Pharmacokinetics and bioavailability of liensinine in mouse blood by UPLC-MS/MS

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ABSTRACT
Liensinine is a bisbenzyltetrahydroisoquinoline alkaloid extracted from lotus (Nelumbo nucifera GAERTNER., Nelumbonaceae), especially in its embryo loti "Lien Tze Hsin" (green embryo of mature seed). A rapid and simple UPLC-MS/MS method was developed to determine liensinine in mouse blood and its application to a pharmacokinetic study. The blood samples were preprocessed by protein precipitation using acetonitrile. Midazolam (internal standard, IS) and liensinine were gradient eluted by mobile phase of methanol and water (0.1% formic acid) in a Waters UPLC BEH C18 column. The multiple reaction monitoring of m/z 611.3 → 206.1 for liensinine and m/z 326.2 → 291.1 for IS with an electrospray ionization (ESI) source was used for quantitative detection. The calibration curve ranged from 0.5 to 400 ng/mL (r > 0.995). The accuracy ranged from 92.2 to 108.2%, the precision of intra-day and inter-day was less than 14%, and the matrix effect was between 100.0% and 109.6%, the recovery was better than 71.0%. The developed UPLC-MS/MS method was successfully used for a pharmacokinetic study of liensinine in mice after oral (5 mg/kg) and intravenous administration (1 mg/kg), and the absolute availability of liensinine was 1.8%.

KEYWORDS
liensinine, mice, UPLC-MS/MS, determination, bioavailability

INTRODUCTION
Liensinine is a bisbenzyltetrahydroisoquinoline alkaloid extracted from lotus (Nelumbo nucifera GAERTNER., Nelumbonaceae), especially in its embryo loti "Lien Tze Hsin" (green embryo of mature seed). According to reports in the literature, liensinine can inhibit the expression of inflammatory factors tumor necrosis factor α (TNF-α) and interleukin 1β (IL-1β) after cerebral ischemic perfusion in rats; in addition, it can also increase superoxide the activity of substance dismutase (SOD) and glutathione (GSH) reduces the content of malondialdehyde (MDA) and improves focal cerebral ischemia-reperfusion injury in rats. Pro-inflammatory factors such as TNF-α, IL-6, IL-1β can promote the excessive accumulation of oxygen free radicals and cause oxidative stress response.

Pharmacokinetics, as a science that uses mathematical analysis to deal with the dynamic processes of drugs in the body [1, 2], has great theoretical value and is an important part of pharmacy. To better understand the pharmacokinetics, an analytical method for the determination of liensinine in biological fluids is necessary. There have several literatures been reported for determination of liensinine in rat plasma and its pharmacokinetic study [3–6]. Therefore, it was necessary to develop a UPLC-MS/MS method for determination of liensinine in mouse blood and its application for the pharmacokinetics.
EXPERIMENTAL

Chemicals and animals

Liensinine and midazolam (IS) (both purity >98%) were obtained from Chengdu Munster biotechnology Co. Ltd (Chengdu, China). HPLC grade formic acid, acetonitrile and methanol were obtained from Merck (Darmstadt, Germany). Milli-Q water system was purchased from Millipore Sigma (Burlington, MA, USA). Twelve Institute of Cancer Research (ICR) mice (male, 20–22 g) were from Laboratory Animal Center of Wenzhou Medical University (Wenzhou, China).

Instrument and condition

An ACQUITY H-Class UPLC equipped with a XEVO TQS-micro triple quadrupole mass spectrometry (Waters Corp, Milford, MA, USA) with electrospray ionization (ESI), was used for analysis mouse blood sample. UPLC BEH C18 (1.7 μm, 2.1 × 50 mm) column was used for separation, it maintained at 30 °C. The mobile phase consisted of methanol and water (0.1% formic acid). The flow rate was set at 0.4 mL/min. The gradient conditions with as follows: 0–0.2 min, 10% methanol; 0.2–1.4 min, 10–80% methanol; 1.4–2.0 min, 80% methanol; 2.0–2.1 min, 80–10%, methanol; 2.1–4.5 min, 10% methanol.

The mass condition was set as below: dry gas (nitrogen) of 900 L/h, capillary voltage of 2 kV, the temperature of source ionization of 150 °C, the temperature for drying gas of 450 °C.

The quantitative analysis was operated in an ESI positive mode and multiple reaction monitoring (MRM), m/z 611.3 → 206.1 for liensinine (cone voltage 46 v, collision voltage 28 v) and m/z 326.2 → 291.1 for IS (cone voltage 30 v, collision voltage 24 v).

Calibration standards

The stored solutions of liensinine (1.0 mg/mL) and midazolam (1.0 mg/mL) were prepared in methanol and water (1:1, v/v). Working standard solutions were diluted from the stored solution of liensinine by methanol, including 5, 20, 50, 200, 500, 1,000, 2,000, and 4,000 ng/mL. The working standard solution (100 ng/mL) of midazolam was diluted with methanol from the stored solution.

Calibration standards of liensinine were spiked to blank mouse blood to concentration of 0.5, 2, 5, 20, 50, 100, 200, and 400 ng/mL. Three quality control (QC) samples were also prepared in same way as calibration standards (1, 45 and 360 ng/mL). All the samples were stored at −20 °C.

Sample preparation

Acetonitrile (100 μL, containing IS 100 ng/mL) was added into 20 μL blood, mixed for 1.0 min, and centrifuged at 13,000 rpm for 10 min. The supernatant (2 μL) was injected into UPLC-MS/NS for analysis.

Method validation

The UPLC-MS/MS method validation performed according to the US Food and Drug Administration (FDA) bioanalytical guidelines [7].

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Fig. 1. Chemical structure and mass spectrum of liensinine (A) and midazolam (B)
**Selectivity.** The selectivity of the UPLC-MS/MS method was evaluated by analyzing blank mouse blood, the blank mouse blood spiked with liensinine and IS, and a mouse sample.

**Linear.** The standard concentration range is 0.5–400 ng/mL. Under the same conditions, measured the peak area of each peak, draw a standard curve with the peak area against the concentration, and evaluate the linearity of the standard curve.

**Precision and accuracy.** The precision and accuracy were evaluated by measuring mouse blood QC samples at six replicates. Precision was expressed as relative standard deviation (RSD), and the intra-day and inter-day precision were determined by measuring QC samples for three consecutive days. The intra- and inter-day precision were measured between the average value of QC samples and the true value for three consecutive days.

**Recovery and matrix effects.** The recovery was evaluated by comparing the measured peak area of QC samples with the corresponding standard peak area. The matrix effect was evaluated by comparing the peak area of the blank mouse blood with the standard solution of after sample treatment and the peak area of the corresponding standard solution.

**Stability.** The stability of liensinine in mouse blood was investigated by analyzing the QC samples placed under three storage conditions. Including long-term stability (−20 °C, 30 days), short-term stability (2 h at room temperature), freeze-thaw stability (3 consecutive freezing and thawing cycles for 3 days) (−20 °C to room temperature).

**Pharmacokinetic study.** Twelve mice were randomly divided into two groups (n = 6). One group was given liensinine (5 mg/kg) by oral administration and another group was given liensinine (1 mg/kg) by intravenous administration. This study was approved by the Animal Care Committee of Wenzhou Medical University. The blood samples (20 μL) were withdrawn from caudal vein after dosing at 0.083, 0.5, 1, 1.5, 2, 3, 4, 6, 8, 12 h, and stored at −20 °C until analysis.

The pharmacokinetics was fitted by the DSA 2.0 (China Pharmaceutical University, China). Bioavailability% = 100% × AUCpo / AUCiv, where po is for oral administration, iv is for intravenous administration.

**RESULTS**

**Method validation**

Fig. 2 exhibited the UPLC-MS/MS of a blank blood sample, a blank blood sample spiked with liensinine and IS, and a blood sample obtained after oral administration. No interference was found at the retention times of the liensinine and the IS.

The equation of the calibration curve (0.5–400 ng/mL) of liensinine was: \( y = (0.0081 \pm 0.0009) x + (0.0065 \pm 0.00053) \) \((r = 0.9981, n = 6)\), where \( y \) represented the ratio of the peak area of liensinine to that of IS, and \( x \) was the concentration of liensinine. The LLOQ was 0.5 ng/mL with the signal-to-noise ratio (S/N) of 10 for in mouse blood. The precision and accuracy of the LLOQ were 13.3 and 92.2%, respectively.

The accuracy ranged from 92.2 to 108.2%, the precision of intra-day and inter-day was less than 14%, and the matrix effect was between 100.0 and 109.6%, the recovery was better than 71.0%, in Table 1.

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The stability of liensinine in variations condition (room temperature for 2 h, −20 °C for 30 days, and 3 freezing and
thawing cycles) was acceptable, the accuracy was within 87 and 112%, and precision was less than 15%.

**Pharmacokinetic study**

The main pharmacokinetic parameters of liensinine were fitted by the non-compartment model, Table 2. The blood concentration of liensinine was showed in Fig. 3. The bioavailability was 1.8%, which exhibited a good oral absorption.

**DISCUSSION**

The mass spectrometry conditions were optimized. We chose the positive mode for the response of the liensinine was stronger than that in the negative ion mode. Then fragment peaks with relatively high fragments were selected as quantitative ion pairs, $m/z$ 611.3 → 206.1 for liensinine (cone voltage 46 v, collision voltage 28 v) and $m/z$ 326.2 → 291.1 for IS (cone voltage 30 v, collision voltage 24 v), were shown in Fig. 1.

Various mobile phases were tested, such as acetonitrile and 0.1% formic acid in water, acetonitrile and water, acetonitrile and 10 mmol/L ammonium acetate, methanol and 0.1% formic acid in water, methanol and water, and methanol and 10 mmol/L ammonium acetate. The methanol and 0.1% formic acid in water was used as the mobile phase because it achieved the better peak, and suitable retention time. The Waters UPLC BEH C18 column was adopted because the satisfactory peak shape.

Choosing suitable sample treatment method was an important step in methodology [8–10]. The extraction efficiencies of acetonitrile, ethyl acetate, and methanol were compared. The extraction efficiencies of acetonitrile

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**Table 1. The accuracy, precision, matrix effect and recovery of liensinine in mouse blood samples ($n = 6$)**

<table>
<thead>
<tr>
<th>Concentration (ng/mL)</th>
<th>Precision (%)</th>
<th>Accuracy (RSD%)</th>
<th>Matrix effect (%)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Intra-day</td>
<td>Inter-day</td>
<td>Intra-day</td>
<td>Inter-day</td>
</tr>
<tr>
<td>0.5</td>
<td>11.2</td>
<td>13.3</td>
<td>92.2</td>
<td>93.9</td>
</tr>
<tr>
<td>1</td>
<td>3.4</td>
<td>6.9</td>
<td>104.3</td>
<td>102.7</td>
</tr>
<tr>
<td>45</td>
<td>8.5</td>
<td>7.2</td>
<td>97.8</td>
<td>108.2</td>
</tr>
<tr>
<td>360</td>
<td>9.0</td>
<td>5.9</td>
<td>100.7</td>
<td>96.0</td>
</tr>
</tbody>
</table>

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**Table 2. Main Pharmacokinetic study of liensinine after oral and intravenous administration**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Unit</th>
<th>po (5 mg/kg)</th>
<th>iv (1 mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUC(0-t)</td>
<td>ng/mL·h</td>
<td>18.8 ± 2.7</td>
<td>211.2 ± 54.9</td>
</tr>
<tr>
<td>AUC(0-∞)</td>
<td>ng/mL·h</td>
<td>19.1 ± 2.8</td>
<td>227.9 ± 60.1</td>
</tr>
<tr>
<td>MRT(0-t)</td>
<td>h</td>
<td>3.2 ± 0.4</td>
<td>2.6 ± 0.5</td>
</tr>
<tr>
<td>MRT(0-∞)</td>
<td>h</td>
<td>3.4 ± 0.5</td>
<td>3.5 ± 0.9</td>
</tr>
<tr>
<td>$t_{1/2z}$</td>
<td>h</td>
<td>1.9 ± 0.2</td>
<td>3.8 ± 0.8</td>
</tr>
<tr>
<td>$CL_F$</td>
<td>L/h/kg</td>
<td>266.0 ± 41.3</td>
<td>4.7 ± 1.2</td>
</tr>
<tr>
<td>$V_F$</td>
<td>L/kg</td>
<td>708.5 ± 79.9</td>
<td>25.9 ± 11.0</td>
</tr>
<tr>
<td>$C_{max}$</td>
<td>ng/mL</td>
<td>5.3 ± 0.2</td>
<td>169.5 ± 53.5</td>
</tr>
<tr>
<td>Bioavailability</td>
<td></td>
<td>1.8%</td>
<td></td>
</tr>
</tbody>
</table>
(around 71%) were better than methanol (around 60%) and ethyl acetate (around 50%). And the matrix effects of acetonitrile were acceptable (around 100%). Compared with rat, the volume of mouse is relatively unsuitable for taking a lot of blood, so this experiment only needs 20 μL for pharmacokinetic study. Because the volume of blood taken is relatively small, it is not suitable for centrifugation to take serum samples. In this study, the accuracy, precision, matrix effect, and stability of the direct protein precipitation of blood meet the requirements of biological testing, so it is possible to directly use blood samples for detection and analysis.

It was also an important task to select the internal standard during the method establishment process [11–13]. In this experiment, several compounds including midazolam, astragal, rubiadin and narciclasine were compared. It was comprehensively shown that midazolam had a better peak shape, a stable structure, and the peak time was similar to that of liensinine, and it could meet the correction function of the internal standard of the experiment.

UPLC-MS/MS was applied to the quantitative analysis of liensinine in mouse blood, which was much faster than traditional HPLC [14, 15]. The AUC(0–t) of were 211.2 ± 54.9 and 18.8 ± 2.7 ng/mL·h for intravenous and oral administration, it showed that the oral absorption was not good, and the bioavailability was 1.8%. As far as we known, no quantitative analysis and pharmacokinetic study of liensinine in mice has been reported.

Peng et al. developed a simple U-HPLC-MS/MS method for the determination of liensinine and iso-liensinine in rat plasma, and its application to a pharmacokinetic study after intravenous administration of 5.0 mg/kg liensinine and iso-liensinine, the t1/2 was 9.28 ± 3.25 h, CL was 3.25 ± 1.00 L/h kg for liensinine [6]. Hu et al. developed a simple, sensitive, and robust analytical method based on UPLC–MS/MS for the determination of the three alkaloids in rat plasma using carbamazepine as IS. The method was successfully applied to a pharmacokinetic study involving intravenous administration of 5.0 mg/kg liensinine and iso-liensinine and neferine to rats (5.0 mg/kg) [3]. The pharmacokinetic parameters: t1/2 was 9.81 ± 2.39 h, CL was 3.82 ± 0.93 L/h kg for liensinine [5]. Lv developed an UPLC–MS/MS method was developed to determine liensinine in rat plasma using carbamazepine as the IS. The pharmacokinetic parameters were demonstrated as follows: t1/2 was 8.2 ± 3.3 h, CL was 3.0 ± 0.9 L/h kg [5]. In our study, t1/2 was 3.8 ± 0.8 h for intravenous administration for mice (1 mg/kg), and the 266.0 ± 41.3 and 4.7 ± 1.2 L/h/kg for oral and intravenous administration. The pharmacokinetic parameters in mice were different from in rats, this may also be related to different dosages, and the bioavailability was not report in the rats by UPLC-MS/MS.

CONCLUSIONS

A simple and fast UPLC-MS/MS method was developed for determination of liensinine in mouse. The calibration curve ranged from 0.5 to 400 ng/mL (r > 0.995). The method established in this study only used 20 μL blood as a biological sample. The developed method was successfully applied to the pharmacokinetics in mouse blood after oral administration 5 mg/kg) and intravenous administration of liensinine (1 mg/kg), and the bioavailability was 1.8%, it was reported for the first time.

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REFERENCE