LC–MS-MS Determination and Pharmacokinetic Study of the Novel Anti-Tumor Candidate Drug TEOA in Rats

Jifeng Gu, Guoqiang Ding, Nianzu Chen and Zhen Zhang

Introduction

The Actinidiaceae family, which is widely distributed in most regions of China, has traditionally been used to treat hepatitis, edema, rheumatic arthritis, dysentery, lymphoid tuberculosis, gastric and breast cancer in Chinese people (1, 2). The root of Actinidia delicosa, a representative herb in the Actinidiaceae family (3), is the medicinal portion, which possesses numerous, beneficial pharmacological activities, such as anti-tumor and protective effects on acute hepatic injuries (4–6). Furthermore, in recent years, its anti-tumor activities have drawn more attention (7–9).

In previously published literature, several types of compounds have been reported in the Actinidiaceae family, such as triterpenoids, flavones, anthraquinones and polysaccharides (10–12). Moreover, in vitro cytotoxicity tests showed that the compound exhibited potent anti-cancer activities against several human cancer cell lines (9, 13). However, no pharmacokinetic data of TEOA were reported, preliminary animal experiments were conducted using rats as the model to evaluate the pharmacokinetic parameters of the compound, which might provide some preclinical data for further research. Herein, for the first time, a specific, sensitive and robust liquid chromatography–tandem mass spectrometry (LC–MS-MS) method was utilized to assay TEOA in rat plasma after oral administration for pre-clinical pharmacokinetic studies.

Experimental

Chemicals and reagents

TEOA (99.0% purity, approximately 200 mg) was isolated from 10 kg Actinidia delicosa root and its chemical structure was identified by spectroscopic data by the authors’ group (Figure 1). Warfarin, used as the internal standard (IS), was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Deionized ethyl acetate from Shanghai Chemical Reagent Company (Shanghai, China). Deionized water was purified through a Milli-Q apparatus (Millipore, Bedford, MA). Fresh blank rat plasma was harvested and stored at –20°C until analysis.

Instrumentation and LC–MS-MS conditions

All data acquisition was performed with a 1200 Series liquid chromatograph (Agilent Technologies, Waldbronn, Germany) equipped with a degasser, a binary pump, an autosampler, a column compartment and an Agilent 6410 triple quadrupole mass spectrometer (Agilent Technologies), additionally equipped with an atmospheric-pressure chemical ionization (APCI) source and controlled by Agilent Masshunter B.01.03 software. Chromatographic separation was conducted on a C18 Capcell PAK UG120 column (100 × 4.6 mm, i.d., 5 μm; Phenomenex, Torrance, CA), maintained at 30°C. The
mobile phase was methanol–water (90:10, v:v) at a constant flow rate of 0.8 mL/min, and the injection volume was 20 μL.

The MS conditions were: vaporizer, 350 °C; nebulizer pressure, 20 psi; gas temperature, 325 °C; gas flow, 5 L/min; capillary voltage, 4,500 V; capillary current, 4 mA. The APCI source was set in the positive ion mode for acquiring all mass spectrometric data. Multiple-reaction monitoring (MRM) mode was used with specific ion transitions of dehydrated protonated molecule [M + H–H2O]+ to product ion at \( m/z \) 471.3 → 203.1 with 20 eV collision energy for the analyte, and protonated molecule [M + H]+ to product ion at \( m/z \) 309.2 → 163.1 with 18 eV collision energy for IS, with a scan time of 0.2 s per transition.

**Preparation of standard solutions**

Two stock solutions of the analyte (1.00 mg/mL) were prepared in methanol. The serial solutions of the analyte were then gradually diluted with acetonitrile to achieve standard working solutions at concentrations of 60.0, 200, 400, 2,000, 6,000, 20,000 and 40,000 μg/mL. The stock solution of IS was also prepared in methanol (1.00 mg/mL) and diluted with acetonitrile to obtain a working solution of 40,000 ng/mL. All solutions were stored at 4°C until analysis.

**Preparation of calibration curves and quality control samples**

Appropriate amounts of working solutions of the analyte and IS were spiked into 200 μL of blank plasma to obtain final concentrations of 3.00, 10.0, 30.0, 100, 300, 1,000 and 2,000 ng/mL for the analyte and 2,000 ng/mL for IS, respectively. Three levels of quality control (QC) samples in plasma were 10.0 ng/mL (low), 50.0 ng/mL (medium) and 1,800 ng/mL (high) for the analyte. All samples were kept for in a −20°C freezer until analysis.

**Sample preparation**

Plasma samples were thawed at room temperature and vortexed for 30 s. Ten microliters of IS (40,000 ng/mL) solution was added to 200 μL of plasma sample and then vortex-mixed for 30 s. The mixture was extracted with 3 mL of ethyl acetate, vortex-mixed for 3 min, and then centrifuged at 4,000 r/min for 5 min. The upper organic layer was transferred to a neat glass tube and evaporated to dryness using a gentle nitrogen stream in a water-bath maintained at 30°C. The residue was dissolved with 100 μL of methanol, vortex-mixed for 30 s, and 20 μL of the supernatant was then injected onto the LC–MS–MS for analysis.

**Method validation**

A full method validation of the TEOA assay in rat plasma was performed according to the Food and Drug Administration (FDA) guidelines (15). The method was validated for specificity, sensitivity, linearity, accuracy and precision, recovery, matrix effect and stability.

The specificity of the method was studied by comparing the chromatograms of blank rat plasma with the corresponding plasma spiked the analyte and IS.

Linearity was observed from six calibration curves prepared and run on three consecutive days over the range of 3.00–2,000 ng/mL for the analyte. Calibration curves were constructed by plotting the peak area ratios of the analyte to IS versus the concentrations of the calibration standards, and a weighted linear regression model (weighting factor \( 1/x^2 \)) was used to fit the calibration line.

For accuracy and precision of the method, each concentration of QC samples was assayed in six replicates on three consecutive days. Intra-day and inter-day precisions were required to not exceed 15%.

For the extraction recovery, the mean peak areas of the three concentration levels of extracted QC samples were compared to the mean peak areas of the extracted blanks spiked with the corresponding neat solutions.

The matrix effect was determined by the ratio of the amounts of the analyte dissolved with blank matrix extract against those of neat standards. The matrix effect of the IS was determined in a similar way.

Stability was investigated by analyzing the plasma samples at low and high concentration levels under different conditions (\( n = 3 \)). Plasma samples were subjected to storage at ambient temperature for 2 h, to the ready-to-inject samples after extraction treatment in the autosampler for 12 h, to long-term storage conditions at −20°C for eight days and to three freeze–thaw stability experiments.

**Application to pharmacokinetic studies**

All animal studies were approved by the institutional ethics committee prior to the study. Six male Wistar rats (weighing 200 ± 20 g) were purchased from the Experimental Animal Center of Fudan University (Shanghai, China). Rats were housed in a temperature-controlled room (22 ± 2°C) with a 12 h light/dark cycle.

After fasting overnight with free access to water for at least 12 h, rats were administered a single dose of 100 mg/kg body weight of TEOA suspended in 0.5% carboxymethyl cellulose sodium (CMC-Na) aqueous solution orally by gavages. Blood samples were collected in 1.5 mL heparinized polyethylene tubes via the orbital vein at 0, 0.083, 0.25, 0.50, 0.75, 1.0, 1.5, 2.0, 3.0, 5.0, 8.0, 12 and 24 h. Plasma was separated by centrifugation at 3,000 rpm for 10 min and then transferred to clean polyethylene
tubes. The plasma samples were stored below −20°C until LC−MS-MS analysis.

Pharmacokinetics parameters were calculated by noncompartmental analysis of data using Drug and Statistics (DAS) 2.0 software package (Mathematical Pharmacology Professional Committee of China, Shanghai, China).

Results

Method development

Sample pretreatment was performed by liquid–liquid extraction in which ethyl acetate was used as the extraction reagent. A mobile phase consisting of a 90 volume of methanol and a 10 volume of 0.1% formic acid was chosen to perform the study.

For TEOA, the mass spectrum of the dehydrated protonated molecule $[M+\text{H−H}_2\text{O}]^+$ at $m/z$ 471.3 showed the formation of characteristic product ions at $m/z$ 191.1, 203.1, 221.2, 311.1, 407.3 and 425.4 (Figure 1). The most sensitive mass transition was monitored from $m/z$ 471.3 to 203.1 for the assay of TEOA. Similarly, the protonated molecule $[M+\text{H}]^+$ of IS (warfarin, $m/z$ 309.2) showed the formation of characteristic product ions at $m/z$ 121.1, 147.1, 163.1, 173.1 and 251.2. The most sensitive mass transition was from $m/z$ 309.2 to 163.1 for the assay of the IS (Figure 2).

Method validation

Specificity

Representative chromatograms obtained from blank rat plasma, blank plasma spiked with the analyte and IS, and rat plasma at 1.0 h after oral administration of TEOA are shown in Figure 3. No interfering endogenous peaks were observed at the respective retention times ($R_t$) of the analyte and IS ($R_t = 2.25$ min for the analyte and $1.75$ min for the IS).

Linearity and lower limit of quantitation

Calibration curves of the analyte were linear over the concentration range of 3.00–2,000 ng/mL for plasma and the typical equation was $y = 7.5077 \times 10^{-7} \cdot x + 5.0578 \times 10^{-4}$ ($r^2 = 0.9995$). The lower limit of quantitation (LLOQ) for the analyte in plasma was 3.00 ng/mL, with precision and accuracy of 3.1 and 1.9%, respectively.

Precision and accuracy

Intra-day and inter-day precision and accuracy for the analyte from rat plasma QC samples were measured within 7.0 and 2.3%, respectively (Table I). The assay values conformed to the accepted variable limits.
Figure 3. MRM chromatograms: blank plasma (A); blank plasma spiked with 3.00 ng/mL for TEOA and 2,000 ng/mL for IS (B); blank plasma spiked with 2,000 ng/mL for TEOA and IS at 2,000 ng/mL (C); rat plasma sample at 1.0 h after oral administration of TEOA at a dose of 100 mg/kg (D).

Table I

<table>
<thead>
<tr>
<th>Spiked concentration (ng/mL)</th>
<th>Measured concentration (mean ± SD, ng/mL)</th>
<th>Precision (RSD, %)</th>
<th>Accuracy (Relative error, %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intra-day (n = 6)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10.0</td>
<td>10.2 ± 0.3</td>
<td>5.7</td>
<td>2.3</td>
</tr>
<tr>
<td>50.0</td>
<td>50.1 ± 0.7</td>
<td>1.5</td>
<td>0.3</td>
</tr>
<tr>
<td>1,800</td>
<td>1784 ± 96.4</td>
<td>4.8</td>
<td>0.9</td>
</tr>
<tr>
<td>Inter-day (n = 18)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10.0</td>
<td>10.0 ± 0.6</td>
<td>7.0</td>
<td>0.1</td>
</tr>
<tr>
<td>50.0</td>
<td>49.7 ± 0.9</td>
<td>3.6</td>
<td>-0.8</td>
</tr>
<tr>
<td>1,800</td>
<td>1,773 ± 93.1</td>
<td>1.6</td>
<td>-1.5</td>
</tr>
</tbody>
</table>

*Note: Data are expressed in prestudy validation, three days, six replicates per day.

Table II

<table>
<thead>
<tr>
<th>Spiked concentration (ng/mL)</th>
<th>Extraction recoveries (mean ± SD, %)</th>
<th>Matrix effects (mean ± SD, %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TEOA</td>
<td>95.3 ± 5.4</td>
<td>100.5 ± 3.0</td>
</tr>
<tr>
<td>50.0</td>
<td>97.6 ± 1.1</td>
<td>99.6 ± 2.8</td>
</tr>
<tr>
<td>1,800</td>
<td>97.1 ± 4.7</td>
<td>99.4 ± 2.2</td>
</tr>
<tr>
<td>Warfarin</td>
<td>94.7 ± 3.2</td>
<td>102.4 ± 0.2</td>
</tr>
</tbody>
</table>

*Note: Data are mean values ± SD (n = 3).
Extraction recovery
Mean extraction recoveries at three QC concentration levels of the analyte from plasma were 95.3 ± 5.4, 97.6 ± 1.1 and 97.1 ± 4.7%, respectively (Table II, n = 3). The recovery for IS was 94.7 ± 3.2% (n = 3). The results indicated that the method showed high recovery.

Matrix effect
Matrix effects of the analyte at low, medium and high concentration levels were 100.5 ± 3.0, 99.6 ± 2.8 and 99.4 ± 2.2%, respectively. For the IS, the matrix effect was 102.4 ± 0.2%. No significant matrix effect was observed for the analyte.

Stability
The analyte was stable in rat plasma after storage at ambient temperature for 2 h, in the autosampler for 12 h, after storage at −20 °C for eight days and through three freeze–thaw cycles (Table III).

Pharmacokinetic study
The validated assay method was successfully applied to the pharmacokinetic study of the compound of TEOA in rats following oral administration of TEOA (100 mg/kg). The mean plasma concentration–time profile of TEOA is shown in Figure 4 and the primary pharmacokinetic parameters are summarized in Table IV. More pharmacokinetic studies need be conducted in other animal models.

Discussion
To achieve acceptable chromatographic peak shapes and strong MS responses, different compositions of the mobile phase were used to perform several trials. The test results showed that a solvent system containing appropriate acidity could improve the peak shape and permit optimum ionization. Finally, the mobile phase consisting of a 90 volume of methanol and a 10 volume of 0.1% formic acid was chosen to perform the study.

In the preliminary study, both APCI and ESI sources were used for assay development in positive and negative ion modes, respectively. APCI in positive ion mode produced greater sensitivity than ESI. Thus, APCI was adopted for the assay of TEOA.

Based on the authors’ experience, direct protein precipitation with acetonitrile or methanol generally resulted in strong ion suppression (approximately 80% suppression). Due to the relative low polarity of the analyte, liquid–liquid extraction is more effective for preparing a clean sample and intensifying the MS response. Various organic solvents, such as ethyl ether, ethyl acetate and dichloromethane, were chosen to evaluate the recovery and matrix effects. Finally, ethyl acetate was found to be optimal to yield a clean plasma chromatogram and the highest recovery for the analyte and IS.

Conclusion
In summary, a highly specific, sensitive and robust LC–MS-MS method for the rapid quantification of TEOA was developed and validated in rat plasma for the first time. According to the validation procedure, the developed method could be useful with desired precision and accuracy for preclinical studies for the novel anti-tumor candidate drug TEOA. The method was successfully applied to the pharmacokinetic study of TEOA after oral administration of 100 mg/kg TEOA to rats.

### Table III
Stability of TEOA during the Storing and Preparing Procedures

<table>
<thead>
<tr>
<th>Spiked concentration (ng/mL)</th>
<th>Measured concentration (mean ± SD, ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Plasma samples at room temperature for 2 h</td>
</tr>
<tr>
<td>10.0</td>
<td>9.85 ± 5.5</td>
</tr>
<tr>
<td>50.0</td>
<td>50.2 ± 0.8</td>
</tr>
<tr>
<td>1,800</td>
<td>1,816 ± 92.3</td>
</tr>
</tbody>
</table>

Data are mean values ± SD (n = 3).

### Table IV
Pharmacokinetic Parameters of Six Male Wistar Rats at an Oral Dose of 100 mg/kg TEOA

<table>
<thead>
<tr>
<th>Parameters</th>
<th>TEOA Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dose (mg/kg)</td>
<td>100</td>
</tr>
<tr>
<td>C_max (mg/mL)</td>
<td>76.0 ± 25.1</td>
</tr>
<tr>
<td>T_max (h)</td>
<td>2.1 ± 0.5</td>
</tr>
<tr>
<td>AUC (0–t) (µg*h/mL)</td>
<td>365.7 ± 213.2</td>
</tr>
<tr>
<td>MRT (0–t) (h)</td>
<td>4.8 ± 1.9</td>
</tr>
<tr>
<td>t_1/2 (h)</td>
<td>3.0 ± 1.2</td>
</tr>
</tbody>
</table>

*Note: C_max, maximum (peak) plasma concentration; T_max, time to maximum plasma concentration; MRT, mean retention time; AUC, area under curve; t_1/2, half life. Data are mean values ± SD (n = 6).
Acknowledgments

The project was supported by the Clinical Pharmacy Foundation of Shanghai Pharmaceutical Association.

References