Simultaneous Determination of Seven Components from Hawthorn Leaves Flavonoids in Rat Plasma by LC-MS/MS

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In this study, a simple, sensitive, and throughout liquid chromatography–tandem mass spectrometry (LC–MS/MS) method was developed for the simultaneous determination of seven flavonoid compounds, namely, rutin, vitexin-4′-O-glucoside, vitexin-2′-O-rhamnoside, hyperoside, vitexin, shanyenoside A and quercetin in rat plasma after intravenous administration of hawthorn leaves flavonoids (HLF) using lysionotin as an internal standard (IS). The target compounds were extracted using protein precipitation by methanol. The detection was achieved by LC-MS/MS in multiple reaction monitoring mode. The optimal mass transition ion pairs (m/z) for quantitation were 609.3/300.1 for rutin, 593.1/413.2 for vitexin-4′-O-glucoside, 577.3/413.2 for vitexin-2′-O-rhamnoside, 463.2/300.1 for hyperoside, 431.2/311.2 for vitexin, 407.2/245.1 for shanyenoside A, 301.1/151.1 for quercetin and 343.2/313.1 for the IS, respectively. The method was fully validated with respect to specificity, sensitivity, linearity, precision, accuracy, recovery and stability experiments. A sufficiently sensitive and selective LC-MS/MS method was first developed in this study to simultaneously evaluate the pharmacokinetics of seven flavonoids in rat plasma following intravenous administration of HLF.

Introduction

The leaves of *Crataegus pinnatifida* listed in the Chinese Pharmacopoeia are a well-known traditional Chinese medicine for the treatment of qi-stagnancy and blood stasis, chest distress, palpitation, dizziness and hyperlipidemia (1). Hawthorn leaves flavonoids (HLF) are the major bioactive constituents of hawthorn leaves and include many kinds of flavonoids, e.g. rutin, vitexin-4′-O-glucoside, vitexin-2′-O-rhamnoside, hyperoside, vitexin, shanyenoside A and quercetin, etc. (2, 3). There are some published reports of simultaneous quantitations of two or four flavonoid constituents including rutin, vitexin, vitexin-4′-O-glucoside and vitexin-2′-O-rhamnoside in biological fluids. A liquid chromatography–ultraviolet detection method has been employed to investigate the pharmacokinetics of vitexin-4′-O-glucoside and vitexin-2′-O-rhamnoside in rat plasma (4). Unfortunately, the limit of quantifications were 230 and 360 ng/ml for vitexin-4′-O-glucoside and vitexin-2′-O-rhamnoside, respectively, which are not sensitive enough for the pharmacokinetic study. Another liquid chromatography–tandem mass spectrometry (LC–MS/MS) method for the determination of rutin, vitexin, vitexin-4′-O-glucoside and vitexin-2′-O-rhamnoside in rat plasma has been developed (5). But the other three main components including hyperoside, shanyenoside A and quercetin have not been assayed from the leaves of *C. pinnatifida* in biological samples by published methods.

In this study, a simple, sensitive, and throughout LC-MS/MS method was first developed and validated for the simultaneous determination of seven flavonoids in rat plasma and further evaluate their pharmacokinetics after intravenous administration of HLF.

Experimental

**Materials and reagents**

Reference standards of rutin, vitexin-4′-O-glucoside, vitexin-2′-O-rhamnoside, hyperoside, vitexin, shanyenoside A and quercetin with purity >98% were isolated and purified in our laboratory and confirmed by comparing the spectroscopic (1H NMR, 13C NMR and MS) data with reported references (6, 7). Lysionotin (internal standard, purity >98%) was purchased from the National Institute for Food and Drug Control (Beijing, China). HPLC-grade methanol and acetonitrile were purchased from Honeywell Burdick & Jackson (Ulsan, Korea), and purified water obtained by means of a Millipore Milli-Q system (Bedford, USA). All the other reagents were of analytical grade. A quantity of HLF was dissolved in 0.9% normal saline containing 10% propylene glycol and then filtered through a 0.2-µm microspore filter. The contents of rutin, vitexin-4′-O-glucoside, vitexin-2′-O-rhamnoside, hyperoside, vitexin, shanyenoside A and quercetin in the resulting HLF solution were 0.19, 7.33, 9.86, 0.16, 0.25, 0.11, and 0.03%, respectively.

**Instruments and analytical conditions**

The LC system consisted of an Agilent Technologies Series 1200 system (Palo Alto, CA, USA) equipped with a degasser, a binary pump, an autosampler and a column compartment. Chromatographic separation was conducted on a ZORBAX SB-C18 column (150 mm × 4.6 mm, i.d., 5 µm; Agilent Technologies; Palo Alto, CA, USA) with a Security-Guard C18 column (4.6 mm × 3 mm, i.d., 5 µm; Phenomenex, Torrance, CA, USA). The mobile phase consisted of acetonitrile (A) and water (B), and the flow rate was kept at 0.8 mL/min, split ratio 50:50 (v/v), 50% to drain and 50% to MS/MS. The gradient conditions of the mobile phase were as follows: 0–2 min, 20% A; 2–6 min, 20–70% A; 6–6.2 min, 70–20% A and then returned to the initial condition. An injector rinse solvent consisting of acetonitrile–water (50:50, v/v) was used.

Mass spectrometric detection was carried out on an Agilent Technologies 6410 triple quadrupole tandem mass spectrometer.
(Palo Alto, CA, USA) with an electrospray ionization (ESI) interface. The parameters of the mass spectrometer were optimized and set as follows: capillary voltage at 4.0 kV, nebulizer pressure at 35 psi and drying gas flow rate at 10 L/min at 300°C. The quantification was performed using multiple reaction monitoring (MRM) of precursor–product ion transitions at \( m/z \) 609.3/300.1 for rutin, \( m/z \) 593.1/413.2 for vitexin-4"-O-glucoside, \( m/z \) 577.3/413.2 for vitexin-2"-O-rhamnoside, \( m/z \) 463.2/300.1 for

![Figure 1. MS/MS spectra of rutin (A), vitexin-4"-O-glucoside (B), vitexin-2"-O-rhamnoside (C), hyperoside (D), vitexin (E), shanyenoside A (F), quercetin (G) and IS (H).](image-url)
hyperoside, m/z 431.2/311.2 for vitexin, m/z 407.2/245.1 for shanyenoside A, m/z 301.1/151.1 for quercetin and m/z 343.2/313.1 for the IS, respectively (Figure 1). The fragmentors and collision energy were set at 155 V, 20 eV for rutin; 150 V, 18 eV for vitexin-4'-O-glucoside; 135 V, 15 eV for vitexin-2"-O-rhamnoside; 135 V, 12 eV for hyperoside; 125 V, 10 eV for vitexin; 140 V, 15 eV for shanyenoside A; 110 V, 12 eV for quercetin and 135 V, 18 eV for the IS, respectively. The run time was 9.0 min, and analytes were eluted at about 4.6–8.2 min.

Preparation of calibration standards and quality control samples
The mixture stock solution and IS working solution were serially diluted with methanol to provide working standard solutions of desired concentrations. The working solutions of standard mixture (10 μL) were spiked into 190 μL of rat blank plasma to obtain final concentrations in the range of 10–800 ng/mL for rutin, 10–9,000 ng/mL for vitexin-4'-O-glucoside or vitexin-2"-O-rhamnoside, 5–300 ng/mL for hyperoside, 8–400 ng/mL for vitexin, 6–200 ng/mL for shanyenoside A and 5–400 ng/mL for quercetin, respectively. The IS working solution (250 ng/mL) was prepared by diluting the IS stock solution with methanol. All solutions were stored at -20°C before analysis.

Quality control (QC) samples at low, medium and high concentrations were 20, 120, 720 ng/mL for rutin; 30, 360, 8100 ng/mL for vitexin-4'-O-glucoside or vitexin-2"-O-rhamnoside; 10, 60, 270 ng/mL for hyperoside; 20, 96, 360 ng/mL for vitexin; 12, 60, 180 ng/mL for shanyenoside A and 10, 60, 360 ng/mL for quercetin, respectively.

Sample preparation
Before analysis, the plasma sample was thawed to room temperature. In a 1.5-mL centrifuge tube, an aliquot of 50 μL of plasma
sample were added with 50 μL of the IS working solution (250 ng/mL, lysionotin) and 100 μL of methanol. The tube was vortex mixed for 1.0 min and centrifuged at 12,000 rpm for 10 min. The supernatant (5 μL) was injected into the LC-MS/MS system for analysis.

**Method validation**

The method validation procedure was performed according to the FDA guidance for bioanalytical method validation (8).

**Specificity**

The specificity of the method was established by comparing chromatograms of six different batches of blank plasma obtained from six rats with those of corresponding standard plasma samples spiked with analytes and IS.

**Calibration curves and sensitivity**

The calibration curves were constructed by plotting the peak area ratios of the analytes versus the IS against the concentrations of the each standard and weight coefficient was $1/x^2$. The lower limit of quantification (LLOQ) was determined as the lowest concentration with a signal-to-noise (S/N) ratio of 10.

**Recovery**

The extraction recoveries for seven analytes were determined by comparing the mean peak areas of six extracted low, medium and high QC samples to mean peak areas of six spike-after-extract samples at the same concentrations.

**Precision and accuracy**

The intraday and interday precision and accuracy were investigated through assaying three concentration levels of QC samples...
(n = 6) on the same day and on three consecutive validation days, respectively.

**Stability**

All stability was determined employing QC samples at three different levels. Evaluation of the freeze–thaw stability was tested after three freeze–thaw cycles (−20 to 20°C). Long-term stability was carried out in plasma stored at −20°C for 3 weeks. Post-preparation stability was studied by determination of the QC plasma samples stored in the autosampler (4°C) for 12 h.

**Pharmacokinetic study**

Male Wistar rats (200–220 g) were obtained from the Animal Center of Jilin University (Jilin, China) for the pharmacokinetic study. All experimental procedures and protocols (number 20140617R03) were approved by the Animal Care and Use Committee of Jilin University and were in accordance with the Guide for the Care and Use of Laboratory Animals. Food was prohibited for 12 h before the experiments but water was freely available. Blood samples (approximately 0.3 mL) were collected from the orbital vein into heparinized 1.5 mL polythene tubes at 0, 0.083, 0.17, 0.33, 0.67, 1, 2, 3, 4, 6, 8 and 12 h after intravenous administration of HLF solution (35 mg/kg) at a dose of 10 mL/kg. The samples were immediately centrifuged at 3000 rpm for 10 min. The obtained plasma was stored at −20°C until analysis. Plasma concentrations of all analytes versus time data for each rat were analyzed by DAS 2.0 Software (China State Food and Drug Administration, Beijing, China).

**Results**

**Method validation**

**Specificity and selectivity**

Figure 2 showed the typical chromatograms of blank plasma, and spiked plasma sample with analytes and the IS. No significant interference was observed from endogenous substances in drug-free rat plasma at their retention times of analytes.

**Linearity and sensitivity**

All correlation coefficients of calibration curves for the eight analytes were higher than 0.9950. The LLOQs of rutin, vitexin-4'-O-glucoside, vitexin-2'-O-rhamnoside, hyperoside, vitexin, shanyenoside A and quercetin were 10, 10, 10, 5, 8, 6 and 5 ng/mL, respectively. It was indicated that this method was sufficient for the pharmacokinetic study.

**Table III**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Rutin</th>
<th>Vitexin-4'-O-glucoside</th>
<th>Vitexin-2'-O-rhamnoside</th>
<th>Hyperoside</th>
<th>Vitexin</th>
<th>Shanyenoside A</th>
<th>Quercetin</th>
</tr>
</thead>
<tbody>
<tr>
<td>t_{1/2a} (h)</td>
<td>0.09 ± 0.02</td>
<td>0.09 ± 0.05</td>
<td>0.05 ± 0.01</td>
<td>0.09 ± 0.01</td>
<td>0.10 ± 0.02</td>
<td>0.11 ± 0.02</td>
<td>0.02 ± 0.02</td>
</tr>
<tr>
<td>t_{1/2b} (h)</td>
<td>1.04 ± 0.57</td>
<td>1.07 ± 0.08</td>
<td>0.84 ± 0.15</td>
<td>0.76 ± 0.24</td>
<td>1.29 ± 0.49</td>
<td>1.20 ± 0.28</td>
<td>0.27 ± 0.08</td>
</tr>
<tr>
<td>AUC_{0-4h} (µg·h/L)</td>
<td>206.14 ± 64.94</td>
<td>25099.07 ± 736.84</td>
<td>1940512 ± 130849</td>
<td>55.58 ± 4.47</td>
<td>561.64 ± 66.01</td>
<td>117.75 ± 9.28</td>
<td>25.72 ± 3.71</td>
</tr>
<tr>
<td>AUC_{0-∞} (µg·h/L)</td>
<td>301.16 ± 72.30</td>
<td>26171.24 ± 761.44</td>
<td>20413.80 ± 1555.78</td>
<td>66.21 ± 4.86</td>
<td>63942 ± 76.37</td>
<td>136.28 ± 8.10</td>
<td>32.08 ± 6.43</td>
</tr>
<tr>
<td>MRT_{0-4h} (h)</td>
<td>0.91 ± 0.39</td>
<td>1.42 ± 0.08</td>
<td>1.11 ± 0.07</td>
<td>0.31 ± 0.09</td>
<td>0.82 ± 0.19</td>
<td>1.01 ± 0.15</td>
<td>0.25 ± 0.04</td>
</tr>
<tr>
<td>MRT_{0-∞} (h)</td>
<td>1.39 ± 0.89</td>
<td>1.44 ± 0.08</td>
<td>1.12 ± 0.07</td>
<td>0.35 ± 0.08</td>
<td>1.05 ± 0.27</td>
<td>1.48 ± 0.25</td>
<td>0.37 ± 0.06</td>
</tr>
<tr>
<td>CL (L/h/kg)</td>
<td>0.27 ± 0.06</td>
<td>0.11 ± 0.03</td>
<td>0.19 ± 0.01</td>
<td>0.99 ± 0.07</td>
<td>0.16 ± 0.02</td>
<td>0.34 ± 0.02</td>
<td>0.36 ± 0.07</td>
</tr>
<tr>
<td>V (L/kg)</td>
<td>0.10 ± 0.01</td>
<td>0.09 ± 0.02</td>
<td>0.11 ± 0.01</td>
<td>0.27 ± 0.04</td>
<td>0.05 ± 0.01</td>
<td>0.24 ± 0.02</td>
<td>0.04 ± 0.03</td>
</tr>
</tbody>
</table>

t_{1/2a}, half-life of rapid distribution phase; t_{1/2b}, half-life of slow distribution phase; AUC, area under the plasma concentration versus time curve; MRT, mean residence time; CL, total body clearance; V, apparent volumes of distribution of the central compartments.
Recovery
The extraction recovery at all QC levels was more than 85% for analytes and IS (data not shown). The data suggested that extraction recovery was consistent and reproducible.

Precision and accuracy
The data for intraday and interday precision and accuracy are listed in Table I. The intraday and interday precision expressed by relative standard deviation (RSD) for all analytes were all within 14.2%, while the accuracy was all within 87.8–112.2% of the actual values at each QC level. The results indicated that the present assay has acceptable accuracy and precision.

Stability
Freeze/thaw, long-term and post-preparation stability data proved that all analytes are stable in rat plasma under three freeze/thaw cycles (−20 to 20°C), at −20°C for 3 weeks, and in the autosampler (4°C) for 12 h (Table II).

Pharmacokinetic study
Following validation, the method was successfully applied to the pharmacokinetic study of the HLF in rats. The mean plasma concentration–time profiles of the seven flavonoid compounds after intravenous administration of HLF are shown in Figure 3. The main pharmacokinetic parameters are listed in Table III. Applying the method, the drug concentration in plasma should be detected until 12 h after administration. After administration of a single dose of 40 mg/kg HLF in rats, these pharmacokinetic data indicated in this study were comparable with previous reports (4, 5, 9), and strongly suggest that our method is useful for clinical pharmacokinetic analysis.

Discussion
To optimize the MS parameters, analyte solutions were directly injected into the ESI source of the mass spectrometer by infusing via a syringe pump. In negative-ion mode, all analytes and the IS gave deprotonated molecular ions, [M–H]−, as the major species for the MRM detection. The mass parameters were optimized based on the maximal intensity of the product ions. In order to obtain maximum sensitivity, parameters such as capillary voltage, source temperature, drying gas flow rate, fragmentor and collision energy were optimized.

Acetonitrile, rather than methanol, can produce shorter chromatographic times, good resolution and acceptable peak shapes for all analytes when acetonitrile was used as the organic mobile phase. The above-described ZORBAX SB-C18 column (150 mm × 4.6 mm, i.d., 5 μm) was proved to be optimal under a gradient elution condition.

Conclusion
In conclusion, for the first time, a sensitive and throughout LC-MS/MS method was developed for the simultaneous determination of seven flavonoid compounds in rat plasma after intravenous administration of HLF. The method showed high sensitivity, linearity, precision, accuracy, recovery and stability. This method will be useful for the determination of seven flavonoids in human in clinical application.

References