Simultaneous Determination of Typhaneoside and Isorhamnetin-3-O-Neohesperidoside in Rats After Oral Administration of Pollen Typhae Extract by UPLC–MS/MS

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Received 10 June 2014; revised 30 August 2014

For the first time, a selective and rapid ultra-performance liquid chromatography method with tandem mass spectrometric (UPLC–MS/MS) detection for simultaneous determination of typhaneoside and isorhamnetin-3-O-neohesperidoside in rat plasma was developed and validated, which was applied to the pharmacokinetic study of Pollen Typhae extract. The separation was carried out on an ACQUITY UPLC™ BEH C18 column with gradient elution using mobile phase including acetonitrile and water (containing 0.1% formic acid). The flow rate was 0.4 mL/min. The detection was conducted by means of electrospray ionization mass spectrometry in negative ion mode with multiple reaction monitoring. The assays were linear over the concentration range of 0.5–100 ng/mL, and the lower limit of quantification was 0.5 ng/mL for typhaneoside and isorhamnetin-3-O-neohesperidoside. The method was validated in terms of intra- and interday precision (<9.37%), accuracy (within ±10.91%), linearity, specificity and stability, and has been successfully applied to a pharmacokinetic study of Pollen Typhae extract in rats after oral administration.

Introduction

Pollen Typhae (named Puhuang in Chinese), which is the pollen of several species of the genus Typha of the family Typhaceae such as T. angustifolia L., T. orientalis Presl., T. latifolia L. and T. minima Presl., plays an important role in the treatment of stranguria, hematuria, dysmenorrheal, metrorrhagia and injuries from falls (1). It was shown that Pollen Typhae possesses various pharmacological activities, such as improving the microcirculation, immunosuppression, raising CAMP levels, lowering cholesterol and anticoagulation (2–4). Little data are available on the pharmacokinetics of simultaneous determination of typhaneoside and isorhamnetin-3-O-neohesperidoside in rat plasma. The method developed in our paper is fast, more suitable for quality control (QC) of Pollen Typhae preparations (12).

In the present study, a rapid, specific and sensitive UPLC–electrospray ionization (ESI)-MS method was developed for simultaneous determination of typhaneoside and isorhamnetin-3-O-neohesperidoside in rat plasma. The method was used to study the pharmacokinetic parameters after oral administration of Pollen Typhae extracts.

Experimental

Chemicals and materials

Typhaneoside (batch no. 111573, purity ≥98.0%), isorhamnetin-3-O-neohesperidoside (batch no. 11571, purity ≥98.0%) and luteolin (IS, batch no. 11520, Figure 1C) were purchased from the National Institute for Food and Drug Control (Beijing, China). Pollen Typhae extract was purchased from Nanjing Zelang Medical Technology Co. (Nanjing, China). Acetonitrile (ACN; HPLC/MS-grade) and methanol (HPLC/MS-grade) were purchased from Fisher (USA). Other reagents were all of analytical grade.

Instrumentation and chromatography

Ultra-performance liquid chromatography was performed on an ACQUITY™ UPLC™ system (Waters Corp., Milford, MA, USA) and an ACQUITY UPLC™ BEH C18 column (100 × 2.1 mm, 1.7 μm) was employed. The column temperature was maintained at 40 °C. Chromatographic separations were achieved with a gradient elution using the mobile phase composed of 0.1% formic acid (HOOCH) in water as solvent A and ACN as solvent B. The gradient conditions of the mobile phase were 0 min 80% A, 1.7 min 68% A, 3.2 min 30% A and 4.0 min 20%A. The flow rate was set at 0.4 mL/min. The injection volume was 3 μL.

Mass spectrometric detection was carried out on a Xevo TQ-S mass spectrometer (Waters Corp.) with an ESI interface. The ESI source was set in negative ionization mode and the quantification mode was multiple reaction monitoring (MRM). The optimized ionization conditions were as follows: capillary voltage, 2.5 kV; cone voltage, 30 V; source temperature, 150°C and desolvation
temperature, 400 °C. Nitrogen was used as desolvation and cone gas with the flow rate of 800 and 150 L/h, respectively. Other parameters were also optimized for maximum sensitivity (Table I).

All data collected were acquired and processed using the MassLynx™ NT 4.0 software with QuanLynx™ program (Waters Corp.).

**Calibration standards and quality control samples**
The separate primary stock solutions of typhaneoside and isorhamnetin-3-O-neohesperidoside were prepared in methanol at concentrations of 20.24, 20.08 and 20.28 µg/mL, respectively, and were kept at −20 °C until analysis. The stock solutions were serially diluted with methanol to provide standard solutions at desired concentrations. Calibration standards of typhaneoside and isorhamnetin-3-O-neohesperidoside at eight concentration levels ranged from 0.506 to 101.2, 0.502 to 100.4 and 0.507 to 101.4 ng/mL were prepared daily by spiking blank plasma with working solutions. QC working solutions were prepared in the same way. Low-, medium- and high-level QC samples were at concentrations of 1, 10 and 80 ng/mL for typhaneoside and isorhamnetin-3-O-neohesperidoside.

**Plasma aliquots preparation**
Aliquots of 0.1 mL of plasma sample and 20 µL of IS working solution (100.08 ng/mL) were put into a 1.5-mL Axygen plastic tube and 10 µL of 30% formic acid was added. The mixture was vortexed for 30 s. Then, 300 µL of ACN was added and the mixture was vortexed for 1 min followed by centrifugation at 12,000 rpm for 10 min at 4 °C. The supernatant was transferred into a clean plastic tube and evaporated to dryness under a gentle nitrogen stream. The residue was reconstituted in 100 µL of methanol solution, vortexed for 1 min and centrifuged at 12,000 rpm for 10 min. After centrifuging, a 3.0-µL aliquot of the solution was injected into the UPLC–MS/MS system.

**Method validation**
The method was validated for selectivity, linearity, accuracy, precision, extract recovery and stability according to the FDA guidelines (13) for validation of bioanalytical methods. Validation runs were conducted on three consecutive days. During routine analysis, each analytical run consisted of a set of calibration standards, a set of QC plasma samples in duplicate at three concentrations and plasma samples to be determined (14).

**Selectivity and matrix effect**
The selectivity of an analytical method is its ability to measure accurately an analyte in the presence of endogenous compounds. Therefore, blank plasma samples obtained from six rats were analyzed according to the sample procedure described above. The corresponding chromatograms were tested for possible interferences at the retention times of typhaneoside, isorhamnetin-3-O-neohesperidoside and the IS. The specificity of the method was investigated by comparing the chromatogram of blank plasma, blank plasma spiked with standard solutions with the samples collected from subjects after administration of Pollen Typhae extracts (15).

Matrix effects on the ionization of analytes were investigated by comparing the peak area of QC replicates at three concentrations in the samples spiked post-extraction (A) with that of analyte standard solutions dried directly and reconstituted with the same volume of initial mobile phase (B). When the peak area ratio of A/B × 100% of the analytes and the internal standard solution was between 85 and 115%, the matrix effect may be considered as negligible (16).

**Linearity and LLOQ**
Calibration curves were prepared by assaying standard plasma samples at eight concentrations of typhaneoside ranging from 0.506 to 101.2 ng/mL, and of isorhamnetin-3-O-neohesperidoside ranging from 0.502 to 100.4 ng/mL. The linearity of each calibration curve was determined by plotting the peak area ratio (y) of typhaneoside /IS and isorhamnetin-3-O-neohesperidoside /IS vs. the nominal concentration (x) of the analytes. The linearity of the calibration curve was evaluated by linear regression analysis and the minimally acceptable correlation coefficient ($r^2$) was ≥0.99. The LLOQ was defined as the lowest concentration on

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**Table I**

Optimized Multiple Reaction Monitoring Parameters for the Detection of Analytes and Internal Standards

<table>
<thead>
<tr>
<th>Compound</th>
<th>Formula/m/z</th>
<th>Parent m/z</th>
<th>Cone voltage</th>
<th>Product ion m/z</th>
<th>Collision energy</th>
<th>Ion mode</th>
</tr>
</thead>
<tbody>
<tr>
<td>Typhaneoside</td>
<td>770.2</td>
<td>769.17</td>
<td>76</td>
<td>314.11</td>
<td>42</td>
<td>ES</td>
</tr>
<tr>
<td>Isorhamnetin-3-O-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>neohesperidoside</td>
<td>624.16</td>
<td>623.19</td>
<td>98</td>
<td>314.12</td>
<td>30</td>
<td>ES</td>
</tr>
<tr>
<td>Luteolin</td>
<td>286</td>
<td>284.97</td>
<td>46</td>
<td>132.90</td>
<td>34</td>
<td>ES</td>
</tr>
</tbody>
</table>

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Figure 1. Chemical structures of typhaneoside (A), isorhamnetin-3-O-neohesperidoside (B) and luteolin (C, IS).
the calibration curve with acceptable precision and accuracy [six replicates with a relative standard deviation (RSD) of <20% and relative error (RE) within ± 20%].

Precision and accuracy
The intraday precision and accuracy were determined by analyzing six QC replicates at three concentrations of 1, 10 and 80 ng/mL for typhaneoside and isorhamnetin-3-O-neohesperidoside on 1 day (n = 6). The interday precision and accuracy were determined by repeating analysis on three consecutive days (n = 6 series per day). The intra- and interday precision and accuracy were separately defined as the RSD and RE (17).

Extraction recovery
The recovery of typhaneoside and isorhamnetin-3-O-neohesperidoside was investigated by comparing the peak areas of extracted

Figure 2. Chromatograms for typhaneoside (Channel 1), isorhamnetin-3-O-neohesperidoside (Channel 2) and IS (Channel 3) in rat plasma samples: (A) a blank plasma sample; (B) a blank plasma sample spiked with analytes; (C) a plasma sample from a rat at 4 h after oral administration of Pollen Typhae extract.
samples spiked with QC working solutions at the three concentration levels (low, medium and high) with those of the samples to which analytes were spiked after the extraction step, which represented 100% recovery.

Stability
Freeze–thaw stability was evaluated after three freeze (−20°C) and thaw (room temperature) cycles before sample preparation. Short-term stability was assessed by analyzing QC plasma samples kept at room temperature for 24 h, which exceeded the routine preparation time of samples. Long-term stability was investigated by assaying QC plasma samples of the three concentration levels after storage at −20°C for 30 days.

Pharmacokinetic study

Animals
Six male Sprague–Dawley rats (weighing 250–300 g) were purchased from Beijing Vital River Laboratory Animal Technology (Beijing, China). They were fasted overnight with access to water before dosing. All animal experiments were approved by the Animal Ethics Committee at the Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences.

Pharmacokinetics
Pollen Typhae extract was given orally as solutions, among which typhaneoside and isorhamnetin-3-O-neohesperidoside were given at a dose of 1.67 and 1.56 mg/kg, respectively. Blood samples (0.5 mL) were collected from the vena orbitalis at 0 (pre-dose), 0.033, 0.083, 0.25, 0.50, 0.75, 1.0, 3.0, 4.0, 8.0 and 10.0 h after administration. The blood samples were immediately centrifuged to separate out plasma and stored at −20°C until analysis.

Data and statistical analysis
All the results were expressed as means ± standard deviation (SD) of six replicates. The pharmacokinetic parameters were analyzed using the Kinetica 4.4 software (Thermo Scientific, USA).

Results

Method validation
Selectivity
Under the optimized UPLC–MS/MS conditions, typhaneoside and isorhamnetin-3-O-neohesperidoside were well separated from interferences in the matrix. Figure 2 shows the chromatographic profiles of blank plasma, blank plasma spiked with typhaneoside, isorhamnetin-3-O-neohesperidoside and IS, and plasma obtained 4 h after oral administration of Pollen Typhae extract. As shown in Figure 2A, the value peak at the retention time of the two compounds was very low, so we can ignore them and the retention times for typhaneoside, isorhamnetin-3-O-neohesperidoside and IS were 1.04, 1.35 and 2.72 min, respectively.

Matrix effects of typhaneoside and isorhamnetin-3-O-neohesperidoside in rat plasma are given in Table II. There was no significant matrix effect on the analytes in this method.

Linearity and LLOQ
Excellent linear relationships and appropriate LLOQ are demonstrated in Table III. The calibration curves were linear in the ranges of 0.506–101.2 ng/mL ($r^2 = 0.999$) for typhaneoside, and 0.502–100.4 ng/mL ($r^2 = 0.997$) for isorhamnetin-3-O-neohesperidoside. Representative regression equations for the calibration curves are $Y = 0.249802X + 0.19483$ and $Y = 0.441999X + 0.352658$ for typhaneoside and isorhamnetin-3-O-neohesperidoside.

Precision and accuracy
Table II demonstrates the precision and accuracy based on the analysis of three batches of QC samples. The intra- and inter-run precisions (RSD) of two analytes were no more than 8.35 and 8.47% for typhaneoside, and 5.85 and 9.37% for isorhamnetin-3-O-neohesperidoside. The RE was 1.44–10.91% for typhaneoside, and −0.26–6.49% for isorhamnetin-3-O-neohesperidoside over the three concentration levels evaluated, indicating acceptable precision and accuracy of the present method.
**Extraction recovery**

The extraction recoveries determined for typhaneoside and isorhamnetin-3-O-neohesperidoside are given in Table IV. At three concentration levels of typhaneoside, the absolute extraction recoveries were 91.93 ± 4.36, 85.94 ± 2.04 and 87.15 ± 3.48%, respectively. The extraction recoveries of isorhamnetin-3-O-neohesperidoside from rat plasma QC samples were 92.83 ± 3.70, 90.44 ± 2.73 and 90.75 ± 2.15% at concentration levels of 1, 10 and 80 ng/mL, respectively.

**Stability**

The stabilities of the analytes in rat plasma sample (n = 6) at different conditions are presented in Table V. The results indicated three analytes in rat plasma samples stable when stored at 20°C for 24 h, −20°C for 30 days and after three freeze–thaw cycles at low, medium and high concentrations, respectively. Therefore, the plasma samples should be processed within 24 h at 20°C or 30 days at −20°C.

**Pharmacokinetic study**

This newly developed method was applied to measure the plasma concentrations of typhaneoside and isorhamnetin-3-O-neohesperidoside in rats following oral administrations of Pollen Typhae extract. The mean plasma concentration–time profiles of the two analytes are illustrated in Figure 3 and its estimated pharmacokinetic parameters are summarized in Table VI. The results indicated that the analytical method is suitable to determine plasma concentrations of the analytes in rat plasma.

Typhaneoside plasma concentration reached a maximum at 0.13 h after administration with an average Cmax of 3.24 ± 2.04 ng/mL. The area under the curve (AUC0–∞) was 7.20 ± 1.20 (ng ng/mL) and mean residence time (MRT) was 3.63 ± 0.89 h. The T1/2 was 2.34 ± 0.75 h.

**Table IV**

<table>
<thead>
<tr>
<th>Analytes</th>
<th>Spiked concentration (ng/mL)</th>
<th>Recovery (%)</th>
<th>RSD (%)</th>
<th>Matix effect (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Typhaneoside</td>
<td>1</td>
<td>91.93 ± 4.36</td>
<td>4.75</td>
<td>85.17 ± 4.46</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>85.94 ± 2.04</td>
<td>2.37</td>
<td>89.12 ± 15.22</td>
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<tr>
<td></td>
<td>80</td>
<td>87.15 ± 3.48</td>
<td>3.99</td>
<td>85.50 ± 1.16</td>
</tr>
<tr>
<td>Isorhamnetin-3-O-neohesperidoside</td>
<td>1</td>
<td>92.83 ± 3.70</td>
<td>4.00</td>
<td>107.97 ± 1.98</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>90.44 ± 2.73</td>
<td>3.06</td>
<td>98.63 ± 4.85</td>
</tr>
<tr>
<td></td>
<td>80</td>
<td>90.75 ± 2.15</td>
<td>2.39</td>
<td>95.30 ± 0.85</td>
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</tbody>
</table>

**Table V**

<table>
<thead>
<tr>
<th>Analytes</th>
<th>Spiked concentration (ng/mL)</th>
<th>Three freeze–thaw cycles</th>
<th>Measured concentration (ng/mL)</th>
<th>RSD (%)</th>
<th>30 days’ storage at −20°C</th>
<th>Measured concentration (ng/mL)</th>
<th>RSD (%)</th>
<th>24 h at room temperature</th>
<th>Measured concentration (ng/mL)</th>
<th>RSD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Typhaneoside</td>
<td>1</td>
<td></td>
<td>0.962 ± 0.076</td>
<td>8.99</td>
<td>0.979 ± 0.986</td>
<td>8.67</td>
<td>0.979 ± 0.030</td>
<td>3.08</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>10</td>
<td></td>
<td>9.93 ± 0.95</td>
<td>9.62</td>
<td>10.4 ± 0.7</td>
<td>7.13</td>
<td>9.85 ± 0.51</td>
<td>5.12</td>
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<tr>
<td></td>
<td>80</td>
<td></td>
<td>80.0 ± 1.0</td>
<td>1.25</td>
<td>80.9 ± 1.7</td>
<td>2.10</td>
<td>80.9 ± 2.5</td>
<td>3.03</td>
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<tr>
<td>Isorhamnetin-3-O-neohesperidoside</td>
<td>1</td>
<td></td>
<td>0.991 ± 0.089</td>
<td>8.95</td>
<td>1.01 ± 0.08</td>
<td>7.75</td>
<td>1.00 ± 0.08</td>
<td>8.20</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>10</td>
<td></td>
<td>10.0 ± 0.2</td>
<td>1.56</td>
<td>9.69 ± 0.28</td>
<td>2.82</td>
<td>10.1 ± 0.8</td>
<td>7.79</td>
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</tr>
<tr>
<td></td>
<td>80</td>
<td></td>
<td>86.5 ± 1.4</td>
<td>1.71</td>
<td>80.1 ± 0.9</td>
<td>1.10</td>
<td>80.5 ± 1.3</td>
<td>1.61</td>
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</table>

**Table VI**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Unit</th>
<th>Typhaneoside</th>
<th>Isorhamnetin-3-O-neohesperidoside</th>
</tr>
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<tbody>
<tr>
<td>Tmax</td>
<td>h</td>
<td>0.3 ± 0.1</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>Cmax</td>
<td>ng/mL</td>
<td>3.2 ± 1.1</td>
<td>175.2 ± 2.5</td>
</tr>
<tr>
<td>AUC0–10</td>
<td>ng h/mL</td>
<td>6.8 ± 1.4</td>
<td>34.4 ± 11.4</td>
</tr>
<tr>
<td>AUC0–∞</td>
<td>ng h/mL</td>
<td>7.2 ± 1.2</td>
<td>36.5 ± 11.7</td>
</tr>
<tr>
<td>T1/2</td>
<td>h</td>
<td>2.3 ± 0.8</td>
<td>2.6 ± 0.6</td>
</tr>
<tr>
<td>MRT</td>
<td>h</td>
<td>3.6 ± 0.9</td>
<td>3.7 ± 1.0</td>
</tr>
</tbody>
</table>

**Discussion**

Typhaneoside and isorhamnetin-3-O-neohesperidoside are considered as two major flavonoids in Pollen Typhae. This is because they not only have higher content than other compounds, but also possess satisfactory effects, such as protecting cells, inhibiting the contractile activity of isolated uteri and analgesic activities. Typhaneoside and isorhamnetin-3-O-neohesperidoside are usually selected as the targets for QC of Pollen Typhae. However, little data were available regarding the pharmacokinetic characterization of typhaneoside and isorhamnetin-3-O-neohesperidoside in rats after oral administration of Pollen Typhae extract. This study is the first UPLC–MS/MS quantitative assay
for investigating the pharmacokinetics of typhaneoside and isorhamnetin-3-O-neohesperidoside in rats. The UPLC separation and MRM detection gave the method high selectivity and consequently high accuracy. There were no interferences from endogenous substances. Compared with earlier published methods on typhaneoside and isorhamnetin-3-O-neohesperidoside (9, 11), this method was found to make an improvement in sensitivity, which was 0.5 ng/mL, 1/20 of the early one. Meanwhile, the injection volume is also lower (3 vs. 10 μL). The improved detection level of the analytes by this method resulted in detection of these analytes till 10 vs. 3 h from earlier method even with a higher dose (5 mg/kg), which is important to characterize elimination phase of these analytes in the pharmacokinetic study. Pharmacokinetic results showed that typhaneoside and isorhamnetin-3-O-neohesperidoside can be detected immediately within 5 min; both the compounds reached a maximum at about 0.3 h, indicated that they may be absorbed rapidly after oral administration, but the elimination of them may also be fast in rats because $T_{1/2}$ was about 2.5 h. These may be useful for the further clinical use and the pharmacological studies of Pollen Typhae.

Conclusions
A novel UPLC–ESI-MS/MS method has been established for simultaneous determination of typhaneoside and isorhamnetin-3-O-neohesperidoside in rat plasma. This method is specific, sensitive and accurate. It has been successfully applied to pharmacokinetic studies of typhaneoside and isorhamnetin-3-O-neohesperidoside in rats following oral administration of Pollen Typhae extract.

Funding
This work was financially supported by the National Natural Science Foundation of China (no. 81173563), the Ministry of National Science and Technique (China, nos 2012ZX09103201-026 and 2009ZX09102-142) and the Innovation Team of Beijing University of Chinese Medicine (no. 2011-CXTD-13).

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