, which are water soluble and tanshinones which are lipophilic. Danshensu (3,4-dihydroxyphenyllactic acid) is one of the major water-soluble constituents in Danshen, and is also a major active component contained in Danshen preparations like Compound Danshen Dipping Pill, which is widely used for the therapy of heart disease. It has been reported that danshensu could protect the cardiac muscle by calcium antagonizing (2–4), reducing oxygen free radical generation (5,6), inhibiting peroxidative damage (7), and preventing platelet aggregation (8,9). The activities of danshensu on other aspects, like anti-tumor (10) and nerve cells injury protection (11) have also been reported. As a result, great research interests have been focused on the therapeutic potential of danshensu.

The previous research on the disposition of danshensu had been focused on the determination of danshensu in animal plasma or serum after administration of Danshen extracts or compound preparations (12–15); however, the investigation of pharmaceutical characteristics of danshensu could be interfered with by other constituents when administering compound preparation or Danshen extracts. Zhao et al. reported the distribution of danshensu in rabbit just at 5 min after intravenous injection of Danshen preparation (16), but the research of the distribution at one time point was insufficient, and intravenous injection was not as meaningful as oral administration, which was adopted in clinical therapy. To our knowledge, there has not been any report on the pharmacokinetic research after oral administration of danshensu solely, or the systematic research on the distribution of danshensu in body.

In the present study, we developed a rapid, sensitive, and selective liquid chromatography (LC) method coupled with liquid–liquid extraction for the determination of danshensu in rat plasma and tissues, and the pharmacokinetics and tissue distribution study of danshensu after a single oral administration of 50.0 mg/kg sodium danshensu to rats.

**Experimental**

**Materials and reagents**

Danshensu was purchased from Sikehua Biotech Co. Ltd. (Sichuan, China) in the form of sodium danshensu. Protocatechuic acid (PA) (the internal standard, see Figure 1) was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China).

High-performance liquid chromatography (HPLC)-grade acetonitrile was purchased from Fisher Scientific (Fair Lawn, NJ). HPLC-grade methanol was obtained from Tianjin Special Chemical Reagents Co. Ltd. (Tianjin, China). Ethyl acetate and...
Animals, drug administration, and sample collection

Male Sprague-Dawley rats (180–220 g) were obtained from the Laboratory Animal Center of Peking University Health Science Center (Beijing, China). Before the experiments were conducted, animals were kept in an environmentally controlled breeding room for 3 days. They were fed with food and water ad libitum, and then fasted overnight before drug administration but with free access to water. A 5 mg/mL of danshensu solution was administrated orally at a dose of 50 mg/kg to rat. Protocols of animal experiments had been approved by the Animal Center of Peking University Health Science Center.

Five rats for each time-point were decapitated, and blood and tissue samples were collected at 3, 6, 10, 15, 20, 30, 45, 60, 90, 150, 240, and 360 min after oral administration. After removal of the blood, tissues including heart, liver, spleen, lung, kidney, and brain were rapidly removed and weighed. Blood samples were centrifugated to obtain the plasma, and tissues were dissected and homogenized by the addition of physiological saline at the ratio of 1:1 (v/v). Deionized water was administered orally to the rats at a dose of 10 mL/kg body for blank plasma and tissues collection. All samples were then immediately stored at approximately –80°C until they were processed and analyzed.

Sample preparation

Biological samples (2 mL of plasma, 2 mL of liver, and the whole of other tissues) were removed to clean tubes and mixed with 50 µL of the internal standard solution (10.0 µg/mL of PA). After vortex mixing to homogenize the sample, 10% (v/v) hydrochloric acid was added to pH 2.0, followed by vortex mixing for 2 min. Triple amount of ethyl acetate to the volume of biological samples was added to each tube, and extraction was performed by vortex mixing the tubes for 5 min. After centrifugation at 9000 rpm for 5 min, supernatant equal to 80% of the volume of ethyl acetate was transferred to a clean test tube, and the residue was resolved in ethyl acetate and extracted again with the same method. Then the two supernatants were combined and dried under a flow of nitrogen gas at 35°C, and the residue was reconstituted in 200 µL of methanol–0.1% TFA (1:1). After filtering through a membrane (0.45 µm pore size), a 20 µL aliquot was injected into the HPLC system for analysis.

Calibration standard and quality control sample

Different concentrations of working solution (50 µL) and internal standard solution (50 µL) were added to the blank plasma or tissue homogenates to prepare plasma and tissue standard solutions, respectively. Then plasma and tissue homogenates were treated using the sample preparation. Quality control samples, which were used in the validation procedures, were similarly prepared at low, medium, and high concentrations for danshensu in blank plasma and tissue homogenates. All the samples were processed as previously described.

Validation procedure

Calibration curve and limit of quantitation

The calibration curves were acquired by plotting the peak area ratio of danshensu: PA against the concentration of calibration standards. Each calibration curve contained six concentration levels. The spiked standard samples were analyzed in three separate analytical runs. The results were fitted to linear regression analysis using 1/x² as weighting factor. The calibration curve had to have a correlation coefficient (r²) of 0.99 or better. The acceptance criterion for each back-calculated standard concentration was 10% deviation from the nom-
inal value. The limit of quantification (LOQ) was defined as the lowest concentration when accuracy and RSD were within 80–120% and ± 15%, respectively.

Accuracy and precision
The accuracy and precision of the method were assessed by intra-day and inter-day validation. The intra-day accuracy and precision were calculated by analyzing the QC samples at low, medium, and high concentration levels in five replicates in one day. The inter-day accuracy and precision were performed by replicate analysis of QC samples on three consecutive days. Precision was expressed as relative standard deviation (RSD) and accuracy was expressed as percentage accuracy [(mean measured concentration/spiked concentration) × 100%].

Recovery
The recovery for danshensu was assessed at three different concentrations. Recovery was expressed by comparing plasma or tissue samples spiked with danshensu with control samples at high, medium, and low concentrations.

Stability
The stabilities of danshensu were investigated under various conditions. The short-term storage stabilities and long-term storage stabilities of danshensu were analyzed at high, medium, and low levels stored at room temperature for 24 h and −20°C for 7 days, respectively. The freeze-thaw stabilities were determined after three freeze and thaw cycles. The stabilities of danshensu were determined by comparing the mean concentration with the initial concentration of freshly prepared samples before storage.

Results and Discussion
Chromatography
Representative chromatograms of blank biological samples, biological samples spiked with danshensu reference solution, and biological samples 10 min after oral dosing of danshensu to rats are shown in Figures 2 and 3. Danshensu

Figure 2. HPLC chromatograms of danshensu in rat plasma. A: blank plasma; B: blank plasma spiked with 1: danshensu, 2: internal standard; C: plasma sample 10 min after oral dosing of danshensu to rats.
and the internal standard could be well separated from the endogenous substances. According to the literature and the previous work of our group, the fixed phase of Zorbax Extend C₁₈ column was better to determine the phenolic acids, and adding acid to the mobile phase could achieve better separation for phenolic acids because it reduces the ionization of phenol, phenolic hydroxyl, and carboxyl groups (1,17). Other parameters like column temperature, flow rate, and detection wavelength were investigated, and symmetric peaks and better separation could be achieved at 20°C with the flow rate at 0.8 mL/min, with a detection wave at 288 nm.

Solid-phase extraction (SPE) and organic precipitation were commonly applied to remove the endogenous interference from plasma and tissue samples to achieve a better extraction procedure with high recovery (18). According to the literature, the recovery of SPE was lower than that of liquid–liquid extraction, which might be caused by the loss of acid constituents during washing (15). Meanwhile, the method of tissue proteins precipitation was investigated using methanol; however, it was found that danshensu and internal standard could not be well detected by HPLC. Thus, liquid–liquid extraction was employed as a simple and sensitive method for sample preparation. By investigating the pH condition and extraction solvents, danshensu was tested to be stable at the pH condition of 2.0, and higher recovery could be obtained after extracting twice with ethyl acetate.

<table>
<thead>
<tr>
<th>Biosamples</th>
<th>Standard curves</th>
<th>( r^2 )</th>
<th>Linear ranges (µg/g)*</th>
<th>LOQ (µg/g)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma</td>
<td>( y = 0.6385x + 0.0055 )†</td>
<td>0.9964</td>
<td>0.025–1.500</td>
<td>0.015</td>
</tr>
<tr>
<td>Heart</td>
<td>( y = 0.2419x – 0.0039 )</td>
<td>0.9929</td>
<td>0.200–1.000</td>
<td>0.150</td>
</tr>
<tr>
<td>Liver</td>
<td>( y = 0.3042x + 0.0043 )</td>
<td>0.9975</td>
<td>0.200–1.000</td>
<td>0.150</td>
</tr>
<tr>
<td>Lung</td>
<td>( y = 0.2741x + 0.0039 )</td>
<td>0.9921</td>
<td>0.100–1.500</td>
<td>0.055</td>
</tr>
<tr>
<td>Kidney</td>
<td>( y = 0.4395x – 0.0151 )</td>
<td>0.9970</td>
<td>0.175–15.750</td>
<td>0.100</td>
</tr>
<tr>
<td>Brain</td>
<td>( y = 0.3442x + 0.0012 )</td>
<td>0.9973</td>
<td>0.100–0.600</td>
<td>0.060</td>
</tr>
</tbody>
</table>

* The unit in plasma is µg/mL.

† \( y = \text{peak area ratio (analyte/internal standard); } x = \text{concentration of compound in rat plasma (µg/mL).} \)

Figure 3. HPLC chromatograms of danshensu in rat heart. A: blank heart tissue; B: blank heart tissue spiked with 1: danshensu, 2: internal standard; C: heart sample 10 min after oral dosing of danshensu to rats.
Calibration curve and limit of quantitation

The standard curves of danshensu constructed with peak-area ratio to analyte concentration in plasma or tissue homogenates and the limit of quantification (LOQ) are listed in Table I. The correlation coefficients ($r^2$) of calibrations of biosamples were higher than 0.99.

Accuracy and precision

The inter- and intra-day precisions were assessed with high, medium, and low concentration levels. The inter-day precisions expressed as relative standard deviation (RSD) in three consecutive days were 0.57–1.07% in plasma, 1.04–3.74% in heart, 2.97–14.47% in liver, 3.45–5.08% in lung, 0.35–2.02% in kidney, and 1.54–6.40% in brain. The intra-day precisions were 2.21–6.00% in plasma, 7.07–9.96% in heart, 4.64–14.65% in liver, 4.93–9.95% in lung, 1.51–2.71% in kidney, and 2.93–8.67% in brain. The intra-day accuracy ranged from 91.68–114.00% and inter-day accuracy ranged from 86.54–116.26% (Table II). All data were below the threshold of Ch.P (19).

Recovery

Recovery was expressed by comparing plasma or tissue samples spiked with danshensu with control samples at high, medium, and low concentrations. The mean recoveries of danshensu ranged from 80.19–117.39%.

Stability

Danshensu in plasma and tissue samples were found to be stable at room temperature for 24 h and –20°C for 7 days. Meanwhile, the biological samples were also found to be stable through three freeze-thaw cycles for at least 6 h. The bias before and after storage in all QC samples was within the method limits.

Application to pharmacokinetic study

The present method was applied to analyze the plasma samples after oral administration of danshensu to rats. Figure 4 shows the mean concentration-time profiles of danshensu in plasma. It suggested that danshensu was rapidly absorbed and slowly eliminated from body.

It is interesting that three peaks were observed at 10, 30, and 90 min after oral administration. According to the literature, the multiple-peaks might have resulted from the rapidly biliary secretion and intestinal resorption via enterohepatic circulation (20,21). Pan et al. (15) also reported the multiple-peaks after oral administration of the compound Salvia recipe, but $T_{max}$ and $C_{max}$ varied from our results. However Luo et al. (14) found only one peak in dog plasma after administration of Danshen extracts. Meanwhile, previous works reported contrary results while giving Danshen extracts or compound Danshen dropping pill. Hong et al. (13) reported that the pharmacokinetic process of danshensu in rats could be described as a two-compartment model, but Yan et al. (22) reported that in the rabbit it was a one-compartment model.
Although the difference of animals should be considered, these results demonstrated that other constituents in the compound Danshen recipe might interfere with the transportation of danshensu, for most of the water-soluble constituents in danshen could be thought of as polymers constructed by danshensu and other small molecules (1,23), and these polymers might be decomposed in body (24). Therefore, it was significant to illuminate its transportation in the body by the sole administration of danshensu, and the assay described here was successfully applied to the pharmacokinetic study of danshensu with high accuracy and sensitivity.

**Tissue distribution**

The concentrations of danshensu in heart, liver, spleen, lung, kidney and brain were detected with the established method; however in spleen, the concentration of danshensu was too low to quantify. Figure 5 shows the concentration versus time profiles of danshensu in tissues after oral dose to rats. In accordance with that in plasma, three peaks were found in all tissues. Danshensu could be detected at 3 min post-dosing and reach the first peak within 15 min, indicating that danshensu was rapidly absorbed and distributed in these tissues. Meanwhile, danshensu was also detected in the brain, showing that danshensu could pass the blood–brain barrier, but was rapidly eliminated and was below the limit of detection after 45 min. The maximum concentration observed in kidney demonstrated that danshensu could be excreted via kidney in the form of prototype slowly but in great amount.

No report on the study of tissue distribution of danshensu after oral administration could be found. Zhao et al. (16) reported the tissue distribution of danshensu at 5 min after intravenous injection of the compound preparations containing danshensu, and the distribution of danshensu in tissues was reported in descending order of lung, liver, heart, brain and spleen. In our study, the amount of danshensu in tissues was in descending order of kidney, lung, heart, liver, brain, and spleen. Although danshensu was used to treat heart diseases, tissue specificity was not observed by the results, illustrating that danshensu might strongly bind to proteins in the heart and, thus, relate to pharmacological activity (21). This result was consistent with the current pharmacological research mentioned in the Introduction Section.

**Conclusion**

This was the first report on the pharmacokinetic study and tissue distribution of danshensu after its oral administration of sodium danshensu to rats. An HPLC–UV method coupled with liquid–liquid extraction was established for the analysis of biological samples, and the results proved that the new method was validated and successfully applied to the study of the pharmacokinetics and tissue distribution of danshensu with adequate sensitivity and accuracy.

Danshensu was absorbed rapidly and eliminated slowly and was distributed to heart, liver, lung, kidney, and brain steadily, and three peaks could be observed from the concentration-time profile in plasma and tissues.

| Table II. Analytical Results of Intra-Day and Inter-Day Variability |
|---------------------|---------------------|---------------------|
| **Samples** | **Intra-day** | **Inter-day** |
| | **Spiked** (µg/g)* | **Measured** (µg/g),† | **Accuracy‡** | **Spiked** (µg/g) | **Measured** (µg/g) | **Accuracy** |
| Plasma | 0.125 | 0.123 ± 0.001 | 98.01 | 0.125 | 0.121 ± 0.007 | 96.52 |
| | 0.500 | 0.570 ± 0.003 | 114.00 | 0.500 | 0.536 ± 0.019 | 107.11 |
| | 1.000 | 1.496 ± 0.010 | 99.73 | 1.000 | 1.527 ± 0.034 | 101.78 |
| Heart | 0.250 | 0.233 ± 0.009 | 93.33 | 0.250 | 0.216 ± 0.022 | 86.54 |
| | 0.500 | 0.497 ± 0.005 | 99.47 | 0.500 | 0.444 ± 0.042 | 88.71 |
| | 1.000 | 1.047 ± 0.025 | 104.66 | 1.000 | 0.951 ± 0.067 | 95.05 |
| Liver | 0.250 | 0.231 ± 0.033 | 92.64 | 0.250 | 0.253 ± 0.037 | 101.29 |
| | 0.500 | 0.507 ± 0.025 | 101.45 | 0.500 | 0.581 ± 0.048 | 116.26 |
| | 1.000 | 1.006 ± 0.030 | 100.56 | 1.000 | 1.074 ± 0.050 | 107.43 |
| Lung | 0.250 | 0.237 ± 0.012 | 94.64 | 0.250 | 0.239 ± 0.024 | 95.46 |
| | 0.750 | 0.775 ± 0.031 | 103.28 | 0.750 | 0.731 ± 0.046 | 97.36 |
| | 1.500 | 1.640 ± 0.057 | 109.33 | 1.500 | 1.448 ± 0.071 | 96.49 |
| Kidney | 0.525 | 0.483 ± 0.010 | 91.93 | 0.525 | 0.491 ± 0.013 | 93.59 |
| | 4.200 | 4.364 ± 0.052 | 100.35 | 4.200 | 4.428 ± 0.067 | 105.43 |
| | 15.750 | 15.549 ± 0.054 | 98.73 | 15.750 | 15.175 ± 0.230 | 96.35 |
| Brain | 0.200 | 0.183 ± 0.012 | 91.68 | 0.200 | 0.189 ± 0.016 | 94.75 |
| | 0.400 | 0.389 ± 0.006 | 97.14 | 0.400 | 0.408 ± 0.012 | 102.08 |
| 0.600 | 0.621 ± 0.014 | 103.43 | 0.600 | 0.601 ± 0.030 | 100.09 |

* The unit in plasma is µg/mL.
† (Mean ± SD).
‡ Accuracy (%) = (mean of measured concentration/spiked concentration) × 100.
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