Simultaneous determination of baicalin, wogonoside, baicalein, wogonin, oroxylin A and chrysin of *Radix scutellariae* extract in rat plasma by liquid chromatography tandem mass spectrometry

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**A B S T R A C T**

A liquid chromatography tandem mass spectrometry (LC–MS/MS) method was developed for the simultaneous determination of baicalin, wogonoside, baicalein, wogonin, oroxylin A and chrysin in rat plasma, using naringin as an internal standard. After acidifying with HCl, plasma samples were pretreated by liquid–liquid extraction with acetone. Chromatographic separation was accomplished on a Hypersil Gold-C\(_{18}\) analytical column (2.1 × 150 mm, 5\(\mu\)m) utilizing a gradient elution profile and a mobile phase consisting of (A) 0.1% formic acid in water and (B) acetonitrile. Detection was performed by multiple reaction monitoring (MRM) mode using electrospray ionization in the positive ion mode. All analytes showed good linearity over the investigated concentration range (\(r > 0.9900\)). The lower limit of quantification was 0.5 ng/ml for baicalin, wogonoside, wogonin and oroxylin A, and 1.0 ng/ml for baicalein and chrysin. Intra-day and inter-day precisions (%RSD) were less than 15% and accuracy (%RE) ranged from −6.7% to 5.8%. The validated method was successfully applied to investigate the pharmacokinetics of the major flavonoids of *Radix scutellariae* extract after oral administration to rats.

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**1. Introduction**

*Radix scutellariae* (Huangqin), the dried roots of *Scutellaria baicalensis* Georgi, is a well-known traditional Chinese medicine (TCM) used for the treatment of inflammation, pyrexia, jaundice, diarrhea and hepatitis, etc. [1–3]. Flavonoids are considered to be the main active constituents of *R. scutellariae*. Baicalin, wogonoside, baicalein, wogonin, oroxylin A and chrysin (structures in Fig. 1), as the major flavonoid glycosides and aglycones had been investigated intensively, were found to exert anti-inflammatory, antioxidant, anti-hepatitis B virus, anti-tumor, anti-allergic and anti-lytic properties [4–8].

Up to now, several analytical methods, including HPLC–UV [9,10], HPLC–ECD [11], UPLC–MS [12], LC–MS [13] and LC–MS/MS [14–16], have been developed for the determination of baicalin, wogonoside, baicalein, wogonin and oroxylin A in biological samples. However, as far as we are aware, no paper was reported on the pharmacokinetic studies of baicalin, wogonoside, baicalein, wogonin, oroxylin A and chrysin simultaneously. Little information is available related to the analytical method or pharmacokinetic profile of chrysin. Walle et al. [17] has investigated the disposition and metabolism of chrysin by HPLC–UV, but the research only aimed directly at administration of single substance and the representative chromatogram is not provided. Since chrysin is pharmacologically active in *R. scutellariae*, it was deemed necessary to develop a method for the analysis of this flavonoid in plasma.

In this study, a rapid and sensitive liquid chromatography–tandem mass spectrometry (LC–MS/MS) method for simultaneous quantification of baicalin, wogonoside, baicalein, wogonin, oroxylin A and chrysin in rat plasma was developed. The method has been successfully applied to a pharmacokinetic study of *R. scutellariae* extract in rats.

**2. Experimental**

**2.1. Chemicals and materials**

Baicalin, baicalein, wogonin, chrysin and naringin (I.S.) (purity > 98%) were received from the National Institute for Drug Control of China (Beijing, China). Wogonoside and oroxylin A (purity > 98%) were purchased from Delta Co. (Anhui, China). Acetonitrile, acetonitrile and formic acid of HPLC grade were obtained from Tedia Co. (Fairfield, USA), and other chemicals were of analytical...
Fig. 1. Product ion mass spectra of [M+H]+ ions of (A) baicalin, (B) wogonoside, (C) baicalein, (D) wogonin, (E) oroxylin A, (F) chrysin and (G) naringenin (I.S.) in positive mode.

2.2. Apparatus and LC–MS/MS conditions

The LC–MS/MS system was composed of a Surveyor™ HPLC system and a TSQ Quantum triple quadrupole mass spectrometer equipped with an electrospray ionization (ESI) source (Thermo Finnigan, USA). Data acquisition was performed with Xcalibur 1.3 software.

grade. Deionized water was prepared by Milli-Q system (Millipore, MA, USA).

The dried powder of R. scutellariae extract was supplied by Tianjin Tasly Pharmaceutical Co. Ltd (Tianjin, China). For oral administration, the concentrations of baicalin, wogonoside, baicalein, wogonin, oroxylin A and chrysin were determined by HPLC–UV described in this paper.
Chromatographic separation was achieved on a Hypersil Gold-C_{18} column (150 × 2.1 mm i.d., 5 μm, Agilent, USA) protected by Luna C_{18} guard column (5 μm, Phenomenex, USA) at room temperature. The mobile phase consisted of 0.1% formic acid in water (A) and acetonitrile (B) using a gradient elution of 30–30% B at 0–3.0 min; 30–60% B at 3.0–3.5 min; 60–60% B at 3.5–9.0 min; 60–30% B at 9.0–12.0 min. The flow rate was 0.3 ml/min, and the injection volume was 20 μl.

Mass spectrometer was operated in the positive mode. Quantification was obtained using multiple reaction monitoring (MRM) mode at m/z transitions of 447 → 271 for baicalin, 461 → 285 for wogonoside, 271 → 123 for baicalin, 285 → 270 for wogonin and oxoroxin A, 255 → 153 for chrysin and 273 → 153 for naringin (I.S.), respectively (Fig.1). The standard solution (1 μg/ml) of each analyte and I.S. was used to optimize the MS/MS operating conditions by direct infusion. The MS parameters were as follows: spray voltage, 4.0 kV; heated capillary temperature, 350 °C; sheath gas (nitrogen), 45 psi; auxiliary gas (nitrogen): 15 psi; collision gas (argon) pressure, 1.5 mTorr; collision energy, 25 eV for baicalin, wogonin, oxoroxin A and naringin (I.S.), 20 eV for wogonoside and 35 eV for baicalin and chrysin, respectively.

2.3. Preparation of standard and quality control samples

Stock solutions were prepared by dissolving the reference standards (1.0 mg/ml for baicalin; 100 μg/ml for wogonoside, baicalen, wogonin, oxoroxin A and chrysin) and I.S. (100 μg/ml for naringin) in methanol. A series of standard mixture working solutions were obtained freshly by further diluting the stock solutions in methanol. The I.S. solution (100 ng/ml) was obtained by diluting the stock solution in methanol. All solutions were stored at 4 °C.

Calibration standards were prepared by spiking appropriate amount of the standard solutions in 50 μl blank plasma to yield final concentrations of 100, 300, 1000, 3000, 10000, 20000, 50000 ng/ml for baicalin; 4, 12, 40, 120, 400, 800, 2000 ng/ml for wogonoside; 6, 18, 60, 180, 600, 1200, 3000 ng/ml for baicalen; 10, 30, 100, 300, 1000, 2000, 5000 ng/ml for wogonin; 1, 3, 10, 30, 100, 200, 500 ng/ml for oxoroxin A and 1, 3, 10, 30, 100, 200, 500 ng/ml for chrysin, respectively. Quality control (QC) samples were prepared containing baicalin (300, 3000, 40000 ng/ml), wogonoside (12, 120, 1600 ng/ml), baicalen (18, 180, 2400 ng/ml), wogonin (30, 300, 4000 ng/ml), oxoroxin A (3, 30, 400 ng/ml) and chrysin (3, 30, 400 ng/ml) in the same manner. The spiked samples were processed according to the following section.

2.4. Sample preparation

The plasma (50 μl) was spiked with 50 μl of I.S. solution (100 ng/ml), 50 μl of 0.5 M hydrochloric acid in methanol solution. The mixture was extracted with 600 μl of acetonitrile by vortex-mixing for 5 min. After centrifugation at 4000 × g for 5 min, the organic layer was pipette-transferred and evaporated to dryness in vacuo at 40 °C. The residue was dissolved with 100 μl of 30% acetonitrile by sonication and centrifuged. The aliquots of 20 μl was injected into the LC–MS/MS.

2.5. Method validation

Selectivity was tested by comparison of blank plasma from six individual rats with corresponding spiked plasma samples.

Calibration curves were constructed from the peak-area ratios of each analyte to IS versus plasma concentrations using a 1/x^2 weighted linear least-squares regression model. The lower limit of quantification (LLOQ) was defined as the final concentration producing a signal-to-noise (S/N) ratio larger than 10.

Intra-day and inter-day precisions were evaluated by evaluated measured results of QC samples at low, medium and high concentrations. Accuracy was determined as the percentage deviation of the mean detected concentrations from the nominal concentrations.

Extraction recoveries were determined by comparing the peak area obtained from plasma samples spiked before extraction with those from plasma samples spiked after extraction. The matrix effect was investigated by comparing the absolute peak area of control plasma extracted and then spiked with a known amount of drug to that of neat standard samples at equivalent concentrations. The same procedure was performed for I.S.

Stability of QC samples was assessed by analyzing samples stored at −20 °C for 1 month, subjected to three freeze (−20 °C) to thaw (room temperature) cycles, and stored at room temperature for 4 h. Samples were considered stable with the deviation from the nominal concentration within ±15.0%.

2.6. Application of the method and pharmacokinetic study

Six male Wistar rats, weighing 200 ± 20 g, were supplied by Vital River Lab Animal Technology Co., Ltd (Beijing, China). The rats were kept under controlled environmental conditions (temperature 22 ± 2 °C; humidity 50 ± 10%) with free access to the standard laboratory food and water. After fasted for 12 h, the rats were administrated R. scutellariae extract (in which the concentrations of baicalin, wogonoside, baicalen, wogonin, oxoroxin A and chrysin were 21.1, 1.88, 9.76, 8.22, 0.62 and 0.23 mg/ml) at an oral dose of 8 ml/kg. Blood samples (200 μl) were collected from the abdominal vein prior to dosage (0 min) and at 0.083, 0.167, 0.33, 0.67, 1.0, 2.0, 4.0, 6.0, 8.0, 10.0, 12.0, 24.0 and 30.0 h thereafter. Following centrifugation (4000 × g for 10 min), plasma samples were stored at −20 °C until analysis. All pharmacokinetic parameters were processed by noncompartmental analysis using DAS 2.0 software (Chinese Pharmacological Society).

3. Results and discussion

3.1. Method development

According to the direct full-scan ESI mass spectra, the ion intensities of baicalin, wogonoside, baicalen, wogonin, oxoroxin A, chrysin and I.S. obtained in positive mode were much stronger than those in negative mode. Product ion mass spectra of the six major flavonoids of R. scutellariae extract and I.S. are shown in Fig. 1. Baicalin and wogonoside are flavonoid glucuronides and their fragments were the result of the protonated ion [M+H]^+ losing its glucuronic acid moiety. For baicalen, the trihydroxyphenyl moiety at m/z 123 was the major product ion from the protonated ion [M+H]^+. Wogonin and oxoroxin A are isomers with identical product ion at m/z 270, which corresponded to the loss of methyl group from the precursor ion [M+H]^+. For chrysin, the Retro-Diels-Alder (RDA) fragmentation was observed to be the most abundant. In addition, the fragment of naringin (I.S.) was due to the loss of glycon of the protonated ion [M+H]^+. The MRM transitions at 447→271, 461→285, 271→123, 285→270, 285→270, 255→153 and 273→153 were selected to analyze baicalin, wogonoside, baicalen, wogonin, oxoroxin A, chrysin and naringin (I.S.), respectively.

As mentioned, wogonin and oxoroxin A have identical precursor and product ions, and the ionization of baicalin produced baicalen ion as well as [M+H]^+. Therefore, full chromatographic separation was critical to avoid any possible isobaric interferences. Different mobile phases were investigated to optimize analytical performance, and acetonitrile was found to improve the resolution of
wogonin and oroxylin A. The addition of formic acid was proved to enhance the sensitivity and to get better peak shape. Representative MRM chromatograms for each analyte are shown in Fig. 2. Satisfactory recoveries were achieved by virtue of liquid–liquid extraction with acetone. Due to the stability of baicalin and baicalein [18], plasma acidification with HCl before extraction could help enhance the extraction efficiency.

3.2. Method validation

3.2.1. Specificity

The detection of six flavonoids and I.S. by MRM was highly selective with no interference. Typical chromatograms are shown in Fig. 2. The retention times of baicalin, wogonoside, naringin (I.S.), baicalein, wogonin, chrysin and oroxylin A were 2.39, 4.08, 7.24, 8.11, 9.48, 9.57 and 9.83 min, respectively.

3.2.2. Linearity and LLOQs

The linear ranges, regression equations, LLOQs, and correlation coefficients of all analytes are shown in Table 1. The correlation coefficients (r) of typical calibration curves were all higher than 0.9947, which exhibited good linearity. The LLOQs for baicalin, wogonoside, baicalein, wogonin, oroxylin A and chrysin were 0.5, 0.5, 1.0, 0.5, 0.5 and 1.0 ng/ml with coefficient of variation 11.7%, 10.3%, 9.16%, 12.3%, 9.19% and 12.1%, respectively.
Table 1
Regression data and LLOQs of the multi-components determined.

<table>
<thead>
<tr>
<th>Components</th>
<th>Linear range (ng/ml)</th>
<th>Linear regression equation</th>
<th>Correlation coefficient (r)</th>
<th>LLOQ (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baicalin</td>
<td>100–50 000</td>
<td>y = 0.0413x + 0.8713</td>
<td>0.9970</td>
<td>0.5</td>
</tr>
<tr>
<td>Wogonoside</td>
<td>4–2000</td>
<td>y = 0.2152x + 0.1782</td>
<td>0.9961</td>
<td>0.5</td>
</tr>
<tr>
<td>Baicalein</td>
<td>6–3000</td>
<td>y = 0.0312x + 0.0594</td>
<td>0.9947</td>
<td>1.0</td>
</tr>
<tr>
<td>Wogonin</td>
<td>10–5000</td>
<td>y = 0.1172x + 0.3065</td>
<td>0.9964</td>
<td>0.5</td>
</tr>
<tr>
<td>Oroxylin A</td>
<td>1–500</td>
<td>y = 0.2920x + 0.0726</td>
<td>0.9970</td>
<td>0.5</td>
</tr>
<tr>
<td>Chrysin</td>
<td>1–500</td>
<td>y = 0.0077x – 0.0029</td>
<td>0.9955</td>
<td>1.0</td>
</tr>
</tbody>
</table>

Table 2
Precision, accuracy and extraction recoveries of baicalin, wogonoside, baicalein, wogonin, oroxylin A and chrysin for LC–MS/MS method.

<table>
<thead>
<tr>
<th>Components</th>
<th>Spiked (ng/ml)</th>
<th>Intra-day concentration measured (ng/ml)</th>
<th>Precision (%, RSD)</th>
<th>Accuracy (%, RE)</th>
<th>Inter-day concentration measured (ng/ml)</th>
<th>Precision (%, RSD)</th>
<th>Accuracy (%, RE)</th>
<th>Extraction recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baicalin</td>
<td>300</td>
<td>295.2 ± 26.9</td>
<td>9</td>
<td>−1.6</td>
<td>292.0 ± 25.5</td>
<td>4.9</td>
<td>−2.7</td>
<td>69.7 ± 5.6</td>
</tr>
<tr>
<td></td>
<td>3000</td>
<td>3021 ± 128</td>
<td>4.2</td>
<td>0.69</td>
<td>3000 ± 224</td>
<td>8.0</td>
<td>0.015</td>
<td>75.5 ± 3.1</td>
</tr>
<tr>
<td></td>
<td>40 000</td>
<td>37463 ± 1224</td>
<td>3.3</td>
<td>−6.3</td>
<td>38800 ± 2299</td>
<td>7.5</td>
<td>−3.0</td>
<td>71.2 ± 3.7</td>
</tr>
<tr>
<td>Wogonoside</td>
<td>12</td>
<td>12.21 ± 1.01</td>
<td>8.2</td>
<td>1.8</td>
<td>12.32 ± 0.29</td>
<td>2.0</td>
<td>2.7</td>
<td>70.9 ± 4.6</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>124.4 ± 6.5</td>
<td>5.2</td>
<td>3.7</td>
<td>121.3 ± 7.8</td>
<td>8.4</td>
<td>1.1</td>
<td>78.1 ± 3.2</td>
</tr>
<tr>
<td></td>
<td>1600</td>
<td>1527 ± 44</td>
<td>2.9</td>
<td>−4.6</td>
<td>154.6 ± 66</td>
<td>4.3</td>
<td>−3.3</td>
<td>77.8 ± 4.5</td>
</tr>
<tr>
<td>Baicalein</td>
<td>18</td>
<td>17.19 ± 1.55</td>
<td>9</td>
<td>−4.5</td>
<td>17.26 ± 1.38</td>
<td>5.2</td>
<td>−4.1</td>
<td>78.6 ± 6.1</td>
</tr>
<tr>
<td></td>
<td>180</td>
<td>172.1 ± 10.6</td>
<td>6.2</td>
<td>−4.4</td>
<td>175.6 ± 12.7</td>
<td>13</td>
<td>−2.5</td>
<td>81.3 ± 2.4</td>
</tr>
<tr>
<td></td>
<td>2400</td>
<td>2298 ± 114</td>
<td>5.0</td>
<td>−4.2</td>
<td>2422 ± 152</td>
<td>12</td>
<td>1.0</td>
<td>80.1 ± 3.2</td>
</tr>
<tr>
<td>Wogonin</td>
<td>30</td>
<td>29.97 ± 2.53</td>
<td>8.4</td>
<td>−0.091</td>
<td>30.30 ± 2.60</td>
<td>3.7</td>
<td>1.0</td>
<td>90.8 ± 1.9</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>300.7 ± 21.2</td>
<td>7.0</td>
<td>0.23</td>
<td>299.6 ± 17.5</td>
<td>6.2</td>
<td>−0.14</td>
<td>93.1 ± 2.4</td>
</tr>
<tr>
<td></td>
<td>4000</td>
<td>3731 ± 179</td>
<td>4.8</td>
<td>−6.7</td>
<td>3793 ± 183</td>
<td>3.6</td>
<td>4.8</td>
<td>91.2 ± 3.8</td>
</tr>
<tr>
<td>Oronixin A</td>
<td>3</td>
<td>3.040 ± 0.401</td>
<td>13</td>
<td>1.3</td>
<td>3.173 ± 0.349</td>
<td>10</td>
<td>5.8</td>
<td>85.2 ± 3.9</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>31.18 ± 3.19</td>
<td>10</td>
<td>4.0</td>
<td>31.18 ± 2.84</td>
<td>9</td>
<td>3.9</td>
<td>94.3 ± 4.2</td>
</tr>
<tr>
<td></td>
<td>400</td>
<td>407.4 ± 32.3</td>
<td>7.9</td>
<td>1.8</td>
<td>408.0 ± 33.9</td>
<td>8.8</td>
<td>2.0</td>
<td>90.2 ± 2.8</td>
</tr>
<tr>
<td>Chrysin</td>
<td>3</td>
<td>2.861 ± 0.315</td>
<td>11</td>
<td>−4.6</td>
<td>2.996 ± 0.287</td>
<td>10</td>
<td>−0.13</td>
<td>80.2 ± 4.9</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>25.08 ± 2.80</td>
<td>10</td>
<td>−3.1</td>
<td>29.94 ± 2.37</td>
<td>6.1</td>
<td>−0.20</td>
<td>89.5 ± 3.4</td>
</tr>
<tr>
<td></td>
<td>400</td>
<td>377.6 ± 25.1</td>
<td>6.7</td>
<td>−5.6</td>
<td>378.1 ± 32.4</td>
<td>7.9</td>
<td>−5.5</td>
<td>87.4 ± 3.7</td>
</tr>
</tbody>
</table>

3.2.3. Precision and accuracy
A summary of precision and accuracy of the method is shown in Table 2. The intra-day and inter-day precisions ranged from 2.9% to 13% and 2.0% to 13%, respectively, while the accuracy ranged from −6.7% to 5.8%. The results indicated that the assay had remarkable reproducibility with acceptable accuracy and precision.

3.2.4. Extraction recovery and matrix effect
As shown in Table 2, the extraction recoveries of six flavonoids were in the range 64.1–98.5%, with RSD less than 11%. The mean extraction recovery of the I.S. was 83.1 ± 3.9%.

The observed matrix effects ranged from 97.3 to 101.5% for baicalin, 88.1 to 103.7% for wogonoside, 93.1 to 106.9% for baicalein. 86.9 to 98.1% for wogonin, 87.6 to 95.5% for oroxylin A and 98.6 to 108.1% for chrysin, indicating a neglectable matrix effect on the ionization of the analytes.

3.2.5. Stability
Results of the stability tests showed that all analytes were stable in plasma samples for 1 month at −20 °C (RE: −4.4% to 12%, RSD < 8.4%), within three freeze-thaw cycles (RE: −9.4% to 10%, RSD < 13%), and for 4 h at room temperature (RE: −7.2% to 8.2%, RSD < 11%).

3.3. Results of pharmacokinetic study
The developed assay was sensitive enough to measure all the six compounds in rat plasma after oral administration of R. scutellariae extract simultaneously. The mean plasma concentration–time profiles of these compounds are shown in Fig. 3. The estimated pharmacokinetic parameters are listed in Table 3. Baicalin, wogonoside, baicalein, wogonin, oroxylin A and chrysin concentration–time profile conformed to a two-compartment pharmacokinetic model. From the figure, it showed that the six flavonoids exhibited rapid absorption and bimodal phenomenon in plasma concentration–time profiles, which were in parallel with the literature [4,13–15,17]. The first peak occurred within 45 min and the second at 8–12 h. Evidence was provided that the first absorption site of baicalin, and possibly other flavonoids, was probably due to the directly absorption, while glucuronidation,
enteric circulation and enterohepatic circulation may contributed to the second peak [9]. The concentration of wogonoside was lower than other compounds except chrysin, which might be due to its lower content in the extract. The difference of $t_{1/2}$ indicated that glycosides, baicalin and wogonoside were eliminated relatively more easier than aglycons (baicalein, wogonin, oroxylin A and chrysin).

4. Conclusion

For the first time, an LC–MS/MS assay for simultaneous determination of baicalin, wogonoside, baicalein, wogonin, oroxylin A and chrysin in rat plasma was developed and validated. The method was simple, sensitive and selective, and was successfully applied to an oral pharmacokinetic study of six flavonoids of *R. scutellariae* extract.

References


