Determination and Pharmacokinetics of WGA in Rat Plasma by LC–MS After Oral Administration of *Xanthoceras sorbifolia* Bunge Extract

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**Abstract**

3-\(O-(3-O\text{-angeloyl}-6-O\text{-}\beta\text{-D-glucopyranosyl}-\beta\text{-D-glucopyranosyl}-28-O\text{-(2-\(\alpha\)-L-rhamnopyranosyl-6-O}\text{-}\beta\text{-D-glucopyranosyl)-16-deoxybarringtogenol A (WGA)}\) is a potential anti-AD (Alzheimer’s disease) active compound isolated from the husks of *Xanthoceras sorbifolia* Bunge. A rapid and accurate high-performance liquid chromatography–mass spectrometry (LC–MS) method was developed and validated for the quantification of WGA in rat plasma. Digoxin was used as internal standard (IS). Sample preparation was performed by liquid–liquid extraction using ethyl acetate-isopropanol (1:1, v/v). HPLC separation was carried out using a Venusil MP C18 column (150 mm \(\times\) 4.6 mm, 5 \(\mu\)m). Isocratic elution was performed using methanol: water (70:30, v/v) as the mobile phase, at a flow rate of 0.8 mL/min. Analysis was performed in selected ion monitoring mode with a positive electrospray ionization interface. No endogenous interference was observed at the retention time of the analyte because of the high specificity of selected ion monitoring mode. The assay was validated to demonstrate the selectivity, linearity, recovery, accuracy, precision and stability. The lower limit of quantification (LLOQ) was 10.0 ng/mL. The developed and validated method has been successfully applied to the quantification and pharmacokinetic study of WGA in rats after oral administration of *X. sorbifolia* Bunge extract.

**Introduction**

*Xanthoceras sorbifolia* Bunge (yellowhorn) is a large woody shrub native to northern China. It has been recognized as a small ornamental tree and an important energy plant for bio-diesel (1). In the past few decades, extensive areas of yellowhorn plantations have been established in northern China to combat desertification. The seeds, husks, leaves and flowers are edible and have been used as folk medicines to treat rheumatic arthritis and nocturnal enuresis in children (2, 3). Recently, it also showed a promise as a treatment of Alzheimer’s disease (AD). The crude extract of yellowhorn could significantly ameliorate the impairment of learning and memory in animal models induced by amyloid-\(\beta\) protein (A\(\beta\)) (4–7). The production and the aggregation of A\(\beta\) have been believed to be important pathological mechanism of AD (8, 9). Further phytochemical investigations showed that the characteristic angeloyl (Ang)-substituted oleananes from yellowhorn are responsible for its anti-AD activity (10–15). Recently, we identified an angeloyl oleanane, 3-\(O-(3-O\text{-angeloyl-6-O}\text{-}\beta\text{-D-glucopyranosyl)-}\beta\text{-D-glucopyranosyl-28-O\text{-(2-\(\alpha\)-L-rhamnopyranosyl-6-O}\text{-}\beta\text{-D-glucopyranosyl)-16-deoxybarringtogenol A (WGA)}\) from the husks of this plant (16). Isolated as a major compound, WGA has been assumed to be one of the anti-AD determinants. As a relatively large
molecule (molecular mass: 1,350.7), there is a great concern about its bioavailability in the early period of drug development. However, there is a lack of information on the pharmacokinetics of the angeloyl oleananes found from this plant. Therefore, it is necessary to develop a rapid and accurate bioanalytical method to quantify WGA in biological fluids and then apply to the bioavailability study of WGA in animals. Thus, the aim of the present work is to develop a rapid and accurate bioanalytical method for the quantification of WGA in rat plasma using liquid chromatography–mass spectrometry (LC–MS). The developed method was further validated and then applied to a pharmacokinetic study of WGA in rats after oral administration of yellowhorn extract. Yellowhorn produces an array of Ang-oleanane analogs structurally similar to WGA, such as xanthoceraside, sorbifoside A and B (17). Therefore, this study is also expected to help in understanding the pharmacokinetic profiles of these saponins in rats.

**Experimental**

**Materials, reagents and animals**

Husks of *X. sorbifolia* were obtained from the Wudan Forest Farm in Wengniute County, Inner Mongolia, China. A voucher specimen (No. 0154620) was deposited in the Herbarium of Northeast China at the Institute of Applied Ecology, Chinese Academy of Sciences. WGA (purity > 98.5%, Figure 1) was isolated and identified in our laboratory (Department of Pharmaceutical Analysis, Shenyang Pharmaceutical University, Shenyang, PR China) (17). Digoxin (IS, used as an internal standard (IS), Figure 1) with purity of greater than 99% was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China).

HPLC grade methanol was purchased from Fisher Scientific (Pittsburgh, PA, USA). Other reagents were of analytical grade and obtained from Dikma Technology Inc. (Beijing, China). Purified water was obtained from a Millipore Milli-Q system (Milford, MA, USA).

Male Wister rats weighing 220–250 g were provided by the Experimental Animal Center of Shenyang Pharmaceutical University (Shenyang, China), and kept in an environmentally controlled breeding room (temperature: 22 ± 2°C, humidity: 50 ± 10%) for 7 days before starting the experiments. They were fed with a standard laboratory food and water ad libitum and fasted for 12 h with free access to water prior to the experiments. The animal study was carried out in accordance with the Guidelines for Animal Experimentation of Shenyang Pharmaceutical University and the protocol was approved by the Animal Ethics Committee of the institution.

**Instruments and LC–MS conditions**

Shimadzu 2010 series HPLC tandem mass spectrometer equipped with an LC-10ADvp binary pump, an on-line degasser, an autosampler and a column temperature controller were used for all analyses. The data were processed using Shimadzu software (version 3.0). Chromatographic separations were performed on a Venusil MP C18 column (150 mm × 4.6 mm, 5 μm particle size) protected by a C18 guard column (12.5 mm × 4.6 mm, 5 μm) at 30°C. Isocratic elution was performed using methanol: water (70:30, v/v) as the mobile phase. The flow rate was set at 0.8 mL/min with 25% of the eluent split into the inlet of the mass spectrometer. Aliquots of 10 μL were injected into HPLC system for analysis. Mass spectra were acquired using a mono-quadrupole mass spectrometer coupled with an electrospray ionization source (ESI). Nitrogen was used as the sheath and auxiliary gas to assist nebulization with the flow rate settled at 1.5 L/min. All mass spectra were acquired in the positive ion mode with capillary voltage at 1.75 kV, curved desolvation line temperature at 250°C and block temperature at 200°C. Analysis was carried out by selected ion monitoring at [M + 2Na]^{2+} m/z 698.50 for WGA and at
[M + Na]+ m/z 803.45 for IS, and the full-scan mass spectra of WGA and IS after injection in mobile phase are shown in Figure 2.

Preparation of *X. sorbifolia* Bunge extract

Dried husks of *X. sorbifolia* (200 g) were extracted three times with 1.6 L of 70% (v/v) ethanol under reflux for 2.0 h. The extraction solutions were concentrated under reduced pressure. The intragastric solution was prepared by dissolving the resulting ethanol crude extract in 200 mL water. The decoction was stored in the refrigerator at 4°C.

Preparation of calibration standards and QC samples

Stock solution of WGA was prepared in methanol at the concentration of 1.003 mg/mL, stored at 4°C and was further diluted with methanol to working concentrations.

Stock solution of IS was prepared at the concentration of 330.0 μg/mL in methanol and diluted with methanol to the working solution containing 132.0 ng/mL of digoxin.

Calibration standards were prepared by addition of working standards of WGA to blank plasma giving final concentrations of 10.03, 25.08, 50.15, 100.3, 250.8, 501.5 and 1,003 ng/mL.

The quality control (QC) samples were prepared from separate stock solutions in blank plasma at the concentrations of 25.08, 250.8 and 802 ng/mL. The spiked plasma samples were stored at −20°C prior to analysis.

Sample preparation

The plasma (200 μL) was spiked with 20 μL of methanol and 20 μL of IS solution (digoxin, 132.0 ng/mL), followed by vortexing of 30 s. The mixture was then extracted with 1.0 mL of ethyl acetate-isopropanol (1:1, v/v) with vortexing for 5 min. After centrifugation at 16,000 × g for 5 min, the upper layer was transferred to a test tube.

![Figure 3. Chromatograms of blank plasma samples (Upper), blank plasma added with WGA and IS (Middle) and plasma sample from a rat (0.75 h after oral administration of *X. sorbifolia* Bunge extract) (Lower).](https://academic.oup.com/chromsci/article-abstract/56/1/68/4555264)

Table I. Precision and Accuracy for the Analysis of WGA in Rat Plasma (n = 6)

<table>
<thead>
<tr>
<th>Concentration (ng/mL)</th>
<th>Intra-day (n = 6)</th>
<th>Inter-day (n = 3 × 6)</th>
<th>Recoveries</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Precision (RSD, %)</td>
<td>Accuracy (RE, %)</td>
<td>Precision (RSD, %)</td>
</tr>
<tr>
<td>-----------------------</td>
<td>-------------------</td>
<td>-------------------</td>
<td>-------------------</td>
</tr>
<tr>
<td>25.08</td>
<td>3.7</td>
<td>5.1</td>
<td>9.2</td>
</tr>
<tr>
<td>250.8</td>
<td>7.1</td>
<td>−2.6</td>
<td>11.1</td>
</tr>
<tr>
<td>802</td>
<td>6.6</td>
<td>−6.6</td>
<td>4.7</td>
</tr>
</tbody>
</table>
tube and evaporated to a dryness under a stream of nitrogen. Then, the residue was reconstituted in 100 μL mobile phase, 10 μL of which was used for LC–MS analysis.

Method validation
The method was validated in accordance with the currently accepted US-FDA Bioanalytical Method Validation Guidance (18) with respect to selectivity and carry-over, calibration curve, lower limit of quantification (LLOQ), accuracy and precision, recovery, matrix effect and stability.

The specificity of the assay for the analyte versus endogenous substances in the matrix was tested by comparing the lowest concentration in the calibration curve standards with reconstructions prepared using drug-free plasma from six rats. The carry-over was assayed by measuring the response of double blank plasma, double upper limit of quantification (ULOQ), double blank plasma and repeated the last two steps three times. It was considered negligible if the measured peak area was <20% of LLOQ at the same day.

The linearity of the assay was assessed by analyzing the calibration curves using least-squares linear regression of the peak area ratios of the analyte to the IS versus the nominal concentration of the calibration standard with a weighed factor (1/x²).

The LLOQ was defined as the lowest concentration on the calibration curve at which the signal-to-noise ratio was above 10 with an acceptable accuracy within ±20% and the precision below 20%.

QC samples at low, medium and high concentration were analyzed on three separate occasions with six replicates at each concentration per occasion to determine the accuracy and precision.

Precision was defined as the relative standard deviation (RSD%) while accuracy was defined as relative error (RE%).

The extraction recovery of WGA was evaluated by comparing the peak areas from blank plasma samples spiked before extraction with those from blank plasma samples spiked after extraction at three QC concentrations in a set of six replicates.

The matrix effect was assessed at three QC concentrations in six replicates using the ratios of peak areas of the blank plasma samples spiked after extraction to those of pure standard solutions containing analyte at the same concentrations.

Stability studies in plasma samples were also conducted at three QC levels in several different storage conditions: at −20°C for 7 days, after three freeze–thaw cycles, at room temperature for 4 h and 12 h after prepared at 4°C.

Application to the pharmacokinetic study
The validated method was applied to determine the WGA in rat plasma samples after oral administration of X. sorbifolia extract. Six rats were fasted for 12 h, with free access to water prior to the experiments. The X. sorbifolia extract were administered to each rat by oral gavage using a stomach tube at a dose of 10 mL/kg (equivalent to 4.4 mg WGA per kg bodyweight). Blood samples of about 0.5 mL were collected into heparinized centrifuge tubes from the suborbital vein at 0, 0.083, 0.17, 0.33, 0.5, 0.75, 1.0, 2.0, 3.0, 4.0, 6.0, 8.0, 10.0 and 12.0 h after oral administration. Following centrifugation (4,000 rpm, 5 min), plasma samples were transferred to polypropylene tubes and stored at −20°C until analysis.

The pharmacokinetic parameters of WGA were calculated by non-compartmental analysis of plasma concentration versus time data using DAS 2.1 software package (Chinese Pharmacological Society). All data were presented as means ± SD. P < 0.05 was considered statistically significant for all the tests.

Results
LC–MS optimization
Under the electrospray ionization conditions chosen, greater sensitivity was achieved for WGA and IS in the electrospray positive ionization (ESI+) source. The positive ion full-scan mass spectra of WGA and IS indicated that the most abundant ions are [M + 2Na]²⁺ for WGA and [M + Na]⁺ for IS. So, the quantitative analysis was carried out in SIM mode at [M + 2Na]²⁺ m/z 698.50 for WGA and [M + Na]⁺ m/z 803.45 for IS, respectively.

The selection of mobile phase is important for improving peak shape, detection sensitivity and shortening running time. The methanol–water and acetonitrile-water systems were investigated. Methanol was used as the organic modifier because it provided

- Figure 4. Plasma concentration-time curve of WGA in rats plasma after oral administration of X. sorbifolia Bunge extract.

Table II. Mean pharmacokinetic parameters of WGA in rats plasma after oral administration of X. sorbifolia Bunge extract (n = 6)

<table>
<thead>
<tr>
<th>No.</th>
<th>T_{max} (h)</th>
<th>C_{max} (μg mL⁻¹)</th>
<th>AUC₀⁻∞ (μg h mL⁻¹)</th>
<th>AUC₀⁻∞ (μg h mL⁻¹)</th>
<th>T_{1/2} (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.0</td>
<td>0.6710</td>
<td>1.803</td>
<td>1.830</td>
<td>2.0</td>
</tr>
<tr>
<td>2</td>
<td>1.0</td>
<td>0.3417</td>
<td>1.627</td>
<td>1.857</td>
<td>3.9</td>
</tr>
<tr>
<td>3</td>
<td>0.75</td>
<td>0.6069</td>
<td>2.042</td>
<td>2.228</td>
<td>3.7</td>
</tr>
<tr>
<td>4</td>
<td>2.0</td>
<td>0.6164</td>
<td>1.242</td>
<td>1.273</td>
<td>2.4</td>
</tr>
<tr>
<td>5</td>
<td>1.0</td>
<td>0.5405</td>
<td>1.179</td>
<td>1.256</td>
<td>2.8</td>
</tr>
<tr>
<td>6</td>
<td>3.0</td>
<td>0.3325</td>
<td>1.526</td>
<td>1.751</td>
<td>2.9</td>
</tr>
<tr>
<td>Mean</td>
<td>1.5</td>
<td>0.5182</td>
<td>1.570</td>
<td>1.699</td>
<td>2.9</td>
</tr>
<tr>
<td>SD</td>
<td>0.9</td>
<td>0.1463</td>
<td>0.3295</td>
<td>0.3745</td>
<td>0.8</td>
</tr>
</tbody>
</table>
higher responses and lower background noise than acetonitrile. Using the mobile phase described in “Instruments and LC–MS conditions” section, WGA and IS were well separated with a total running time of 12 min.

Method validation

Selectivity and matrix effect
No endogenous interference was observed at retention times of WGA and the IS from the six different rat plasma samples, demonstrating the high selectivity of the method. Representative chromatograms of blank rat plasma, blank rat plasma spiked with WGA and IS, rat plasma sample collected at 0.75 h after administration of X. sorbifolia extract are shown in Figure 3.

For matrix effect, all the ratios were between 90 and 110%, indicating that no significant matrix effect was observed for WGA or IS.

Linearity and LLOQ
The calibration curves showed good linearity over the concentration range of 10.03–1,003 ng/mL in rat plasma. Typical linear regression equation of the calibration curves was $y = 1.182 \times 10^{-2}x - 1.670 \times 10^{-3}$ ($r = 0.9930$), where $y$ is the peak area ratio of the analyte to the IS, and $x$ is the plasma concentration of the analyte. The LLOQ of the assay was 10.0 ng/mL in rat plasma.

Precision, accuracy and recovery
The intra-day, inter-day precision and accuracy of WGA are summarized in Table I. All the results of the tested samples were within the acceptable criteria (RSD%: 15; RE%: ±15).

The extraction recoveries of WGA from rat plasma were 98.5 ± 8.8, 97.0 ± 9.5 and 94.5 ± 7.8% at concentrations of 25.08, 250.8 and 802 ng/mL, respectively (Table I) and the extraction recovery of IS was 85.2 ± 13.3%, indicating that the recoveries of WGA and IS were consistent, precise and reproducible at different concentration levels in various plasma samples.

Stability
The measured concentration for WGA at each QC levels deviated within 15.0%, which demonstrated that WGA was stable in plasma samples at room temperature for 4 h, at –20°C for at least 7 days, after three freeze and thaw cycles, and at 4°C in autosampler for 12 h after prepared.

Pharmacokinetic study

After oral administration of X. sorbifolia extract to individual rats ($n = 6$), the plasma drug concentration-time curves (mean ± SD) of WGA is presented in Figure 4 and the corresponding pharmacokinetic parameters are listed in Table II, respectively.

At the early stage of the method development, the supernatant was separated out and evaporated to dryness under vacuum at 40°C after vortex-mixing and centrifugation. It was found that WGA in the residue could be completely reconstituted with the mobile phase. To obtain symmetric peak shape, the supernatant was diluted with mobile phase before being injected into the LC–MS system. Protein precipitation (PPT) generally resulted stronger matrix effects than LLE, so LLE was used for sample preparation.

WGA is a potential anti-AD active compound isolated from the husks of X. sorbifolia Bunge. A rapid and accurate high-performance LC–MS method was developed and validated for the quantification of WGA in rat plasma. The assay was validated to demonstrate the selectivity, linearity, recovery, accuracy, precision and stability. The method has been successfully applied to the quantification and pharmacokinetic study of WGA in rats after oral administration of X. sorbifolia Bunge extract. This study is also expected to help in understanding the pharmacokinetic profiles in rats of the WGA-like saponins rich in yellowthorn.

Conclusion

A simple, robust and efficient LC–MS method for the determination of WGA in rat plasma has been developed and validated. The method has been successfully applied to the pharmacokinetic study of WGA in the rats after oral administration of X. sorbifolia extract. The LLOQ (up to 10 ng/mL) enabled a full description of the rat pharmacokinetics of WGA. The assay described here demonstrates that WGA can be detected accurately in the plasma, allowing its pharmacokinetic profile to be elucidated directly.

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