Simultaneous Determination of Two Epimeric Furofuran Lignans (Sesamin and Asarinin) of *Asarum heterotropoides* Extract in Rat Plasma by LC/MS/MS: Application to Pharmacokinetic Study

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A rapid, sensitive and selective liquid chromatography-tandem mass spectrometry was developed to determine two epimeric furofuran lignans (sesamin and asarinin) simultaneously from *Asarum heterotropoides* extract in rat plasma. Simple protein precipitation with acetonitrile was performed to extract analytes by using alantolactone as an internal standard. Chromatographic separation was achieved using a DIKMA Diamonsil C18 analytical column (4.6 mm × 150 mm, i.d., 5 μm) by isocratically eluting with a mobile phase consisting of methanol/5 mM ammonium acetate/formic acid (75:25:0.1, v/v/v) at a flow rate of 0.8 mL/min. Tandem mass spectrometric detection with an electrospray ionization interface was performed by multiple reaction monitoring in positive ionization mode. This method was validated according to specificity, sensitivity, linearity, intra- and inter-day precision (<10.7%) and accuracy (<2.3%) and recovery and stability in a concentration range of 25.0–15 000 ng/mL for sesamin and 5.00–3000 ng/mL for asarinin. This method has been successfully applied in a pharmacokinetic study of *A. heterotropoides* extract containing sesamin and asarinin after this extract was orally administered in rats.

Introduction

*Asarum heterotropoides* (Xixin in Chinese) belongs to Aristolochiaceae and has been used as a traditional medicine in China (1). *A. heterotropoides* is listed in Chinese Pharmacopoeia and commonly used to treat stomatitis, toothache, gingivitis and rheumatic arthralgia (2). Modern pharmacological studies have shown that *Asarum* species exhibit anti-inflammatory, antitussive, anti-allergic, anti-hyperlipidemic and anti-myocardial ischemia properties by enhancing myocardial contractility, anti-arrhythmic activities and others (3, 4).

Phytochemical studies have shown that various types of essential oils, amides, lignans, flavonoids, terpenoids and alkaloids are isolated from *Asarum* species (5, 6). Furofuran lignans are also present in this herb (7). Among these components, sesamin and asarinin (Figure 1) are the main active components present in *A. heterotropoides* root (8). Asarinin is listed in Chinese Pharmacopoeia and used as a quality control (QC) index of crude herbs by the State Food and Drug Administration of China (2). Modern pharmacological tests have revealed that these compounds exhibit several biological activities such as decreasing cholesterol levels (9), exhibiting antihypertensive and antiangiogenic properties (10, 11) and providing immunosuppressive and hepatoprotective activities (12, 13). Asarinin can also inhibit myocardial injury in metabolic syndrome of rats (14) and decrease the possibility of acute heart transplant rejection (15). Therefore, the pharmacokinetic study of sesamin and asarinin may help evaluate the reasonable use of *A. heterotropoides* and its related preparations.

Previous publications reported several methods to analyze sesamin and asarinin in plant materials and Chinese patent medicine by liquid chromatography (LC) with ultraviolet (8, 16, 17), LC with photodiode (18), gas chromatography (GC) (19), GC with mass spectrometry (19) and LC coupled with mass spectrometry (20, 21). In biomedical analysis, an analytical method is used to determine sesamin and asarinin simultaneously in serum samples by LC with photodiode and fluorescent detection (22). However, the application of this method is limited by a long chromatographic run time (32 min), a large volume of serum sample (1.0 mL), time-consuming preparation (>40 min) and insufficient sensitivity to perform an oral pharmacokinetic study of *A. heterotropoides*.

Recently, Song *et al.* performed an ultra-performance LC/MS tandem mass spectrometry (UPLC–MS/MS) to analyze four different lignans simultaneously, including sesamin and asarinin in rat plasma after *Acanthopanax sessiliflorus* extract was orally administered; however, this approach requires more sophisticated instrumentation (23). Given that asarinin and sesamin are the main active components of *A. heterotropoides*, a simple, sensitive and efficient LC/MS/MS method should be developed to quantify the two epimeric lignans simultaneously in plasma samples. In this study, LC/MS/MS was optimized, validated and applied in pharmacokinetic evaluation after *A. heterotropoides* extract was orally administered in rats.

Experimental

Chemicals and materials

The reference samples of sesamin, asarinin and alantolactone as an internal standard (IS) were obtained from MUST Bio-Technology Co., Ltd. (Chengdu, China). Deionized water was prepared through a Milli-Q water purification system (Millipore, Milford, MA, USA). HPLC-grade acetonitrile and methanol were obtained from Fisher (Merk, Darmstadt, Germany), while HPLC-grade formic acid was purchased from (Tedia, Fairfield, USA).

Preparation of *A. heterotropoides* extract

Crude *A. heterotropoides* (100 g) was ground to produce power and extracted twice by refluxing with 1 000 mL of 80% ethanol for 2 h at each time. The extracted solutions were combined and then precipitated with fourfold volumes of dehydrated ethanol.
The upper layer in the ethanol mixture was concentrated under reduced pressure; the obtained aqueous solution was further condensed to reach a final concentration of *A. heterotrropoides* of 0.28 g/mL. The mixture was used for the animal experiments in this study and the two furofuran lignans in the solution were quantitatively determined. The corresponding concentrations of asarinin and sesamin in *A. heterotrropoides* extract were 1.21 and 0.432 mg/mL, respectively.

**Calibration standards and QC samples**

Stock standard solutions (1.00 mg/mL) of sesamin and asarinin were prepared in methanol and were used to prepare working standard solutions. Serial dilutions of sesamin were prepared at different concentrations: 0.250, 0.500, 1.50, 5.00, 15.0, 50.0 and 150 μg/mL. Serial dilutions of asarinin were also prepared at different concentrations: 0.05, 0.100, 0.300, 1.00, 3.00, 10.0 and 30.0 μg/mL. The stock solution of the IS was prepared by dissolving alantolactone in acetonitrile at a concentration of 1.00 μg/mL.

The calibration curves were prepared at different concentrations by diluting the corresponding standard solutions with drug-free human plasma. For sesamin, the concentrations were listed as follows: 25.0, 50.0, 150, 500, 1 500, 5 000 and 15 000 ng/mL; for asarinin, the concentrations were listed as follows: 5.00, 10.0, 30.0, 100, 300, 1 000 and 3 000 ng/mL. QC plasma samples were prepared at three different concentrations. For sesamin, the concentrations were 50.0, 500 and 13 500 ng/mL. For asarinin, the concentrations were 10.0, 100 and 2 700 ng/mL. The plasma samples were stored at −60°C until analysis.

**LC/MS conditions**

The chromatographic separation was performed on a Diamonsil C18 analytical column (4.6 mm × 150 mm, i.d., 5 μm; DIKMA, USA), with a SecurityGuard C18 guard column (4.6 mm × 30 mm, i.d., 5 μm; Phenomenex, USA). The mobile phase used was methanol/5 mM ammonium acetate/formic acid (75:25:0.1, v/v/v). The chromatographic separation was under isocratic conditions with a flow rate of 0.80 mL/min. The column temperature was maintained at 30°C.

MS analysis was performed using a 6460 triple quadrupole mass spectrometer equipped with electrospray ionization source in positive multiple reaction monitoring (MRM) mode. A single precursor-ion product ion transition was monitored for each analyte and the IS. The transitions were: m/z 372.3 → m/z 173.2 for sesamin, m/z 372.3 → m/z 173.2 for asarinin, m/z 250.3 → m/z 105.1 for IS, see Figure 2. The fragmentation energies of both analytes were set at 90 and 115 V and that for IS was at 100 V. The optimized collision energies were 10, 12 and 20 eV for sesamin, asarinin and IS, respectively. Other parameters of the mass spectrometer were as follows: drying-gas temperature 350°C, drying-gas flow 10 L/min, nebulizer pressure 45 psi and capillary voltage 4 000 V.

**Sample preparation**

Acetonitrile (150 μL) and IS solution (100 μL; 1 000 ng/mL) were gradually added to an aliquot of the plasma sample (150 μL). After the solution was vortexed for 2 min, the mixture was centrifuged at 11 000 rpm for 10 min. The total supernatant was transferred to a clean glass tube and then evaporated to dryness with a gentle flow of nitrogen at 45°C. The residues were reconstituted in 100 μL of mobile phase [methanol/water/formic acid (75:25:0.1, v/v/v)] and transferred to autosampler vials; 20 μL of the resulting mixture was injected into the LC/MS/MS system.

**Assay validation**

This method was validated for selectivity, matrix effect, linearity, precision, accuracy, recovery and stability according to the FDA guidance for validation of bioanalytical methods (24).

**Application in a pharmacokinetic study**

Six male Wistar rats (weighing 200 ± 20 g) were purchased from the Animal Center of Shenyang Pharmaceutical University (Shenyang, China). The rats were subjected to fasting for 12 h before the treatment but were provided free access to water during the experiment. All of the animal experiments were performed in accordance with the Guideline for Animal Experimentation of Shenyang Pharmaceutical University, and the protocol (no. 2013-0125) was approved by the Animal Ethics Committee of the Institution.

The validated method was applied in a pharmacokinetic study of sesamin and asarinin in rat plasma after 2.8 g/kg of *A. heterotrropoides* extract (equivalent to 12.1 and 4.32 mg/kg for asarinin and sesamin, respectively) was orally administered. Blood samples (approximately 0.3 mL) were withdrawn into heparinized tubes from the orbital veins after 0, 0.083, 0.25, 0.50, 1, 2, 3, 5, 8, 12, 24, 36 and 48 h. These samples were immediately centrifuged at 3 000 rpm for 10 min. The plasma samples were stored at −60°C until analysis. Pharmacokinetic calculations were performed using the DAS 2.0 statistical software (Pharmacology Institute of China).
Results

Method development

The \([M+NH_4]^+\) ions at \(m/z\) 372.3, 372.3 and 250.3 were used as precursor ions of sesamin, asarinin and IS, respectively. The product ion mass spectra of the two epimeric furofuran lignans in \(A.\) heterotropoides extract and IS are shown in Figure 2. For the isomers of sesamin and asarinin, the most abundant product ions were characterized at \(m/z\) 173.2 in MS/MS scan mode. Thus, the MRM transitions on the positive ionization of sesamin and asarinin were selected at \(m/z\) 372.3 → 173.2 to monitor both lignans. For detection, the MRM transition of the IS was set at \(m/z\) 250.3 → 105.1.

Assay validation

Specificity

Typical chromatograms are shown in Figure 3 for blank plasma, blank plasma spiked with 25.0 ng/mL sesamin, 5.00 ng/mL asarinin and 1 000 ng/mL IS, and rat plasma samples 0.5 h after oral administration of \(A.\) heterotropoides extract. No interfering peak from endogenous substances in plasma was observed at the respective retention time of the two analytes and the IS.

Matrix effects

The matrix effect was calculated by comparing peak areas of analyte spiked after extraction to those of an equivalent concentration of the standard solutions at three QC levels. With regard to matrix effect, the determined values are all between 85 and 115%, which suggested that there is little or no matrix effect on the ionization of the analytes and the IS in this method.

Linearity and lower limit of quantitation

Typical regression equations are \(y = 3.25 \times 10^{-2}x - 4.08 \times 10^{-3}\) over the range 25.0–15 000 ng/mL, correlation coefficient \((r^2) = 0.9925\), for sesamin, and \(y = 2.18 \times 10^{-2}x - 6.56 \times 10^{-4}\) over the range 5.00–3 000 ng/mL, \(r^2 = 0.9958\), for asarinin. Both correlation coefficients exceeded 0.99, indicative of good linearity over these concentration ranges. The lower limit of quantitation (LLOQ) defined as the lowest concentration on the...
calibration curve was 25.0 ng/mL for sesamin and 5.00 ng/mL for asarinin, which could fulfill the analytical requirement that the signal-to-noise ratio >10 and acceptable accuracy and precision within ±20%.

**Precision and accuracy**

Precision and accuracy were evaluated by determining QC samples at three concentration levels in six replicates on three validation days. Table I summarizes the intra- and inter-day precision and accuracy for sesamin and asarinin, obtained by analysis of three QC concentration levels of samples. These data show that all the values were within the acceptable range of ±15%, which implies that the method has good precision and accuracy.

**Recovery**

Extraction recoveries of sesamin and asarinin at three concentrations (50.0, 500 and 13 500 ng/mL, and 10.0, 100 and 2 700 ng/mL, respectively) are 92.3 ± 3.6%, 94.5 ± 6.7% and 90.9 ± 5.5%, and 96.3 ± 1.2%, 97.2 ± 3.7% and 93.1 ± 0.6%, respectively, while the recovery of the IS is 98.8 ± 6.2%. These results indicate that the current processing conditions for plasma sample can provide adequate recoveries for the analytes and IS.

**Stability**

Observations for evaluating stability indicate that the plasma samples were stable under the different storage conditions for plasma samples, including three freeze–thaw cycles (−60 to 20°C), post-preparative stability for 12 h and long-term stability (−60°C for 2 months). The results are presented in Table II.

**Pharmacokinetic study**

The proposed method was successfully applied in the pharmacokinetic study of sesamin and asarinin in male Wistar rats after *A. heterotropoides* extract was orally administered at a dose of 2.8 g/kg. The mean plasma concentration–time curves of sesamin and
Main Pharmacokinetic Parameters of Two Lignans After Asarum heterotropoides Extract Was Orally Administered at a Dose of 2.8 g/kg in Rats (n = 6)

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Sesamin</th>
<th>Asarinin</th>
</tr>
</thead>
<tbody>
<tr>
<td>$t_{1/2}$ (h)</td>
<td>11.81 ± 1.76</td>
<td>6.48 ± 2.32</td>
</tr>
<tr>
<td>$T_{max}$ (h)</td>
<td>2.33 ± 0.52</td>
<td>2.50 ± 0.55</td>
</tr>
<tr>
<td>$C_{max}$ (ng/mL)</td>
<td>577.4 ± 28.6</td>
<td>2341.4 ± 945.8</td>
</tr>
<tr>
<td>MRT (h)</td>
<td>13.05 ± 1.78</td>
<td>8.13 ± 2.11</td>
</tr>
<tr>
<td>MRR (h)</td>
<td>16.23 ± 2.46</td>
<td>8.60 ± 2.59</td>
</tr>
<tr>
<td>AUC$_{0-\infty}$ (ng h/mL)</td>
<td>7.864 ± 1.804.6</td>
<td>18.623.3 ± 6.123.5</td>
</tr>
<tr>
<td>AUC$_{0-\infty}$ (ng h/mL)</td>
<td>8.401.8 ± 1.978.4</td>
<td>18.849.8 ± 6.356.4</td>
</tr>
</tbody>
</table>

Table II.
Stability of Sesamin and Asarinin in Rat Plasma at Three QC Levels

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Nominal conc. (ng/mL)</th>
<th>Three freeze–thaw cycles (−60°C to 20°C)</th>
<th>Post-preparative stability for 12 h</th>
<th>Long-term stability (−60°C for 2 months)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Measured conc. (ng/mL)</td>
<td>Accuracy (%)</td>
<td>Measured conc. (ng/mL)</td>
<td>Accuracy (%)</td>
</tr>
<tr>
<td>Sesamin</td>
<td>50.0</td>
<td>49.4 ± 0.8</td>
<td>−1.1</td>
<td>51.1 ± 1.4</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>521.4 ± 29.1</td>
<td>4.3</td>
<td>509.8 ± 15.1</td>
</tr>
<tr>
<td></td>
<td>13500</td>
<td>14110.8 ± 1 341.5</td>
<td>4.5</td>
<td>13326.4 ± 1 715.7</td>
</tr>
<tr>
<td>Asarinin</td>
<td>10.0</td>
<td>10.9 ± 0.6</td>
<td>8.8</td>
<td>10.5 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>104.0 ± 7.2</td>
<td>4.0</td>
<td>97.7 ± 7.2</td>
</tr>
<tr>
<td></td>
<td>2 700</td>
<td>2 639.0 ± 223.2</td>
<td>−2.3</td>
<td>2 484.9 ± 261.8</td>
</tr>
</tbody>
</table>

Discussion

To obtain a perfect chromatographic separation and good ionization capacity of the isomers of sesamin and asarinin, we optimized different conditions by varying the mobile phase, flow rate and type of analytical columns. A previous study reported that methanol provides higher mass spectral signals of analytes than acetonitrile (23). In our experiment, good mass spectral signals were also obtained using methanol. Formic acid was added to the mobile phase to enhance protonation and ionization of the analytes. Formic acid can also enhance separation resolution because silica surface silanols (C$_{18}$ column) were suppressed to minimize cation-exchange interaction, which may help separate the two epimeric lignans more efficiently. A mobile phase consisting of methanol/5 mM ammonium acetate/formic acid (75:25:0.1, v/v/v) at a flow rate of 0.8 mL/min exhibited the most efficient separation, revealed symmetrical peak shapes and provided proper retention times for both the lignans and the IS.

Protein precipitation methods of the analytes and IS from the plasma samples were investigated during the experiment. Different organic precipitation solvents such as methanol, acetonitrile and trichloroacetic acid were evaluated. Methanol was chosen as the optimal precipitation agent because it allowed the highest recovery among the solvents. Moreover, protein precipitation method was simpler and less time consuming than liquid–liquid extraction. Therefore, this method could be utilized in this study.

Conclusion

In summary, a selective, sensitive and rapid LC/MS/MS method was developed to determine the two epimeric lignans (sesamin and asarinin) in rat plasma simultaneously. The sample was conducted by a simple protein precipitation pretreatment, which provided good recovery and elicited slight matrix effect. The LC/MS/MS method provided excellent separation, good selectivity and sensitivity at LLOQs of 25.0 and 5.00 ng/mL for sesamin and asarinin, respectively. The proposed method has been successfully applied in the pharmacokinetic study of sesamin and asarinin after A. heterotropoides extract was orally administered in male Wistar rats.

References