Original Article
Pharmacokinetics in rats and tissue distribution in mouse of magnoflorine by ultra performance liquid chromatography-tandem mass spectrometry


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Abstract: Magnoflorine is one of the most widespread aporphine alkaloids. In this work, a sensitive and selective ultra performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) method for the determination of magnoflorine in rat plasma and mouse tissue have been developed and validated. After addition of nuciferine as an internal standard (IS), protein precipitation by acetonitrile-methanol (9:1, v/v) was used for samples treatment. Chromatographic separation was achieved on a UPLC BEH C18 column (2.1 mm×100 mm, 1.7 μm) with 0.1% formic acid and acetonitrile as the mobile phase with gradient elution. An electrospray ionization source was applied and operated in positive ion mode; multiple reactions monitoring (MRM) mode was used for quantification using target fragment ions \(^{m/z} 342.8\rightarrow 298.2\) for magnoflorine and \(^{m/z} 296.0\rightarrow 265.1\) for IS. Calibration plots were linear throughout the range 2-2000 ng/mL for magnoflorine in rat plasma and tissue. Mean recoveries of magnoflorine in rat plasma were better than 83.0%. RSD of intra-day and inter-day precision were both less than 9%. The accuracy of the method was between 95.5% and 107.5%. The method was successfully applied to pharmacokinetics and tissue distribution study of magnoflorine. The absolute bioavailability of magnoflorine was reported as 22.6%. The magnoflorine underwent a rapid and wide distribution to tissues; the level of magnoflorine in liver is highest, then followed by heart, spleen and lung. Based on tissue distribution data, a back-propagation artificial neural network (BP-ANN) method was developed and it could be used to predict the concentrations of magnoflorine in tissues.

Keywords: Magnoflorine, pharmacokinetics, tissue distribution, UPLC-MS/MS, rat

Introduction

Magnoflorine, one of the most widespread aporphine alkaloids, exists widely in herbs including Annonaceae, Aristolochiaceae, Berberidaceae, and Euphorbiaceae [1, 2], and showed a wide range of useful biological activities such as antioxidation [3], antidiabetic [4, 5], anti-inflammation [6], antifungal [7], antitumor and antiviral activities [8]. Considering the growing beneficial role of magnoflorine in human health, the details in vivo pharmacokinetic and disposition study of magnoflorine are necessary.

Recently, there have been several method for determination of magnoflorine [9-13]. Tian et al. investigated the pharmacokinetics of magnoflorine in rat plasma after oral and intravenous administration respectively by LC-MS/MS [11]. Xue et al. studied the pharmacokinetics and biotransformation of magnoflorine in rat urine, feces, plasma and bile [12]. Zhou et al. reported the pharmacokinetics of magnoflorine in rat plasma after intravenous administration by HPLC [13]. All of these studies revealed the preliminary data on the pharmacokinetics of magnoflorine, however, up to now, the tissue distribution of magnoflorine still has not been reported.

Therefore, in this study, an ultra-performance liquid chromatography-tandem mass spectrometry with electrospray ionization (UPLC-ESI-MS/MS) method was developed and successfully applied to the quantification of magnoflorine in...
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rat biological samples. The present study provides a full investigation of the pharmacokinetic profiles and tissue distribution of magnoflorine following a single oral and intravenous administration.

Experimental

Chemicals and reagents

Magnoflorine (purity >98%) and nuciferine (IS, purity >98%) were purchased from the Chengdu Mansite Pharmaceutical CO. LTD. (Chengdu, China). LC-grade acetonitrile and methanol were purchased from Merck Company (Darmstadt, Germany). Ultra-pure water was prepared by Millipore Milli-Q purification system (Bedford, MA, USA). Rat blank plasma samples were supplied from drug-free rats (Laboratory Animal Center of Wenzhou Medical University).

Instrumentation and conditions

A UPLC-MS/MS system with ACQUITY I-Class UPLC and a XEVO TQD triple quadrupole mass spectrometer (Waters Corp., Milford, MA, USA), equipped with an electrospray ionization interface, was used for analysis. The UPLC system was comprised of a Binary Solvent Manager and a Sample Manager with Flow-Through Needle. Masslynx 4.1 software (Waters Corp., Milford, MA, USA) was used for data acquisition and instrument control.

Magnoflorine and nuciferine (IS) were separated on an UPLC BEH C18 column (2.1 mm×100 mm, 1.7 μm) maintained at 40°C. The initial mobile phase consisted of 0.1% formic acid and acetonitrile with gradient elution at a flow rate of 0.4 mL/min and an injection volume of 2 μL. Elution was in a linear gradient, where the acetonitrile increased from 30% to 60% between 0 and 1.5 min, then increased to 95% within 0.5 min, maintained at 95% for 0.5 min, then decreased to 30% within 0.1 min, then maintained at 30% for 0.4 min. The total run time of the analytes was 3 min. After each injection, the sample manager underwent a needle wash process, including both a strong wash (methanol-water, 50/50, v/v) and a weak wash (methanol-water, 10/90, v/v).

Nitrogen was used as the desolvation gas (1000 L/h) and cone gas (50 L/h). Ion monitoring conditions were defined as capillary voltage of 2.5 kV, source temperature of 150°C, and desolvation temperature of 500°C. MRM modes of ions m/z 342.8→298.2 for magnoflorine, and m/z 296.0→265.1 for IS were utilized to conduct quantitative analysis.

Calibration standards and quality control samples

The stock solutions of magnoflorine (1.0 mg/mL) and nuciferine (IS) (1.0 mg/mL) were prepared in methanol-water (50:50, v/v). The 0.25 μg/mL working standard solution of the IS was prepared from the IS stock solution by dilution with methanol; working solutions for calibration and controls were prepared from stock solutions in the same manner. All of the solutions were stored at 4°C before use.

Magnoflorine calibration standards were prepared by spiking blank plasma or tissue with appropriate amounts of the working solutions. Calibration plots were offset to range between 2-2000 ng/mL for magnoflorine in rat plasma (2, 5, 10, 20, 50, 100, 200, 500, 1000 and 2000 ng/mL), tissue (5, 10, 20, 50, 100, 200, 500, 1000 and 2000 ng/mL), each by adding 10 μL of the appropriate working solution to 100 μL of blank plasma or tissue, followed by short vortex mixing. Quality-control (QC) samples were prepared in the same manner as the calibration standards, in three different plasma concentrations (4, 900 and 1800 ng/mL). The analytical standards and QC samples were stored at -20°C. The calibration standards and QC samples protein was precipitated by acetonitrile-methanol (9:1, v/v) before UPLC-MS/MS analysis.

Sample preparation

Before analysis, the plasma sample was thawed to room temperature. An aliquot of 10 μL of the IS working solution (0.25 μg/mL) was added to 50 μL of the collected plasma sample in a 1.5 mL centrifuge tube, followed by the addition of 150 μL of acetonitrile-methanol (9:1, v/v). The tubes were vortex mixed for 1.0 min. After centrifugation at 14900 g for 10 min, the supernatant (2 μL) was injected into the UPLC-MS/MS system for analysis.

When magnoflorine was extracted from the tissues, 50 mg of heart, liver, spleen, lung, kidney and brain were weighed accurately and placed in 2.0 mL polystyrene tubes, then 200 μL of acetonitrile-methanol (9:1, v/v) and 10 μL of the IS working solution (0.5 μg/mL) was added.
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The mixture was stored at -80°C for 20 min then grinded for 2 min by a SCIENTZ-48 Tissue Grinder, the grinding parameter was 64 Hz and 1800 r/s. The tubes were vortex mixed for 1.0 min. After centrifugation at 14900 g for 10 min, the supernatant (2 µL) was injected into the UPLC-MS/MS system for analysis.

Method validation

Rigorous tests for selectivity, linearity, accuracy, precision, recovery, and stability, according to the guidelines set by the United States Food and Drug Administration (FDA) [14] and European Medicines Agency (EMA) [15], were conducted in order to thoroughly validate the proposed bioanalytical method. Validation runs were conducted on three consecutive days. Each validation run consisted of one set of calibration standards and six replicates of QC plasma samples.

The selectivity of the method was evaluated by analyzing six lots of different sources of blank rat plasma, blank plasma-spiked magnoflorine and IS, and a rat plasma sample.
Calibration curves were constructed by analyzing spiked calibration samples on three separate days. Peak area ratios of magnoflorine-to-IS were plotted against magnoflorine concentrations. Resultant standard curves were well fitted to the equations by linear regression, with a weighting factor of the reciprocal of the concentration (1/x) in the concentration range of 2-2000 ng/mL. The lower Limit of quantitation (LLOQ) was defined as the lowest concentration on the calibration curves.

To evaluate the matrix effect, blank rat plasma was extracted and spiked with the analyte at 4, 900, and 1800 ng/mL concentrations (n=6). The corresponding peak areas were then compared to those of neat standard solutions at equivalent concentrations, this peak area ratio is defined as the matrix effect. The matrix effect of the IS was evaluated at a concentration of 50 ng/mL in a similar manner.

Accuracy and precision were assessed by the determination of QC samples at three concentration levels in six replicates (4, 900, and 1800 ng/mL) over three days of validation testing. The precision is expressed as RSD.

The recovery of magnoflorine was evaluated by comparing the peak area of extracted QC samples with those of reference QC solutions reconstituted in blank plasma extracts (n=6). The recovery of the IS was determined in the same way.

Carry-over was assessed following injection of a blank plasma sample immediately after 3 repeats of the upper limit of quantification (ULOQ), after which the response was checked for accuracy [16].

Stability of magnoflorine in rat plasma were evaluated by analyzing three replicates of plas-
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**Table 1.** Precision, accuracy, and recovery of magnoflorine for QC samples in rat plasma (n=6)

<table>
<thead>
<tr>
<th>Concentration (ng/mL)</th>
<th>RSD (%)</th>
<th>Accuracy (%)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Intra-day</td>
<td>Inter-day</td>
<td>Intra-day</td>
</tr>
<tr>
<td>4</td>
<td>2.6</td>
<td>8.8</td>
<td>101.7</td>
</tr>
<tr>
<td>900</td>
<td>6.2</td>
<td>4.7</td>
<td>96.1</td>
</tr>
<tr>
<td>1800</td>
<td>2.7</td>
<td>7.4</td>
<td>95.5</td>
</tr>
</tbody>
</table>

**Table 2.** Summary of stability of magnoflorine and IS under various storage conditions (n=3)

<table>
<thead>
<tr>
<th>Condition</th>
<th>Concentration (ng/mL)</th>
<th>RSD (%)</th>
<th>Accuracy (%)</th>
<th>Added</th>
<th>Measured</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ambient, 2 h</td>
<td>4</td>
<td>1.2</td>
<td>102.5</td>
<td>4</td>
<td>4.1</td>
</tr>
<tr>
<td></td>
<td>1800</td>
<td>2.4</td>
<td>99.3</td>
<td>4</td>
<td>1876.7</td>
</tr>
<tr>
<td></td>
<td>(IS) 50</td>
<td>0.5</td>
<td>98.4</td>
<td>4</td>
<td>49.2</td>
</tr>
<tr>
<td>-20°C, 20 days</td>
<td>4</td>
<td>6.7</td>
<td>95.0</td>
<td>4</td>
<td>1979.5</td>
</tr>
<tr>
<td></td>
<td>1800</td>
<td>7.9</td>
<td>110.0</td>
<td>4</td>
<td>1930</td>
</tr>
<tr>
<td></td>
<td>(IS) 50</td>
<td>4.5</td>
<td>106.8</td>
<td>4</td>
<td>53.4</td>
</tr>
<tr>
<td>3 freeze thaw</td>
<td>4</td>
<td>8.7</td>
<td>96.8</td>
<td>4</td>
<td>48.4</td>
</tr>
<tr>
<td></td>
<td>1800</td>
<td>5.6</td>
<td>105.4</td>
<td>4</td>
<td>1896.7</td>
</tr>
<tr>
<td></td>
<td>(IS) 50</td>
<td>7.6</td>
<td>96.8</td>
<td>4</td>
<td>48.4</td>
</tr>
<tr>
<td>Autosampler ambient 24 h</td>
<td>4</td>
<td>2.1</td>
<td>98.3</td>
<td>4</td>
<td>3.93</td>
</tr>
<tr>
<td></td>
<td>1800</td>
<td>3.2</td>
<td>102.5</td>
<td>4</td>
<td>1845.6</td>
</tr>
<tr>
<td></td>
<td>(IS) 50</td>
<td>2.7</td>
<td>101.2</td>
<td>4</td>
<td>50.6</td>
</tr>
</tbody>
</table>

**Figure 3.** Mean plasma concentration time profile after oral (15 mg/kg) and intravenous (5 mg/kg) administration of magnoflorine in rats.

Ma samples at concentrations of 4 or 1800 ng/mL which were all exposed to different conditions. These results were compared with the freshly-prepared plasma samples. Short-term stability was determined after the exposure of the spiked samples to room temperature for 2 h, and the ready-to-inject samples (after protein precipitation) in the HPLC autosampler at room temperature for 24 h. Freeze/thaw stabil-

ity was evaluated after three complete freeze/thaw cycles (-20 to 25°C) on consecutive days. Long-term stability was assessed after storage of the standard spiked plasma samples at -20°C for 20 days. The stability of the IS (50 ng/mL) was evaluated similarly [17, 18].

**Pharmacokinetic study**

All twelve Male Sprague-Dawley rats (200-220 g) were obtained from the Laboratory Animal Center of Wenzhou Medical University to study the pharmacokinetics of magnoflorine. All experimental procedures and protocols were reviewed and approved by the Animal Care and Use Committee of Wenzhou Medical University. Diet was prohibited for 12 h before the experiment but water was freely available. Blood samples (0.2 mL) were collected from the tail vein into heparinized 1.5 mL polythene tubes at 0.0833, 0.25, 0.5, 1, 1.5, 2, 4, 6, 8, 12, and 24 h after oral (15 mg/kg) and intravenous (5 mg/kg) administration of magnoflorine. The samples were immediately centrifuged at 3000×g for 10 min. The plasma as-obtained (50 µL) was stored at -20°C until UPLC-MS/MS analysis. Plasma magnoflorine concentration versus time data for each rat was analyzed by DAS (Drug and Statistics) software (Version 2.0, Wenzhou Medical University).

**Tissue distribution model study**

Thirty mouse were randomly divided into six different groups (n=5) and received magnoflorine 40 mg/kg by oral administration. These thirty mice were euthanized by decapitation at 0.5, 1, 2, 4, 6 and 8 h after dosing, respectively. Tissues including heart, liver, spleen, lung, kidney and brain were obtained, then stored at -20°C until UPLC-MS/MS analysis.

The data of tissue (heart, spleen, liver, lung, kidney, brain) and plasma concentration were employed into the back-propagation artificial...
neural network (BP-ANN) at Matlab R2011a. The input layer consist of six factors and the output is one factor, for example, when blood, brain, heart, spleen, lung and kidney were selected as the input data, then the output was liver. The node numbers of hidden layer were calculated by the formula of $m = \sqrt{n + l + a}$, where $m$ represents the node number in the hidden layer, $n$ represents the node number in the input layer, $l$ represents the node number in the output layer, $a$ represents a constant, for more details refer to [19].

## Results and discussion

### Method development

Mass detector parameters were assessed by infusion of a standard solution directly into the ESI source. In order to optimize MS-MS conditions, the daughter ion spectrum of the [M+H]$^+$ ion was recorded by ramping the capillary voltage and the collision energy. The most prevalent fragment was detected at $m/z$ 343.2 with a capillary voltage of 2500 V, and collision energy of 20 eV. Accordingly, the $m/z$ 343.2 → 298.2 transition was selected for further UPLC-MS/MS analysis in MRM mode (Figure 1).

The mobile phase played a critical role in achieving appropriate ionization and good chromatographic behavior [20-24]. Acetonitrile and water (containing 0.1% formic acid) were chosen as the mobile phase because the combination provides proper retention time and peak shape. Compared to methanol, acetonitrile was chosen as organic phase, as it provides sharper peak shape and lower pump pressure. The total run time for each injection was 3 min.

Efficient removal of proteins and other potential interference in the bio-samples prior to LC-MS analysis was a crucial step in the development of this method [25-33]. The liquid-liquid extraction was tried in our work at first. We tried ethyl acetate, ethyl ether and chloroform as extraction organic solvents, the recovery of them (between 20.2% and 60.3%) were poor. Then the simple protein precipitation by acetonitrile-methanol (9:1, v/v) was employed in our study, with the acceptable recovery (around 83.0% and 89.9%) and the acceptable matrix effect after protein precipitation (around 101.2% and 104.1%).

### Selectivity and matrix effect

Figure 2 shows typical chromatograms of a blank plasma sample, a blank plasma sample spiked with magnoflorine and IS, and a plasma sample. There were no interfering endogenous

## Table 3. Primary pharmacokinetic parameters after oral or intravenous administration of magnoflorine in rats (n=6)

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Oral administration</th>
<th>Intravenous administration</th>
</tr>
</thead>
<tbody>
<tr>
<td>$AUC_{0-\infty}$ (ng/mL×h)</td>
<td>1645.6±551.4</td>
<td>2430.0±636.8</td>
</tr>
<tr>
<td>$AUC_{0-t}$ (ng/mL×h)</td>
<td>2384.9±1465.6</td>
<td>2450.4±645.8</td>
</tr>
<tr>
<td>$t_{1/2}$ (h)</td>
<td>13.2±8.2</td>
<td>1.5±0.7</td>
</tr>
<tr>
<td>$CL$ (L/h/kg)</td>
<td>8.0±3.4</td>
<td>2.1±0.5</td>
</tr>
<tr>
<td>$V$ (L/kg)</td>
<td>122.9±23.6</td>
<td>4.5±2.3</td>
</tr>
<tr>
<td>$C_{max}$ (ng/mL)</td>
<td>181.5±14.5</td>
<td>2453.4±592.3</td>
</tr>
</tbody>
</table>

Absolute Bioavailability, $F$, 22.6

![Figure 4. Mean concentration of magnoflorine in various tissues at 30 min, 1, 2, 4 and 6 h after an oral administration of 40 mg/kg magnoflorine in mouse (n=5, mean ± SD).](image-url)
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substances observed at the retention time of the magnoflorine and IS.

The matrix effect for magnoflorine at concentrations of 4, 900, and 1800 ng/mL were measured to be 104.1%, 101.2% and 103.0% (n=6). The matrix effect for IS (50 ng/mL) was 95.3% (n=6). As a result, matrix effect from plasma was considered acceptable in this method.

Linear regressions of the peak area ratios versus concentrations were fitted over the concentration range 2-2000 ng/mL for magnoflorine in rat tissue. The equation utilized to express the calibration curve was:

\[ Y = 0.000181882 \times X - 0.00031354, r = 0.9976 \text{ for heart}; \]
\[ Y = 0.000165258 \times X - 0.000599872, r = 0.9981 \text{ for liver}; \]
\[ Y = 0.0000959296 \times X - 0.000488783, r = 0.9986 \text{ for spleen}; \]
\[ Y = 0.000088954 \times X - 0.00195514, r = 0.9976 \text{ for lung}; \]
\[ Y = 0.000097305 \times X - 0.00140368, r = 0.9965 \text{ for kidney}; \]
\[ Y = 0.0000128663 \times X - 0.00273223, r = 0.9966 \text{ for brain}. \]

The LLOQ for the determination of magnoflorine in tissue was 2 ng/mL. The precision at LLOQ were between 9.3% and 16.7%, and accuracy was between 87.4% and 113.5%, respectively.

### Precision, accuracy and recovery

The precision of the method was determined by calculating RSD for QCs at three concentration levels over three days of validation tests. Intra-day precision was 7% or less, and inter-day precision was 9% or less at each QC level. The accuracy of the method ranged from 95.5% and 107.5% at each QC level. Mean recoveries of magnoflorine were higher than 83.0%. The recovery of the IS (50 ng/mL) was 90.2%. Assay performance data was presented below in Table 1.

### Carry-over

None of the analytes showed any significant peak (≥20% of the LLOQ and 5% of the IS) in
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blank samples injected after the ULOQ samples. Adding 0.4 min to the end of the gradient elution effectively washed the system between samples, thereby eliminating carry-over [16].

Stability

Results from the auto-sampler showed that the magnoflorine was stable under room temperature, freeze-thaw, and long-term (20 days) conditions, confirmed because the bias in concentrations were within 95.0% and 110.0% of their nominal values (Table 2). To this effect, the stability results of the established method were acceptable for pharmacokinetics and tissue distribution.

Pharmacokinetic study

The method was applied to a pharmacokinetics study in rats. The mean plasma concentration-time curve after oral (15 mg/kg) and intravenous (5 mg/kg) administration of magnoflorine was shown in Figure 3. Primary pharmacokinetic parameters, based on non-compartment model analysis, were summarized in Table 3.

The absolute bioavailability of magnoflorine was 22.6%, which was calculated according to formula: F=AUCpo·Div/AUCiv·Dpo ×100%.

Tissue distribution

The mean concentration-time curves of magnoflorine in plasma, heart, liver, spleen, lung, kidney and brain after oral administrated of 40 mg/kg were shown in Figure 4. The results demonstrated that the magnoflorine underwent a rapid and wide distribution to tissues. The concentration of magnoflorine in liver is highest, then followed by heart, spleen and lung. The concentration of magnoflorine in brain is lowest, which indicated that it is hard to across through blood brain barrier.

Artificial neural network algorithm is a computational model inspired by a brain's central nervous system which is capable of machine learning as well as pattern recognition. BP-ANN has been a widely used artificial modeling methods in medical area [34]. In this study, the BP-ANN model was trained within 100 epochs, the performance parameters were listed in Table 4. The results showed the developed BP-ANN model of magnoflorine achieved an excellent prediction accuracy in six tissues (brain, heart, spleen, liver, lung and kidney) and blood (Figure 5). According to the BP-ANN model, we could predict the different tissue concentrations; it will be helpful for comprehension of their characteristics in vivo distribution.

Conclusion

The UPLC-MS/MS method in this study was validated for selectivity, linearity, accuracy, precision, recovery and stability. The developed and validated UPLC-MS/MS method with an LLOQ of 2 ng/mL and a total run time of 3 min. The method was successfully applied to pharmacokinetics and tissue distribution study of magnoflorine. The absolute bioavailability of magnoflorine was identified at 22.6%. The magnoflorine underwent a rapid and wide distribution to tissues; the concentration of magnoflorine in liver is highest, then followed by heart, spleen and lung. According to these data, a BP-ANN model was developed to predict the concentrations of magnoflorine in tissues.

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Disclosure of conflict of interest

None.

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