Study on Pharmacokinetics of Three Preparations from Levistolide A by LC–MS-MS

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A rapid sensitive analytical method was established and validated to investigate levistolide A in rat plasma by liquid chromatography–tandem mass spectrometry operated in the positive ion mode. Levistolide A (LA) and internal standard (IS) andrographolide (AD), mixed with the plasma sample, were separated on a reversed phase SpurSIL™ C18 5 µm column. The precursor/product transitions (m/z) were 398.5/381.3 for LA and (m/z) 368.0/351.1 for AD. The calibration curve was linear over the range from 5 to 1,250 ng/mL for oral administration and 10–4,000 for intravenous administration with a correlation coefficient (r) ≥0.9993. The lower limit of quantification was 5 ng/mL for LA in plasma. The inter- and intra-day accuracy and precision were less than ±15% of the relative standard deviation. In this study, the developed method is successfully applied to the comparative pharmacokinetic study of LA in rats after oral administration of LA alone, Rhizoma Chuanxiong, and Danggui-Shaoyao-San along with the bioavailability study of LA in rats. Our study shows that low bioavailability (7.5%) is observed after oral administration of LA. Traditional formula compatibility of Danggui-Shaoyao-San could significantly enhance LA bioavailability compared with LA alone and Rhizoma Chuanxiong.

Introduction

Danggui-Shaoyao-San (DSS), consisting Paeonia lactiflora, Angelica sinensis, Rhizoma Chuanxiong, Poria cocos, Atractylodes macrocephala and Alismatis, is a common formula in traditional Chinese medicine. It not only has blood-activating and stasis-eliminating pharmacological effects for the treatment of gynecological disorders, but also has an antidepressant-like effect for the treatment of depression (1, 2). In addition, it possesses the ability for improvement of the cognitive functions and the neuroprotective dysfunctions, leading to an effective improvement of Alzheimer’s patients in Asian countries (3–5). Recently, it is proved to ameliorate deterioration of cognition in SAMP8, especially in female animals. Increasing estradiol (E2), NO and glycine might contribute to the cognitive improvement effect of DSS in female SAMP8 (6). Meanwhile, DSS also contributes to improve myocardial ischemia (7).

Rhizoma Chuanxiong which belongs to Umbelliferae family, as a significant constituent part of DSS, is the dried rhizome of Ligusticum chuanxiong Hort. It is widely used in the prescriptions of traditional Chinese medicines (8). It has been confirmed to have antioxidative effects (9). In addition, it is significantly effective in the treatment of headache and vertigo as well as vitalizing blood circulation to treat cardiovascular diseases, such as angina pectoris, ischemic stroke, migraines, menstrual disorders, amenorrhea, dysmenorrhea, abdominal pain with mass formation, headaches and rheumatic arthralgia (10, 11). Besides, it was reported for dispersal of tissue stasis, removal of chronic inflammation and facilitation of tissue perfusion (12). The elementary biologically active components of Rhizoma Chuanxiong are organic acids, alkaloids and phthalides (9, 13). Moreover, extracts of lobed kudzuvine root and Rhizoma Chuanxiong were investigated for their in vitro of beta-amyloid (Aβ1-42)-aggregation- and acetylcholinesterase-inhibitory activities (10).

Levistolide A (LA), polymerized by two molecular ligustilide, has been demonstrated to be the active components in Rhizoma Chuanxiong (14). It is responsible for antimycobacteria as well as curing cardiovascular diseases (15, 16). In addition, LA is available for sensitizing multidrug resistance medicine cells to various chemotherapy drugs (17). It was also proved that levistolide A and vinorelbine have a synergic anticancer effect on human non-small cell lung carcinoma (18).

To evaluate the determination of LA in plants or pharmaceutical samples, several methods have been reported, which include chromatographic techniques, NMR spectroscopy and tandem mass spectrometry(MS) (16) and high-performance liquid chromatography with diode-array detection electrospray tandem mass spectrometry (HPLC–DAD) (3, 19). However, because of the poor selectivity and more time consumption, it raises the necessity to establish a method with high sensitivity, specificity, reliability and simplification to determine the pharmacokinetic parameters of LA. Zuo et al. identified absorbed components and metabolites of Rhizoma Chuanxiong decoction by ultraviolet (UV) detection, tandem mass spectrometry (MS) and MS/MS (15). However, its analysis condition was unsuited to biological samples. In addition, there are no publications regarding the simultaneous determination of LA, Rhizoma Chuanxiong and DSS extract for the target of LA and the application of the pharmacokinetics so far. Therefore, in this study, a robust and selective HPLC coupled with mass spectrometry (LC–MS) method was used for the quantitation of LA in rat plasma. It may be a turning point in the quantification of LA as well as bioavailability after i.g. and i.v. administration.
Experimental

Instruments and reagents
The samples were analyzed by liquid chromatography–tandem mass spectrometry (LC–MS-MS), which consists of an Agilent 1200 series liquid chromatographic system (containing a G1311A Quart pump, a G1322A degasser, a G1313A automatic sampler (ALS) and a G1316A thermostatted column compartment; Agilent Technologies, USA) interfaced to a API4000 triple quadruple mass spectrometer (Applied Biosystems/MDS-SCIEX, Foster City, CA, USA) and equipped with electrospray ionization (ESI). Data acquiring and processing were carried out on Analyst 1.5.1 software package (Applied Biosystems, MDS-SCIEX, Toronto, Canada), which also controlled the LC–MS-MS system. The separation was achieved on a Spursil C18 column (150 × 4.6 mm, 5 μm; Waters Corp., Milford, MA, USA). Andrographolide (>98% purity, LA) was purchased from Chengdu Herbpurify Co., Ltd (Chengdu, China). Andrographolide (>98% purity, AD) was purchased from National Institutes for Food and Drug Control and used as the internal standard (IS). Figure 1 shows the chemical structures and fragmentation pattern of LA and AD. HPLC-grade methanol was supplied by Merck (Darmstadt, Germany). The purified water used for the LC–MS-MS analysis, which was produced with a Millipore water purification system (Millipore, Bedford, MA, USA). HPLC-grade ammonium acetate was purchased at CNW Technologies GmbH (Germany). All other chemicals and solvents were of analytical grade. Freshly obtained drug-free rat plasma was collected from male Sprague-Dawley (SD) rats (supplied by Medical Experimental Animal Center of Guangzhou University of Chinese Medicine) in our laboratory and reserved at −80°C until use.

Preparation of Rhizoma Chuanxiong extract
One kilogram dry powder of *Rhizoma Chuanxiong* (purchased from Xing Yuanchun Chinese Pharmacy, Guangzhou, China) was immersed overnight in 10-fold volume of 95% ethanol. The mixture was extracted under reflux twice each 2 h. After filtration, the supernatants were desiccated *in vacuo*. Then the dried residue was compounded in 0.3% sodium carboxy methylcellulose (CMC-Na) suspension. Afterward, we obtained an oral solution of *Rhizoma Chuanxiong* decoction with 2 mg/mL of LA in it.

Preparation of Danggui-Shaoyao-San extract
Totally 2,610 g dry powder of DSS including 900 g *P. lactiflora*, 270 g *A. sinensis*, 270 g *Rhizoma Chuanxiong*, 360 g *P. cocos*, 360 g *A. macrocephala* and 450 g *Alium sativum* (purchased from Xing Yuanchun Chinese Pharmacy) was immersed overnight in 10-fold volume of 95% ethanol. The mixture was extracted under reflux twice for 2 h. The supernatants were filtered before evaporating to desiccation *in vacuo*. Then the dried residue was also compounded in 0.3% CMC-Na suspension. Finally, an oral solution of DSS decoction with 2 mg/mL of LA in it was procured.

Preparation of calibration samples and quality control samples
The primary stock solutions of LA (66.6 μg/mL) and the IS AD (230 μg/mL) were prepared in methanol, respectively. The calibration curves were made as follows: one is for oral administration, which was obtained by diluting the mixture of the stock standard solutions with methanol at effective concentrations of 5, 10, 50, 125, 250, 500, 1,250 and 2,500 ng/mL. Another set of the concentration of calibration curve line was as the same manner to prepare the concentrations of 10, 25, 100, 200, 400, 1,000, 2,000 and 4,000 ng/mL for intravenous administration. All solutions were stored at 4°C and were brought to room temperature prior to use. Calibration curve samples for measuring LA were freshly prepared by spiking 10 μL working standard solution with 100 μL drug-free rat plasma. Quality control (QC) samples were separately prepared in the same manner as the calibration samples at low (10 and 25 ng/mL), medium (500 and 1,000 ng/mL) and high (1,000 and 3,000 ng/mL) LA levels for i.g. and i.v., respectively. The IS working solution (AD, 500 ng/mL) was diluted by the primary stock solutions. Further processing of both the calibration curve samples and QC samples were executed as the procedure mentioned in preparation of plasma sample section.

Animals
Thirty-two male SD rats with a weight of 250 ± 10 g were obtained from Laboratory Animal Center of Guangzhou University of Traditional Chinese Medicine. All of the rats were sustained under controlled air-conditioned conditions (temperature, 22 ± 2°C; relative humidity, 50 ± 10%; commutative 12 h light–dark cycles). The rodents were supplied free food and water intake and acclimatized to our animal room for 7 days. The animal investigation was conducted according to the Regulations of the Animal Ethics Committee of Guangzhou University of Traditional Chinese Medicine.

All rats were fasted for 12 h and had free access to water prior to dosing. Thirty-two male SD rats were randomly divided into four groups with eight rats in each. Three groups were by oral administration at the doses of 20 mg/kg of LA. The first group...
was given an *Rhizoma Chuanxiong* solution containing LA at a concentration of 2 mg/mL in 0.30% CMC-Na suspension. The second group was given a DSS solution containing LA at a concentration of 2 mg/mL inside 0.30% CMC-Na suspension. The third one was managed to acquire an LA standard solution with a concentration of 2 mg/mL in 0.30% CMC-Na suspension. Another one group was assigned to receive a dose of 2 mg/kg LA standard solution intravenously, which was prepared in physiologic saline. Serial blood samples (500 mL) were collected into heparinized plastic tubes via the postorbital vein plex from each rat at 0 h (before dosing) and subsequently at 0.083, 0.25, 0.5, 1, 1.5, 2, 4, 6, 8 and 12 h after oral dosing and at 0.033, 0.117, 0.25, 0.5, 0.75, 1, 2, 4, 6, 8 and 12 h after intravenous dosing. The blood samples were rapidly centrifuged at 3000g for 10 min at 4°C to obtain plasma. These plasma samples were frozen at −80°C until LC–MS-MS analysis.

**Preparation of plasma sample**

The solid-phase extraction (SPE) cartridges (ProElut C18 200 mg/3 mL, 50/pk; Dikma Technology, USA) were washed with 3,000 μL of methanol followed by 3,000 μL of water. After the frozen plasma samples (100 μL) were thawed out at room temperature, 10 μL IS (500 ng/mL) and 790 μL water were spiked with it. The resultant mixture was loaded to the SPE cartridges, then washed with 1,000 μL of water. Clean 1.5 mL Eppendorf tubes were positioned under the SPE cartridges and the objective compounds were eluted with 1,000 μL of methanol–1 mM ammonium acetate (90 : 10, v/v) solution. Compounds were separated under isocratic conditions at a flow rate of 0.25 mL/min. The chromatographic run time for each injection was 5 min. The column temperature was set to 35°C using a column heater.

**Liquid chromatographic conditions**

The LC mobile phase was methanol–1 mM ammonium acetate (90 : 10, v/v) solution. Compounds were separated under isocratic conditions at a flow rate of 0.25 mL/min. The chromatographic run time for each injection was 5 min. The column temperature was set to 35°C using a column heater.

**Mass spectrometric experiments**

Typical sets of operation parameters used in this study were under the following conditions: the probe temperature was 400°C, the nebulizer gas (GS1), turbo gas (GS2) and curtain gas (CUR) were nitrogen and separately set to 35, 25 and 15 psi. The ion spray voltage was set at 4,500 V. The sensitivity of detection of both LA and IS in the positive ion mode was found to be higher than that in the negative mode. So positive ESI mode was used, and the ions were performed in the multiple reaction monitoring (MRM) mode, in which we monitored the transition selected for determination. The dwell time was at 200 ms. Optimal compounds parameters including declustering potential (DP), entrance potential (EP), collision energy (CE) and collision exit potential (CXP) were 40.4, 8.9, 11.2 and 15 V and 40.3, 7.2, 11.1 and 12 V for LA and IS, respectively. The corresponding parameters for each analyte are summarized in Table I.

<table>
<thead>
<tr>
<th>Table I</th>
<th>Optimized Mass Parameters for LA and the Internal Standard AD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Analyte</td>
<td>MRM (m/z)</td>
</tr>
<tr>
<td>LA</td>
<td>388.40 → 381.4</td>
</tr>
<tr>
<td>AD</td>
<td>388.20 → 297.10</td>
</tr>
</tbody>
</table>

**Method validation**

The analytical method was validated to satisfy the US Food and Drug Administration (FDA) bioanalytical method validation guidance (20). Validation parameters were discussed in the following.

**Matrix effect**

The matrix effects in rat plasma, except for analytes, may cause ion inhibition or enhancement of the signal. An assessment of matrix effect was determined by comparing peak areas (A) of the standard analytes in spiked blank plasma (five different sources) with the corresponding peak areas (B) obtained by injection of analytes added in the mobile phase at appropriate concentration. The matrix effect for LA was investigated at QC of low, medium and high concentrations, whereas the matrix effect over the IS was determined at a single concentration of 500 ng/mL. The peak area ratio of A/B (as a percentage) was used to measure quantification for the matrix effects. It was identified that the matrix effects did not have an obvious effect if the ratio is <85% or >115% (21).

**Stability**

The stability was tested with untreated QC samples at three concentration levels, i.e., low (10 and 25 ng/mL), medium (500 and 1,000 ng/mL) and high (1,000 and 3,000 ng/mL). LA levels for i.g. and i.v., respectively, using five replicates at each concentration and each storage condition. The long-term stability was assessed for 30 days of storage at −20°C. The freeze–thaw stability was investigated over three freeze–thaw cycles (−20°C to room temperature as one cycle). The post-preparative stability was determined at room temperature for 24 h in the same manner. The stability was expressed by the relative error (RE). Samples confirmed to be stable if assay values exceed the acceptance criteria of accuracy and precision (85–115%) (22).

**Extraction recovery**

The recovery of LA was tested at three QC levels (n = 5), respectively. Recoveries were calculated by comparing the mean peak area of the extracted QC sample with that of the standard solution containing the equivalent amount of analyses (23). The recovery of LA was operated at low (10 ng/mL), medium (500 ng/mL) and high (1,000 and 3,000 ng/mL) LA levels for i.g. and i.v., respectively, using five replicates at each concentration and each storage condition. The long-term stability was assessed for 30 days of storage at −20°C. The freeze–thaw stability was investigated over three freeze–thaw cycles (−20°C to room temperature as one cycle). The post-preparative stability was determined at room temperature for 24 h in the same manner. The stability was expressed by the relative error (RE). Samples confirmed to be stable if assay values exceed the acceptance criteria of accuracy and precision (85–115%) (22).

**Calibration curve**

The calibration curve was a line obtained by spiking pooled drug-free plasma with an appropriate amount of working solution to produce the calibration curve points whose concentration was 5, 10, 50, 125, 250, 500, 1,250 and 2,500 ng/mL for oral administration and 10, 25, 100, 200, 400, 1,000, 2,000 and 4,000 ng/mL for intravenous administration. Besides, IS was 500 ng/mL.
The correlation coefficient (r) of calibration curve was found to be 0.9993 or better.

**Lower limit of quantification**
The lower limit of quantification (LLOQ) was determined as the concentration that can be accurately measured, 10 times the signal-to-noise (S/N) ratio.

**Accuracy and precision**
To determine the accuracy and precision of this method, three QC concentration levels containing low (10 and 25 ng/mL), medium (500 and 1,000 ng/mL) and high (1,000 and 3,000 ng/mL) LA levels for i.g. and i.v., respectively, and they were done in blank plasma for analysis. The measured value was expressed as the mean of the five values. The accuracy was determined by comparing the difference between the nominal value and measured value expressed as the RE. The precision was defined as the coefficient of variation and expressed as relative standard deviation (RSD) (24). For the evaluation of intra-day accuracy and precision, five aliquots of each concentration were analyzed in 1 day. For inter-day accuracy and precision, five replicates of each concentration were evaluated on 3 days. The criteria for acceptability of accuracy and precision should be within ±15% (25).

**Specificity and selectivity**
The assay were assessed by comparing the chromatograms from five different lots of drug-free rat plasma with those mixed with respective standards to detect if there any interferences at the LC peak region for LA and IS from endogenous plasma components.

![Representative LC–MS-MS chromatograms of blank plasma (A), LLOQ of LA and IS spiked with blank plasma (5 and 10 ng/mL, respectively) (B), blank plasma spiked with 1016 ng/mL of LA and 500 ng/mL of IS (C) and rat plasma at 1.5 h following a single oral administration of DSS at 20 mg/kg (D).](image-url)
All the plasma samples were processed and analyzed using the same procedure and LC–MS conditions as mentioned.

**Pharmacokinetic analysis**

The relevant pharmacokinetic analysis was performed as follows: the maximum concentration ($C_{\text{max}}$) and the time-to-maximum concentration ($T_{\text{max}}$) were determined by the experimental data. Other pharmacokinetic parameters maintaining biological half-life ($t_{1/2}$) and total body clearance (CI) were calculated by using NONMEM Program version 1.1 (Globomax Inc., Ellicott City, MD, USA). The elimination rate constant ($k$) was estimated from the terminal linear portion of the log plasma concentration–time curve, and the half-life ($t_{1/2}$) of the drug was obtained by 0.693/$k$. The area under the plasma concentration–time curve (AUC) from time zero to the last measurable time point ($AUC_{0\rightarrow \infty}$) was estimated using the trapezoidal rule-extrapolation method (26).

**Results**

**Liquid chromatography**

It was essential in the method development for parameters optimization such as selection of types of reversed-phase chromatographic column and organic solvent, composition of the mobile phase, flow rate, etc. Reversed-phase chromatographic column containing Spurisl$^{\text{TM}}$ C18 5 $\mu$m column (150 x 2.1 mm; Dikma, USA), Gemini 5 $\mu$m C18 110A (50 x 2 mm 5 $\mu$m; Phenomenex, USA) and Analytical ODS 5 $\mu$m (4.6 x 150 mm; Agilent, USA) were tested to improve peak shape. Finally, a Spurisl$^{\text{TM}}$ C18 5 $\mu$m column (150 x 2.1 mm; Dikma, USA) was suitable for the chromatographic separation because under the current LC conditions, the column provided excellent results in terms of response, retention time and peak shapes. The mobile phase such as methanol, ammonium acetate and formic acid along with altered flow rates (in the range of 0.15–0.3 mL/min) were tested for complete chromatographic resolution of levistolide A and IS. It was also concluded that the optimal mobile phase consisted of methanol—1 mM ammonium acetate (90:10, v/v) at a flow rate of 0.25 mL/min, because methanol and ammonium acetate were able to get the lowest background noise and the best resolution. The chromatographic run time for one injection was 5 min. The method exhibited the baseline separation of LA and IS were approximately at 2.0 and 3.0 min with a good peak shape and symmetry, respectively (Figure 2). Figure 2 shows representative LC–MS-MS chromatograms of blank plasma, LLOQ of LA and IS spiked with blank plasma (5 and 10 ng/mL, respectively), blank plasma spiked with 70 ng/mL of LA and 150 ng/mL of IS and rat plasma at 1.5 h following a single oral administration of DSS at 20 mg/kg.

**Mass spectrometry**

In our study, to develop an optimal MS-MS method for detection of LA in biological samples, a comparison between the ionization of LA under the ESI negative and positive ion modes was carried out. Our research results showed that a better response with good sensitivity, reproducibility and fragmentation as well as stronger intensity of the most abundant molecule ion for LA was produced in the positive ion mode than that in the negative mode. Under the same MS detector conditions, AD (IS), as a result of its similarity in chemical structure to LA, could be ionized efficiently as well. Thus, the positive ion mode was chosen for analysis of LA throughout this study. Furthermore, precursor ions were fragmented and unique product ions were measured in MRM, which enabled selective detection of all compounds simultaneously. The sensitive ions in the full-scan positive ion mass spectra were 398.5 [M+NH$_4$]$^+$ → 381.3 [M+NH$_4$–OH]$^+$ for LA and 368.0 [M+NH$_4$]$^+$ → 351.1 [M+NH$_4$–OH]$^+$ for AD (IS). The transitions of $m/z$ 398.5 precursor ion to the $m/z$ 381.3 were used for quantification for LA. Similarly, for AD $m/z$ 368.0 precursor ion to the $m/z$ 351.1 was also used for the quantification of the analytes.

**Validation procedures**

**Matrix effect**

In this work, the matrix effects of plasma samples were evaluated by determining QC samples under the optimized assay conditions. The results are presented in Table II, all of them were

**Table II**

<table>
<thead>
<tr>
<th>Route of administration</th>
<th>LA concentration (ng/mL)</th>
<th>MC (mean ± SD) (ng/mL)</th>
<th>Precision (%)</th>
<th>Accuracy (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>i.g.</td>
<td>10</td>
<td>10.26 ± 0.65</td>
<td>6.30</td>
<td>2.53</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>467.54 ± 10.75</td>
<td>2.30</td>
<td>6.84</td>
</tr>
<tr>
<td></td>
<td>1,000</td>
<td>989.76 ± 45.53</td>
<td>4.60</td>
<td>1.03</td>
</tr>
<tr>
<td>i.v.</td>
<td>25</td>
<td>23.45 ± 0.75</td>
<td>3.20</td>
<td>6.61</td>
</tr>
<tr>
<td></td>
<td>1,000</td>
<td>943.12 ± 42.44</td>
<td>4.50</td>
<td>6.03</td>
</tr>
<tr>
<td></td>
<td>3,000</td>
<td>2879.43 ± 37.43</td>
<td>1.30</td>
<td>4.19</td>
</tr>
</tbody>
</table>

MC, measured concentration.

**Table III**

<table>
<thead>
<tr>
<th>Route of administration</th>
<th>LA concentration (ng/mL)</th>
<th>Three freeze–thaw cycles</th>
<th>Short-term stability for 24 h in plasma (mean ± SD)</th>
<th>Long-term stability for 30 days at –70°C (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>MC (mean ± SD) (ng/mL)</td>
<td>Precision (%)</td>
<td>Accuracy (%)</td>
</tr>
<tr>
<td>i.g.</td>
<td>10</td>
<td>9.97 ± 0.47</td>
<td>4.7</td>
<td>0.30</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>494.32 ± 12.85</td>
<td>2.6</td>
<td>1.15</td>
</tr>
<tr>
<td></td>
<td>1,000</td>
<td>938.47 ± 54.43</td>
<td>5.8</td>
<td>6.56</td>
</tr>
<tr>
<td>i.v.</td>
<td>25</td>
<td>24.14 ± 1.04</td>
<td>4.3</td>
<td>3.56</td>
</tr>
<tr>
<td></td>
<td>1,000</td>
<td>973.91 ± 13.63</td>
<td>1.4</td>
<td>2.68</td>
</tr>
<tr>
<td></td>
<td>3,000</td>
<td>2901.34 ± 69.63</td>
<td>2.4</td>
<td>3.40</td>
</tr>
</tbody>
</table>

MC, measured concentration.
within the acceptable limit, which indicated that no significant ion suppression or enhancement from the plasma matrix was observed on the detection.

Stability
The stability experiments were aimed at testing the possible conditions to which the samples might be exposed during storage and handling. The post-preparative stability indicated that LA was stable at room temperature for 24 h. LA could also withstand three cycles of the freeze–thaw process for QC samples. QC samples were stable when stored frozen at −20°C for at least 30 days. All results of the stability tests are summarized in Table III, indicated no appreciable degradation occurred for routine analysis.

Extraction recovery
The extraction recovery was determined in six replicates by comparing the peak areas of the extracted plasma at three QC levels with those obtained from the direct injection of standard solutions without preparation at the same concentrations. Liquid/liquid extraction has been commonly applied in biological fluids by different solvents. Therefore, we tested the extraction recoveries of LA using different organic solvents including acetonitrile, methanol and their mixtures. But we observed bad peak shapes after that. Subsequently, SPE was investigated as samples pre-treatment technique. Considering their lipophilic characterization, the extracted recovery with Dikma C18-SPE column was high. Table IV summarizes the results.

Calibration curve, linearity and LLOQ
The calibration curves were established by eight calibration standards for oral administration and intravenous administration, respectively. The calibration standard curve had a reliable reproducibility over the standard concentrations across the calibration range. The calibration curve for oral administration was prepared by a typical equation $y = 0.0024x + 0.0037$, and the calibration curve for i.v. administration was obtained by a typical equation $y = 0.002x + 0.005$, the best fit of peak area ratios of LA to IS ($y$) versus the plasma concentration of LA ($x$, ng/mL). The average correlation coefficient ($r$) was $>0.9993$, exhibited to be linear over the calibration range for LA. The LLOQ samples of six different rodents’ plasma was defined as the LLOQ in the calibration curve that can be accurately measured. The LLOQ for LA were analyzed and found to be 5.0 ng/mL, with an accuracy of 108.8% and precision of 8.4%. The LLOQ for IS was selected to be 10.0 ng/mL, with an accuracy of 104.2% and precision of 9.2%, respectively. The LLOQ was based on S/N ≥10, where the limit is sufficient for rat pharmacokinetic studies following oral administration and i.v. administration of LA.

### Accuracy and precision
The intra-day accuracy and precision in rat plasma were evaluated using the low, medium and high QC ($n = 5$) spiked plasma samples. One reproduction of the QC samples at each concentration level from three separate validation batches was used to determine the inter-day precision. As summarized in Table V, the obtained results suggested that the method was judged to have a satisfactory accuracy and precision.

### Selectivity and specificity
The selectivity and specificity were assessed by comparing the typical chromatographic profiles of five different batches of drug-free rat plasma with the corresponding spiked plasma samples spiked with LA and the IS. The retention times of LA and the IS were ~2.0 and 3.0 min. No endogenous substances were observed to interfere with LA and the IS under the specified LC–MS-MS conditions.

### Pharmacokinetic study
The newly validated method was successfully applied to the pharmacokinetics of LA in rat plasma after i.v. and i.g. administration at a dose of 2 and 20 mg/kg, respectively. Furthermore, the mean plasma concentration–time profile of LA after oral administration and intravenous administration to rats is shown in Figure 3. The major pharmacokinetic parameters were calculated by a non-compartmental model and are listed in Table VI.

<table>
<thead>
<tr>
<th>Route of administration</th>
<th>LA concentration (ng/mL)</th>
<th>MC (mean ± SD) (ng/mL)</th>
<th>RSD (%)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>i.g.</td>
<td>10</td>
<td>9.65 ± 0.50</td>
<td>5.18</td>
<td>96.5</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>478.24 ± 37.30</td>
<td>7.80</td>
<td>95.6</td>
</tr>
<tr>
<td></td>
<td>1,000</td>
<td>975.42 ± 27.31</td>
<td>2.80</td>
<td>97.5</td>
</tr>
<tr>
<td>i.v.</td>
<td>25</td>
<td>26.13 ± 1.67</td>
<td>6.39</td>
<td>104.5</td>
</tr>
<tr>
<td></td>
<td>1,000</td>
<td>962.41 ± 40.42</td>
<td>4.20</td>
<td>96.2</td>
</tr>
<tr>
<td></td>
<td>3,000</td>
<td>2973.35 ± 371.67</td>
<td>12.50</td>
<td>99.1</td>
</tr>
</tbody>
</table>

MC, measured concentration.

### Table V Intra- and Inter-Day Precision and Accuracy for Analysis of LA in Plasma

<table>
<thead>
<tr>
<th>Route of administration</th>
<th>LA concentration (ng/mL)</th>
<th>Intra-day ($n = 5$)</th>
<th>Inter-day ($n = 5$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MC (mean ± SD) (ng/mL)</td>
<td>Precision (%)</td>
<td>Accuracy (%)</td>
</tr>
<tr>
<td></td>
<td>MC (mean ± SD) (ng/mL)</td>
<td>Precision (%)</td>
<td>Accuracy (%)</td>
</tr>
<tr>
<td>i.g.</td>
<td>10</td>
<td>9.76 ± 1.33</td>
<td>13.63</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>484.51 ± 12.62</td>
<td>2.50</td>
</tr>
<tr>
<td></td>
<td>1,000</td>
<td>963.25 ± 27.93</td>
<td>2.90</td>
</tr>
<tr>
<td>i.v.</td>
<td>25</td>
<td>24.69 ± 0.91</td>
<td>3.69</td>
</tr>
<tr>
<td></td>
<td>1,000</td>
<td>937.17 ± 47.86</td>
<td>5.11</td>
</tr>
<tr>
<td></td>
<td>3,000</td>
<td>2976.64 ± 116.09</td>
<td>3.90</td>
</tr>
</tbody>
</table>

MC, measured concentration.
Discussion

The statistical analysis between three oral groups was performed using ANOVA. Compared with the LA group and the *Rhizoma Chuanxiong* group, the AUC_{0→∞} values of the DSS group were remarkably improved (*P* < 0.05). Furthermore, the *T*_{max} of the DSS was longer than that of the LA group and the *Rhizoma Chuanxiong* group. The higher AUC_{0→∞} values and the longer *T*_{max} always mean that the analytes could stay longer and be absorbed better in the body, leading to a better effect on improving the absorption of LA, which may prolong its residence time and get the large AUC_{0→∞} values, and thus prolong its efficacy.

It is also commonly reported for the pharmacokinetics of traditional Chinese medicine (TCM) that the coexisting constituents in herbal extracts may serve as substrates of inducers or inhibitors of cytochrome enzymes (27). It has been proven that CYP3A4, CYP2C9 and CYP1A2 are the major isoenzymes participating in *in vitro* metabolism of ligustilide, which is one kind of essential ingredients belonging to *Rhizoma Chuanxiong* and *A. sinensis* (14, 23). In addition, it has been verified that CYP3A plays a primary role in tetramethylpyrazine metabolism in rats (28). The ingredients like ligustilide and tetramethylpyrazine in DSS may act as substrates of inhibitors or inducers of cytochrome enzymes and thus may affect on the pharmacokinetics of LA (29). However, further research studies are required to verify these relationships.

The oral bioavailability calculated by (AUC i.g./dose)/(AUC i.v./dose) of LA in rats was ~7.5%. It was reported that an army of effective ingredients in herbs that possess low bioavailability (30) may be attributable to extensive first-pass effect or the solubility of drugs (31–33), or even to be affected by coexisting constituents in the herbal extracts. For example, it was also proved that *P. lactiflora* Pall could reduce the plasma concentration and bioavailability of ligustilide, the dimer of LA; however, the *P. cocos–A. macrocephala–Alismatis* group contributes to increase its bioavailability (34). More studies to elucidate this hypothesis are warranted.

Because DSS is used in blood-activating and stasis-eliminating drugs, it is possible that the increase in ligustilide elimination (*P* < 0.05) may be an immediate result of hepatic blood flow change (35). Meanwhile, *A. sinensis* (36) of DSS could be responsible for activating blood circulation and *R. Chuanxiong* is proved to have effect on the influence for the blood velocity (32, 37), so that they could help to accelerate the elimination of LA. Nevertheless, to date there is no certain evidence to support this contention. Moreover, in most of the cases, clearance of LA and ligustilide was significantly higher than that of the hepatic blood flow in rats (38), implying that in addition to widespread metabolism, other factors might also contribute to the fast elimination of LA and ligustilide from systemic circulation.

This study demonstrates that important information for investigating the pharmacokinetics and oral bioavailability of LA, *Rhizoma Chuanxiong* and DSS extract *in vivo*. The results were complex and require more detailed research works. Further studies concerning the relationship among the pharmacokinetics of LA and other ingredients of DSS should be performed to obtain more effective information for clinical practice after the administration of DSS. The results of such studies should contribute to the feasibility of the effective therapeutic usage of TCM (39).

![Figure 3. Mean plasma concentration–time profile of LA (filled diamonds), *Rhizoma Chuanxiong* (filled squares), DSS (filled triangles) in rat plasma after oral administration to rats (n = 6) (A), and the mean plasma concentration–time profile of LA (filled circles) in rat plasma after intravenous administration to rats (n = 6) (B).](image-url)

**Table VI.**

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Parameters (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>k</em>₁ (h⁻¹)</td>
</tr>
<tr>
<td>LA (i.g.)</td>
<td>0.183 ± 0.02</td>
</tr>
<tr>
<td>DSS (i.g.)</td>
<td>0.250 ± 0.01*</td>
</tr>
<tr>
<td>Chuanxiong (i.g.)</td>
<td>0.217 ± 0.03</td>
</tr>
<tr>
<td>LA (i.v.)</td>
<td>0.289 ± 0.02*</td>
</tr>
<tr>
<td>LA (i.v.)</td>
<td>0.5 ± 0.05</td>
</tr>
<tr>
<td>LA (i.v.)</td>
<td>2.0 ± 0.10*</td>
</tr>
<tr>
<td>LA (i.v.)</td>
<td>0.75 ± 0.06</td>
</tr>
<tr>
<td>LA (i.v.)</td>
<td>3988.5 ± 319.04*</td>
</tr>
</tbody>
</table>

*ki*, rate of elimination; *ka*; rate of absorption; *kb*; rate of distribution.

*P* < 0.05 versus the LA (i.g.) group.
Conclusions
In this study, a rapid, simple, reproducible and suitable LC–MS–MS method was developed and validated for the quantification of LA with AD as IS in rat plasma with high recovery and minimal matrix effect. The assay utilized a SPE as sample clean-up procedure and a reversed-phase separation with sufficient selectivity and sensitivity. The results suggest that the method is able to be used in the drug metabolism and pharmacokinetic study of LA and other Chinese medicinal preparations containing LA, which is worthy of further studies. The accurate and precise method has been successfully applied to compare the pharmacokinetic behaviors between oral and intravenous groups, and the oral bioavailability was calculated, which can be a reference of pharmacology research related to *Rhizoma Chuanxiong*. DSS is convenient to be dosed and with low risks. In the meanwhile, it not only has a favorable absorption after oral intake, but also lasts a longer efficacy.

Acknowledgments
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