Short communication

Simultaneous determination of mangiferin and neomangiferin in rat plasma by UPLC–MS/MS and its application for pharmacokinetic study

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**A B S T R A C T**

In this study, a sensitive and rapid ultra performance liquid chromatography tandem mass spectrometry (UPLC–MS/MS) method was developed to determine mangiferin and neomangiferin in rat plasma simultaneously. Chromatographic separation was carried out on an Acquity UPLC BEH C18 column and mass spectrometric analysis was performed using a Xevo TQD triple quadrupole mass spectrometer coupled with an electrospray ionization (ESI) source. The MRM transitions of \(m/z\) 423.2 → 303.1 and \(m/z\) 585.0 → 273.1 were used to quantify for mangiferin and neomangiferin, respectively. The linearity of this method was found to be within the concentration range of 5–2000 ng/mL for mangiferin, and 2–1000 ng/mL for neomangiferin in rat plasma, respectively. Only 3.0 min was needed for an analytical run. This assay was used to support a preclinical study to investigate the pharmacokinetics of mangiferin and neomangiferin in rats.

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1. Introduction

*Anemarrhena asphodeloides* Bunge. (Asparagaceae) yields *Rhizoma anemarrhena*, which has been commonly used in Asian countries for hundreds of years as Traditional Chinese Medicine (TCM) and officially listed in the Chinese Pharmacopoeia. Modern research found that *Rhizoma anemarrhena* has the bioactive effects of anti-pathogenic microorganism, hypoglycemic effects, anti-inflammatory, antipyretic effects and anti-platelet aggregation [1,2]. Mangiferin and neomangiferin are the major bioactive constituents of *Rhizoma anemarrhena*. The chemical structures of mangiferin and neomangiferin are shown in Fig. 1. It has been shown that mangiferin and neomangiferin have many beneficial biological activities, including anti-inflammatory, anti-oxidant, and anti-diabetic effects [3–5]. Moreover, recent studies have indicated that mangiferin may lower triglycerides and total cholesterol levels [6–8], offer neuroprotection [9,10], promote urate excretion [11] and inhibit the development of tumor [12–14]. In addition, neomangiferin has beneficial effect on high fat diet-induced non-alcoholic fatty liver disease [15].

Up to now, there have been several analytical methods for the simultaneous determination of mangiferin and neomangiferin in biological samples, mainly including LC–MS/MS [16–18]. However, to our knowledge, their pharmacokinetics in biological fluid are not elucidated clearly. Even above LC–MS/MS publications had reported the methods for the simultaneous determination of mangiferin and neomangiferin with other substances in rats after consumption of crude herb extracts, their pharmacokinetic properties after given with pure substances still remain unknown so far [16–18]. Moreover, these methods generally had poor assay specificity and long run times. Since a very large number of samples are generated in pharmacokinetic assays, most of the above mentioned methods do not meet the criteria for this kind of study.

As a result of recent advances in analysis techniques, ultra performance liquid chromatography coupled with tandem mass spectrometry (UPLC–MS/MS) has emerged as an efficient analytical tool with improved sensitivity, selectivity, and specificity. In the present work, a highly rugged, selective and rapid UPLC–MS/MS method has been developed and fully validated as per the USFDA guidelines for the simultaneous measurement of mangiferin and neomangiferin in rat plasma using diazepam as internal standard.

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The method was free from endogenous matrix interference and was successfully applied to a pharmacokinetic study in rats.

2. Materials and methods

2.1. Chemicals and reagents

Mangiferin (purity 98.0%) and neomangiferin (purity 98.0%) were purchased from Chengdu Mansite Pharmaceutical Co., LTD. (Chengdu, China). Diazepam (internal standard, IS, purity 98.0%) was obtained from Sigma (St. Louis, MO, USA). Formic acid was analytical grade and purchased from the Beijing Chemical Reagents Company (Beijing, China). Acetonitrile and methanol were of HPLC grade and were purchased from Merck Company (Darmstadt, Germany). HPLC grade water was obtained using a Milli Q system (Millipore, Bedford, MA, USA).

2.2. UPLC–MS/MS conditions

Liquid chromatography was performed on an Acquity ultra performance liquid chromatography (UPLC) unit (Waters Corp., Milford, MA, USA) with an Acquity BEH C18 column (2.1 mm × 50 mm, 1.7 μm particle size) and inline 0.2 μm stainless steel frit filter. A gradient program was employed with the mobile phase, combining solvent A (0.1% formic acid in water) and solvent B (acetonitrile) as follows: 10–10% B (0–0.5 min), 10–95% B (0.5–1.0 min), 95–95% B (1.0–2.0 min), 95–10% B (2.0–2.1 min), 10–10% B (2.1–3.0 min). The flow rate was 0.40 mL/min and the injection volume was 6 μL. The column and sample temperature were maintained at 40 °C and 4 °C, respectively.

A Xevo TQD triple quadruple mass spectrometer equipped with an electrospray ionization (ESI) source (Waters Corp.) was used for mass spectrometric detection. The quantitative analysis of mangiferin and neomangiferin in rat plasma was performed using multiple reaction monitoring (MRM) method. The MRM transitions were m/z 423.2 → 423.1, m/z 585.0 → 273.1, and m/z 285.1 → 193.2 for mangiferin, neomangiferin and IS, respectively. The Masslynx 4.1 software (Waters Corp.) was used for data acquisition and instrument control.

2.3. Standard solutions, calibration standards and quality control (QC) sample

The stock solutions of mangiferin and neomangiferin used to make the calibration standards and quality control (QC) samples were prepared by dissolving 10 mg each compound in 10 mL methanol to obtain a concentration of 1.00 mg/mL of each compound. The stock solutions were further diluted with methanol to obtain working solutions at several concentration levels. Calibration standards and QC samples in plasma were prepared by diluting the corresponding working solutions with blank rat plasma. Final concentrations of the calibration standards were 5, 10, 20, 50, 100, 200, 500, 1000 and 2000 ng/mL for mangiferin, and 2, 5, 10, 20, 50, 100, 200, 500 and 1000 ng/mL for neomangiferin in rat plasma, respectively. The concentrations of QC samples in plasma were 10, 60, and 1600 ng/mL for mangiferin, and 4, 80, and 800 ng/mL for neomangiferin, respectively. IS stock solution was made at an initial concentration of 1.00 mg/mL. The IS working solution (300 ng/mL) was made from the stock solution using acetonitrile for dilution. All stock solutions, working solutions, calibration standards and QCs were immediately stored at −80 °C.

2.4. Sample preparation

Before analysis, the plasma sample was thawed to room temperature. In a 1.5 mL centrifuge tube, an aliquot of 200 μL of the IS working solution (300 ng/mL in acetonitrile) was added to 100 μL of collected plasma sample. The tubes were vortex mixed for 1.0 min and made 100 μL of mixed solution. It was immediately centrifuged at 10,000 g for 10 min. The supernatant was transferred to a 0.5 mL Eppendorf tube and evaporated to dryness in a vacuum centrifuge. The residue was reconstituted with 100 μL of a mobile phase (acetonitrile:water = 70:30). After vortexing, it was centrifuged at 10,000 g for 5 min. The supernatant was transferred to a 0.5 mL Eppendorf tube and evaporated to dryness in a vacuum centrifuge. The residue was reconstituted with 100 μL of a mobile phase (acetonitrile:water = 70:30). After vortexing, it was centrifuged at 10,000 g for 5 min.

Fig. 1. The chemical structures of the analytes in the present study: (A) mangiferin; (B) neomangiferin.

Fig. 2. Representative chromatograms of mangiferin, neomangiferin and IS in rat plasma samples: (A) a blank plasma sample; (B) a blank plasma sample spiked with mangiferin, neomangiferin and IS; (C) a plasma sample from a rat 0.5 h after an intravenous co-administration of mangiferin and neomangiferin.
Table 1
Recovery and matrix effect of mangiferin, neomangiferin and internal standards (n = 6).

<table>
<thead>
<tr>
<th>Analytes</th>
<th>Concentration added (ng/mL)</th>
<th>Recovery (%)</th>
<th>Matrix effect (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mean ± SD</td>
<td>RSD (%)</td>
</tr>
<tr>
<td>Mangiferin</td>
<td>10</td>
<td>82.5 ± 3.3</td>
<td>4.0</td>
</tr>
<tr>
<td></td>
<td>160</td>
<td>89.9 ± 3.8</td>
<td>4.2</td>
</tr>
<tr>
<td>Neomangiferin</td>
<td>4</td>
<td>85.1 ± 3.0</td>
<td>3.5</td>
</tr>
<tr>
<td></td>
<td>80</td>
<td>80.8 ± 3.5</td>
<td>4.4</td>
</tr>
<tr>
<td></td>
<td>800</td>
<td>85.2 ± 3.4</td>
<td>4.0</td>
</tr>
<tr>
<td>IS</td>
<td>300</td>
<td>83.0 ± 3.2</td>
<td>3.8</td>
</tr>
</tbody>
</table>

and spun in a centrifuge at 15,000g for 10 min. The supernatant (6 μL) was injected into the UPLC–MS/MS system for analysis.

2.5. Method validation

Before using this method to determine mangiferin and neomangiferin in plasma samples, the method was fully validated for specificity, linearity, sensitivity, precision, accuracy, recovery, matrix effect and stability according to the United States Food and Drug Administration (FDA) guidelines for the validation of a bianalytical method [19].

A selectivity study is designed to investigate whether endogenous constituents and other substances existing in samples will interfere with the detection of analytes and IS. Selectivity was studied by comparing the chromatograms of six different batches of blank rat plasma with the corresponding spiked plasma.

Calibration curves were prepared according to Section 2.4. The linearity of each calibration curve was determined by plotting the peak area ratio (y) of analytes to IS versus the nominal concentration (x) of analytes with weighted (1/x²) least square linear regression. The sensitivity of the method was determined by quantifying the lower limit of quantification (LLOQ). The LLOQ was defined as the lowest acceptable point in the calibration curve which was determined at an acceptable precision and accuracy.

The extraction recovery was evaluated by comparing peak areas obtained from extracted spiked samples with those of the post-extracted spiked samples at corresponding concentrations. The extraction efficiency of the analytes was determined by analyzing six replicates of QC samples at three concentration levels.

The matrix effect was evaluated by comparing the peak areas of the post-extracted spiked QC samples with those of corresponding standard solutions. The matrix effect of the analytes was determined by analyzing six plasma samples at three concentration levels. The extraction recovery and matrix effect of IS were determined using the same procedure at a single concentration of 300 ng/mL.

For the evaluation of intra-day precision and accuracy, six replicates QC samples were analyzed at three concentration levels on the same day. For the evaluation of inter-day precision and accuracy, three replicates of QC samples were analyzed at three concentration levels on six consecutive days. Precision was expressed as% relative standard deviation (RSD) and accuracy was expressed as% relative error (RE) between the measured and nominal value. The precision for QC samples was within 15%, and accuracy between 15 and 15%.

The stability experiments were performed to evaluate the stability of the analytes in rat plasma under the following conditions: short-term stability at room temperature for 4 h; long-term stability at −80 °C for 35 days; three freeze (−80 °C) − thaw (room temperature) cycles on consecutive days. The extracted QC samples kept in the autosampler at 4 °C for 24 h were analyzed to evaluate post-preparation stability. All stability testing in plasma was determined by analyzing five replicates of QC samples at three concentration levels. The determined concentrations were compared with the nominal values.

2.6. Pharmacokinetic study

Male Sprague-Dawley rats (180–220 g) were obtained from Laboratory Animal Center of Henan University of Science and Technology (Luoyang, China) used to study the pharmacokinetics of mangiferin and neomangiferin. All experimental procedures and protocols were were reviewed and approved by the Animal Care and Use Committee of Henan University of Science and Technology and were in accordance with the Guide for the Care and Use of Laboratory Animals. Diet was prohibited for 12 h before the experiment but water was freely available. Blood samples (0.3 mL) were collected from the tail vein into heparinized 1.5 mL polythene tubes at 0.083, 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 6, and 8 h after intravenous administration of mangiferin (3.0 mg/kg) and neomangiferin (3.0 mg/kg). The samples were immediately centrifuged.

Table 2
Precision and accuracy of method for the determination of mangiferin and neomangiferin in rat plasma (n = 6).

<table>
<thead>
<tr>
<th>Analytes</th>
<th>Concentration added (ng/mL)</th>
<th>Intra-day</th>
<th>Inter-day</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>RSD (%)</td>
<td>RE (%)</td>
</tr>
<tr>
<td>Mangiferin</td>
<td>10</td>
<td>8.2</td>
<td>9.6</td>
</tr>
<tr>
<td></td>
<td>160</td>
<td>5.6</td>
<td>6.2</td>
</tr>
<tr>
<td>Neomangiferin</td>
<td>4</td>
<td>3.1</td>
<td>−2.9</td>
</tr>
<tr>
<td></td>
<td>80</td>
<td>9.5</td>
<td>−10.8</td>
</tr>
<tr>
<td></td>
<td>800</td>
<td>6.4</td>
<td>−7.5</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Mangiferin</th>
<th>Neomangiferin</th>
</tr>
</thead>
<tbody>
<tr>
<td>t1/2 (h)</td>
<td>1.08 ± 0.09</td>
<td>0.89 ± 0.09</td>
</tr>
<tr>
<td>Cmax (ng/mL)</td>
<td>1152.34 ± 116.21</td>
<td>478.37 ± 69.22</td>
</tr>
<tr>
<td>AUCL∞ (ng/mL)</td>
<td>2032.53 ± 261.58</td>
<td>1007.25 ± 196.33</td>
</tr>
<tr>
<td>Vd (L/kg)</td>
<td>2.35 ± 0.45</td>
<td>3.96 ± 0.99</td>
</tr>
<tr>
<td>CL (L/h)</td>
<td>1.50 ± 0.18</td>
<td>3.09 ± 0.70</td>
</tr>
<tr>
<td>MRTE (h)</td>
<td>1.14 ± 0.18</td>
<td>1.43 ± 0.10</td>
</tr>
</tbody>
</table>
at 4000g for 8 min. The plasma obtained (100 μL) was stored at −80°C until analysis. Plasma mangiferin and neomangiferin concentrations versus time data for each rat was analyzed by DAS (Drug and statistics) software (Version 2.0, Shanghai University of Traditional Chinese Medicine, China).

3. Results and discussion

3.1. Method development and optimization

The chromatographic conditions were optimized to improve peak shape, increase signal intensity of the analytes and shorten run time. The mobile phase systems of acetonitrile-water and methanol-water at different proportions were tested. The response of the analytes was obviously greater in acetonitrile-water mobile phase than that in methanol-water. The ionization in ESI mode occurs in the solution state, and the additives to the mobile phase may have a significant influence on the ionization of the analytes. With addition of formic acid (0.1%) to the mobile phase, the peak intensity and peak symmetry of the analytes and IS were all dramatically improved. Best chromatographic separations were achieved at 40°C with a gradient elution program consisting of acetonitrile-0.1% formic acid water at a flow rate of 0.40 mL/min with an acceptable run time of 3.0 min.

To obtain better extraction efficacy and less endogenous interference, various sample extraction approaches including protein precipitation and liquid–liquid extraction with different solvents were investigated. Above all, several solvent combinations were tested for liquid–liquid extraction of the analytes and IS. However, liquid–liquid extraction was not a viable option because it is time consuming and complicated. Subsequently, we used the protein precipitation reagents including acetonitrile and methanol, which provided satisfactory extraction efficiency. In the end, acetonitrile was chosen as the extraction solvent because of its higher extraction efficiency than methanol.

3.2. Specificity

Fig. 2 shows a typical chromatogram for the blank plasma (Fig. 2A), blank plasma spiked with analytes and IS (Fig. 2B), and rat plasma obtained 0.5 h after intravenous administration of mangiferin and neomangiferin (Fig. 2C). As shown in Fig. 2, there were no obvious endogenous interferences under the described chromatographic conditions.

3.3. Linearity and sensitivity

The calibration curves were created by plotting the peak area ratios of the various analytes to internal standard versus nominal concentration of the analytes standards. Both calibration curves were regressed using a linear equation with a weighting factor of 1/x. The coefficient of correlation of all the calibration curves was more than 0.99. The typical regression equation of these curves was calculated as follows: mangiferin, y = (0.9535x + 1.9534, r^2 = 0.9925); neomangiferin, y = (1.6745 x + 1.0451, r^2 = 0.9939). As shown, all the standard calibration curves showed good linearity within the range using least squares regression analysis. The LLOQ values were 5.0 ng/mL for mangiferin and 2.0 ng/mL for neomangiferin, respectively.

3.4. Recovery and matrix effect

The extraction recoveries and matrix effect of mangiferin and neomangiferin ranged from 80.8 to 89.9% and 93.0 to 107.1%, respectively. The extraction recovery and matrix effect of IS were 83.0% and 103.2%, respectively. The detailed results are presented in Table 1. The matrix effect on the ionization of the analytes and IS was not obvious under these conditions. These data indicated that the sample preparation method was satisfactory and resulted in little appreciable matrix effect for the analytes and IS.

3.5. Precision and accuracy

The intra- and inter-day precisions and accuracies of low, medium and high QC levels of the analytes are summarized in Table 2. The assay values for both intra- and inter-day were found to be within the accepted variable limits. The intra- and inter-day precisions of the analytes were within the range of 3.1–9.5% and 3.0–9.6%, respectively. The inter- and intra-day accuracies of the analytes were within the range of −10.8 to 9.6% and −8.8 to 10.3%, respectively. The results showed that the method was accurate and precise for the determination of the two analytes in rat plasma.

3.6. Stability

The stability of the two analytes in plasma was investigated by analyzing five replications of QC samples at three concentration levels after short-term storage (room temperature, 4 h), at 4°C for 24 h after preparation, three freeze-thaw cycles, and long-term storage (−80°C, 35 days). The results were found to be within the assay variability limits (±15%).
3.7. Application of the method in a pharmacokinetic study

The validated UPLC–MS/MS method was applied successfully in a single dose pharmacokinetic study of mangiferin (3.0 mg/kg) and neomangiferin (3.0 mg/kg) in rats. All the samples were assayed by the validated method. Representative chromatograms for mangiferin and neomangiferin in rat samples are shown in Fig. 2C. The mean plasma concentration time profiles of mangiferin and neomangiferin in rats are presented in Fig. 3. In addition, the main pharmacokinetic parameters are listed in Table 3.

4. Conclusions

An UPLC–MS/MS method for the simultaneous determination of mangiferin and neomangiferin in rat plasma was developed and validated. The method affords the sensitivity, accuracy and precision needed for quantitative measurements of mangiferin and neomangiferin in rat plasma. It was also successfully applied in a preclinical pharmacokinetic study in rats.

References