Simultaneous determination of the bioactive components in rat plasma by UPLC-MS/MS and application in pharmacokinetic studies after oral administration of Radix Scutellariae extract

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Abstract

A highly sensitive and rapid ultraperformance liquid chromatography–tandem mass spectrometry (UPLC-MS/MS) method has been developed and validated for simultaneous quantification of the four main bioactive compounds, i.e. baicalin, baicalein, wogonoside and wogonin, in rat plasma after oral administration of Radix Scutellariae extract. Clarithromycin was used as an internal standard (IS). Plasma samples were processed by protein precipitation with methanol. The separation was performed on an Acquity BEH C18 column (100 × 2.1 mm, 1.7 μm) at a flow rate of 0.4 mL/min, using 0.1% formic acid–acetonitrile as mobile phase. The MS/MS ion transition ions monitored were 447.5 → 270.1 for baicalin, 270.1 → 168.1 for baicalein, 461.2 → 284.0 for wogonoside, 284.2 → 168.1 for wogonin and 748.5 → 158.1 for IS. Method validation was performed according to US Food and Drug Administration guidelines and the results met the acceptance criteria. The lower limit of quantification (LLOQ) achieved was 1.13 ng/mL for baicalin, 1.23 ng/mL for baicalein, 0.82 ng/mL for wogonoside and 0.36 ng/mL for wogonin. The calibration curves obtained were linear (r > 0.99) over the concentration range ~ 1–1000 ng/mL. The intra- and inter-day precision was <15% and the accuracy was within ±14.7%. After validation, this method was successfully applied to a pharmacokinetic study of Radix Scutellariae extract.

KEYWORDS

baicalin, baicalein, pharmacokinetics, UPLC-MS/MS, wogonin, wogonoside

1 INTRODUCTION

Radix Scutellariae, a key ingredient herb in many compound preparations such as Xiao-Chai-Hu Decoction and Huang-Lian-Jie-Du Decoction, and has been widely used in traditional Chinese medicine for the treatment of inflammation, fever, hepatitis, allergic diseases, hypertension, etc. (Wang, Deng, & Que, 1998). Previous studies indicated that flavonoids including baicalin, baicalein, wogonoside and wogonin were the main bioactive components of Radix Scutellariae. Furthermore, baicalin was used as a phytochemical marker for the quality control of Radix Scutellariae in the Chinese Pharmacopoeia, and wogonoside is also a major flavonoid (Qi, Zhou, Wang, & Zhu, 1998; Wu, Sun, & Liu, 2005). Pharmacological studies indicated that baicalin and wogonoside shared many beneficial activities with Radix Scutellariae with respect to anti-inflammatory, anti-allergic, anti-oxidant and hepatoprotective properties (Chou, Chang, Li, Wong, & Yang, 2003; Jang et al., 2003; Kim et al., 2005; Lim, 2003). Baicalein and wogonin, which exist in the aglycone form, have almost the same pharmacological activities as baicalin and wogonoside, respectively. However, there are few data on the pharmacokinetic profiles of baicalin, wogonoside, baicalein and wogonin of Radix Scutellariae extract in rats. It is generally assumed that baicalin is poorly absorbed from the gastrointestinal tract in its native form and hydrolyzed by microflora enzymes (bacterial β-glucuronidase) in the gut to its aglycone baicalein in human and rat (Yim et al., 2004; Zuo et al., 2002). Similar to baicalin, wogonoside is first metabolized by microflora enzymes and subsequently circulated and excreted mainly in conjugated form following intake of
Radix Scutellariae extract in rat and human (Chen, Wang, Du, & Zhong, 2002; Zuo et al., 2002). Therefore, simultaneous determination of the four active ingredients in rat plasma for investigating the pharmacokinetics of Radix Scutellariae extract is essential.

Nowadays, several analytic methods for the separation and quantification of flavonoids in Radix Scutellariae are generally based on chromatographic techniques such as high-performance liquid chromatography (HPLC) with electrochemical or ultra-violet detection or coupled to mass spectrometry (Deng, Yang, & Mou, 2008; Di, Feng, & Liu, 2006; Jia et al., 2010; Kotani, Kojima, Hakamata, & Kusu, 2006; Lai, Hsiu, Tsai, Hou, & Chao, 2003). Unfortunately, the existing methods have one or more drawbacks, such as long chromatographic separation, interference from reagents and increased dead volume. However, ultra-performance liquid chromatography (UPLC), widely applied in pharmaceutical analysis, breaks through the bottleneck of chromatographic science and is better than traditional or optimized HPLC owing to its ultra-resolution, high analysis speed and high sensitivity (Zhang et al., 2014). Recently, UPLC coupled to quadrupole time-of-flight mass spectrometry (Q-TOF-MS) or trap-TOF-MS has been increasingly explored and applied to the analysis of natural products, which will be beneficial to the development of herb medicine research.

The goal of current investigation was to develop a sensitive and rapid UPLC-MS/MS method for the quantification of baicalin, wogonoside, baicalein and wogonin in rat plasma simultaneously. The method was applied to a pharmacokinetic study of the above four compounds after oral administration of Radix Scutellariae extract to rats. Furthermore, the technology could provide many advantages for the analysis of complex compounds in herbal formulations (Lai, Hsiu, Chen, Hou, & Chao, 2003).

2 | EXPERIMENTAL

2.1 | Materials and reagents

The reference standards of baicalin, baicalein, wogonoside and wogonin standard substance, whose chemical structures were shown in Figure 1, were purchased from Shanghai Winherb Medical S&T Development Co. Ltd (Shanghai, China). Acetonitrile was of HPLC grade and obtained from Tedia Company Inc. (Fairfield, OH, USA); formic acid and methanol were obtained from Merck KGaA (Darmstadt, Germany); ultra-pure water was purified using an EPED super purification system (Nanjing, China). Distilled water was used for the extraction and preparation of samples. Other regents and chemicals were of analytical grade.

2.2 | UPLC-MS/MS instrument and conditions

Chromatographic analysis was performed on an Acquity UPLC system (Waters Corp., Milford, MA, USA), consisting of a binary pump solvent management system, an online degasser and an autosampler. The sample was separated on an Acquity UPLC BEH C18 column (100 × 2.1 mm, 1.7 μm) and eluted with a gradient of solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in acetonitrile). The gradient program increased B from 25 to 45% in 0−10 min, B returned to 25% at 10.1 min and was maintained for 20 min to analyze the next sample. The flow rate for all analyses was 0.4 mL/min. All separations of standards and serum samples were performed at room temperature. All sample extracts were maintained in the autosampler at 4°C while awaiting injection. All injection volumes were 10 μL.

Mass spectrometry detection was performed using a Xevo Triple Quadrupole MS (Waters Corp., Milford, MA, USA) equipped with an electrospray ionization source (ESI). The ESI source was set in positive ionization mode. The parameters in the source were set as follows: capillary voltage, 3.5 kV; source temperature, 150°C; desolvation gas flow, 1000 L/h; desolvation temperature, 550°C; and cone gas flow, 50 L/h. The quantification was performed using multiple reaction monitoring (MRM), and the cone voltage and collision energy was optimized for each analyte; selected values were given in Table 1. Dwell time was automatically set by MassLynx (Waters Corp., Milford, MA, USA).

2.3 | Radix Scutellariae extract preparation

The roots of Radix Scutellariae were purchased from Weiyuan county of Gansu Province, China. A total of 15.0 g Scutellaria baicalensis roots which were chopped into pieces about 1 cm long were immersed into

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**Figure 1** Chemical structures of baicalin, baicalein, wogonoside and wogonin in radix Scutellariae extract

**Table 1** Precursor/product ion pairs and parameters for MRM of compounds used in this study

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Retention time (min)</th>
<th>[M + H]+ (m/z)</th>
<th>MRM transitions</th>
<th>Cone voltage (V)</th>
<th>Collision energy (eV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baicalin</td>
<td>2.04</td>
<td>447.1</td>
<td>447.1 → 270.1</td>
<td>4.0</td>
<td>24.0</td>
</tr>
<tr>
<td>Bacicalein</td>
<td>2.64</td>
<td>270.1</td>
<td>270.1 → 168.1</td>
<td>12.0</td>
<td>14.0</td>
</tr>
<tr>
<td>Wogonoside</td>
<td>2.37</td>
<td>461.2</td>
<td>461.2 → 284.0</td>
<td>13.0</td>
<td>16.0</td>
</tr>
<tr>
<td>Wogonin</td>
<td>3.17</td>
<td>284.2</td>
<td>284.2 → 168.1</td>
<td>15.0</td>
<td>20.0</td>
</tr>
<tr>
<td>Clatithromycin</td>
<td>6.58</td>
<td>748.5</td>
<td>748.5 → 158.1</td>
<td>28.0</td>
<td>18.0</td>
</tr>
</tbody>
</table>

MRM, Multiple reaction monitoring.
150 mL water for 1 h, and then extracted under thermal reflux for 1 h twice. The extract was filtrated out by analytical filter paper and evaporated to dryness in a rotary evaporator R-210 (BUCHI Ltd. Labortechnik AG, Switzerland) at 60°C under reduced pressure. Finally, the dried residue was dissolved in water to form a final concentration of 0.3 g/mL (equivalent to dry weight of raw materials), which was orally administrated to rats. The supernatant was filtered through a 0.22 μm membrane, and an aliquot of 5.0 μL was injected for UPLC-MS/MS analysis.

2.4 | Contents of baicalin, wogonoside, baicalein and wogonin in radix Scutellariae extract

To calculate the administered dose, the contents of the four compounds in Radix Scutellariae extract were quantitatively analyzed. The extract powder was ultra-sonicated with 70% ethanol for 1 h; the suspension was then diluted 100 times. After centrifuging at 13,000 rpm for 10 min, the supernatant was ready for analysis. The contents of baicalin, wogonoside, baicalein and wogonin in the extract were measured quantitatively using the same conditions described above. The contents of above four components were 11.2, 6.18, 13, 263 ng/mL for baicalin; 3.60, 11.3, 112.5, 262 ng/mL of baicalein; and 112, 6.18, 1.04 and 0.21% in Radix Scutellariae extract, respectively.

2.5 | Preparation of calibration standards, quality control and internal standard

Stock solutions were prepared by dissolving various accurate amounts of standards in methanol solution: 363 μg/mL of baicalin; 350 μg/mL of wogonoside; 262 μg/mL of baicalein; and 112 μg/mL of wogonin. The above stock solutions were placed in a 10 mL volumetric flask and diluted with methanol solution to the scale line. Then the mixed standard solution was formulated containing 36.3 μg/mL of baicalin, 35.0 μg/mL of wogonoside, 26.2 μg/mL of baicalein and 11.2 μg/mL of wogonin. The internal standard (IS) stock solution (clarithromycin) of 237.0 μg/mL was also prepared in methanol. IS working solution (2.37 μg/mL) was prepared by diluting the stock solution with methanol. Calibration samples in plasma were prepared by mixing solutions of standard mixture, IS and methanol with rat blank plasma to obtain final concentrations in the range of 1.13–1089 ng/mL for baicalin, 1.23–1080 ng/mL for wogonoside, 0.82–787.5 ng/mL for baicalein, 0.36–337 ng/mL for wogonin and 13.4 ng/mL for IS, respectively. All solutions were stored at −20°C before use. Quality control (QC) samples at low, medium and high concentrations (1.13, 36.3, 363 ng/mL for baicalin; 1.23, 36.0, 360 ng/mL for wogonoside; 0.82, 26.3, 263 ng/mL for baicalein; 0.36, 11.3, 112.5 ng/mL for wogonin) were prepared by diluting a new mixture stock solution with the following concentrations: 3.63 μg/mL for baicalin; 3.60 μg/mL for wogonoside; 2.63 μg/mL for baicalein; and 1.13 μg/mL for wogonin.

2.6 | Validation procedures

In accordance with the US Food and Drug Administration bioanalytical method validation guidance, the method was validated in terms of specificity, selectivity, calibration curve, sensitivity, matrix effect, accuracy, precision and stability.

2.6.1 | Specificity and selectivity

The specificity of the method was evaluated by preparing and analyzing six different batches of rat plasma samples to investigate the potential interferences at the LC peak region for analytes and IS. The chromatogram of a blank plasma sample was compared with those obtained with a sample at the lower limit of quantification (LLOQ). The signal intensity at this concentration was at least five times higher than that of blank plasma samples.

2.6.2 | Linearity and LLOQ

The linearity of each calibration curve was determined by plotting the peak area ratio (y) of analytes to IS vs the nominal concentration (x) of analytes with weighted (1/x²) least square linear regression. The lower limit of detection (LLOD) was defined as the lowest concentration with a signal-to-noise ratio of at least 2-fold, and the LLOQ was the concentration giving a signal-to-noise ratio at least 5-fold with acceptable accuracy within 20% deviation and precision between 80 and 120%.

2.6.3 | Accuracy and precision

Three concentrations (high, medium and low) of baicalin, wogonoside, baicalein and wogonin standard stock solutions were added to plasma to obtain control samples, and were determined in five separate runs on the same day for intra-day and on three consecutive days for the inter-day accuracy variation. The accuracy was expressed as relative error (RE) within 85–115% from the nominal values, and the precision as relative standard deviation (RSD) within ±15% except for LLOQ, where it should be within 80–120% for accuracy and <20% precision.

2.6.4 | Extraction recovery and matrix effect

The extraction recoveries of baicalin, wogonoside, baicalein and wogonin standard stock solutions were added to plasma to obtain control samples, and were determined in five separate runs on the same day for intra-day and on three consecutive days for the inter-day accuracy variation. The accuracy was expressed as relative error (RE) within 85–115% from the nominal values, and the precision as relative standard deviation (RSD) within ±15% except for LLOQ, where it should be within 80–120% for accuracy and <20% precision.

2.6.5 | Stability

Three QC samples (five samples for each) were tested for pretreatment, post-treatment, three freeze–thaw cycles and long-term stabilities. Pre-treatment stability was assessed by exposing QC samples at room temperature for 4 h. Post-treatment was evaluated by placing QC samples in the autosampler at 4°C for 24 h. For freeze–thaw cycle stability assessment, QC samples were repeatedly frozen and thawed for three cycles at −70–20°C. Long-term stability was carried out via placing QC samples at −70°C for 2 weeks.

2.7 | Pharmacokinetic study

The study was in accordance with the guidelines of the Committee on the Care and Use of Laboratory Animals in China. Six male Sprague–Dawley rats, weighing 250–280 g, were supplied by Shanghai SLAC Lab. Animal Co. Ltd (Shanghai, China). The rats were maintained
in an air-conditioned animal quarter at a temperature of 22 ± 2°C and a relative humidity of 50 ± 10%. The animals were acclimatized to the facilities for 5 days, and then fasted with free access to water for 12 h prior to each experiment. Radix Scutellariae extract was administered to rats by way of the traditional oral gavage route yielded satisfactorily at a dose of 4.5 g extract/kg body weight once. Blood (0.2 mL) was drawn into heparinized hemospasia tube at 0, 5, 15, 30, 60 min and 2, 4, 6, 8, 24 h post-dose and centrifuged at 13,000 rpm for 5 min at 4°C. Plasma samples were then transferred to Eppendorf tubes and stored at −80°C until analysis.

3 | RESULTS AND DISCUSSION

3.1 | Method optimization

On the purpose of achieving short retention times and symmetric peak shape, several combinations of acetonitrile, methanol, formic acid and acetic acid were applied to optimize the mobile phase. It was found that the acetonitrile–water system provided good separation with a low baseline. Moreover, the use of 0.1% formic acid in the water phase helped attain a higher response and better peak sensitivity for the analytes. Hence acetonitrile–0.1% formic acid with above gradient elution was employed and selected as the mobile phase with an appropriate retention time and low background noise.

A standard solution along with the mobile phase was directly infused into the mass spectrometer to optimize mass conditions. Baicalin, wogonoside, baicalein and wogonin responded better in positive ion mode compared with negative ion mode, and higher sensitivity was observed by ESI than ACPI. The results of the precursor ion full-scan spectra revealed that the most abundant ions were protonated molecular ions [M + H]+ for all the analytes. Therefore [M + H]+ ions were selected as the parent ions to perform the intellistart procedure (embedded in Masslynx) to seek for the best daughter ions, in which other parameters such as capillary and cone voltage, flow rate of desolvation gas and cone gas, and dwell time were optimized. All of the MRM transitions and parameters applied in the study are listed in Table 1.

3.2 | Sample preparation

To 200 μL plasma sample was added 20 μL of IS solution and 600 μL methanol. The supernatant was concentrated at 37°C for a rotary instrument after 10 min of 13,000 rpm centrifugation. The residue was reconstituted in 200 μL of methanol and centrifuged at 13,000 rpm for 10 min, then the supernatant was used for analysis.

FIGURE 2 Typical multiple reaction monitoring chromatograms of: (a) black (drug-free) plasma sample; (b) blank plasma spiked with four analytes in LLOQ and IS – (1) IS, (2) baicalin, (3) baicalein, (4) wogonoside, (5) wogonin; and (c) plasma sample obtained after oral administration of radix Scutellariae extract – (1) IS, (2) baicalin, (3) baicalein, (4) wogonoside, (5) wogonin
3.3 Method validation

3.3.1 Specificity and selectivity

Figure 2 shows the representative MRM chromatograms of the blank rat plasma (A), blank rat plasma spiked with the analytes at LLOQ and IS (B) and an in vivo plasma sample obtained at 15 min after oral administration of Radix Scutellariae extract (C). The retention times were about 2.04, 2.64, 2.37 and 3.17 min for baicalin, baicalein, wogonoside and wogonin, respectively. No endogenous peaks were observed at the retention times of analytes and IS.

3.3.2 Linearity and LLOQ

The calibration curve of baicalin was \( y = 0.029x + 0.010 \) (\( r = 0.9963 \)), \( y = 0.033x + 0.011 \) (\( r = 0.9984 \)) for baicalein, \( y = 0.019x + 0.014 \) (\( r = 0.9972 \)) for wogonoside and \( y = 0.022x + 0.004 \) (\( r = 0.9989 \)) for wogonin in rat plasma. The lowest concentrations with RSD <20% were taken as LLOQs and were found to be 1.13 ng/mL for baicalin, 1.23 ng/mL for baicalein, 0.82 ng/mL for wogonoside and 0.36 ng/mL for wogonin, respectively. No endogenous peaks were observed at the retention times of analytes and IS.

3.3.3 Precision and accuracy

The accuracy data in the present study ranged from 65.1 to 94.2% (RE), and the intra- and inter-day precisions were 6.7-14.7% and 6.3-13.1% (RSD), respectively. All of the assay values were found to be within the accepted variable limits, indicating that the established method was precise and accurate. All results were shown in Table 2.

3.3.4 Extraction recovery and matrix effect

The extraction recovery and matrix effect were evaluated by analyzing QC samples at low, medium and high concentrations with five replicates. As detailed in Table 3, the mean recovery of the analytes and IS was within 60.86-100.83%, and the corresponding matrix effect ranged from 77.11 to 115.05%, which showed that methanol is a feasible and appropriate medium for the analytes and IS extraction, and moreover, there was no measurable matrix effect on the ionization of analytes and IS.

### TABLE 2 Precision and accuracy data of the analytes in rat plasma

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Theoretical concentration (ng · mL(^{-1}))</th>
<th>Intra-day (n = 5)</th>
<th>Inter-day (n = 15)</th>
<th>Accuracy (%) (n = 15)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baicalin</td>
<td>363</td>
<td>336.30 ± 27.16</td>
<td>11.5</td>
<td>325.45 ± 24.12</td>
</tr>
<tr>
<td></td>
<td>36.3</td>
<td>33.04 ± 2.21</td>
<td>6.7</td>
<td>30.09 ± 2.53</td>
</tr>
<tr>
<td></td>
<td>1.13</td>
<td>1.02 ± 0.15</td>
<td>14.4</td>
<td>0.94 ± 0.19</td>
</tr>
<tr>
<td>Baicalein</td>
<td>360</td>
<td>313.04 ± 25.20</td>
<td>8.3</td>
<td>306.36 ± 37.07</td>
</tr>
<tr>
<td></td>
<td>36.0</td>
<td>35.47 ± 3.80</td>
<td>9.4</td>
<td>31.29 ± 2.44</td>
</tr>
<tr>
<td></td>
<td>1.23</td>
<td>0.84 ± 0.14</td>
<td>14.7</td>
<td>0.71 ± 0.09</td>
</tr>
<tr>
<td>Wogonoside</td>
<td>262.5</td>
<td>256.71 ± 20.54</td>
<td>8.0</td>
<td>226.71 ± 18.59</td>
</tr>
<tr>
<td></td>
<td>26.3</td>
<td>25.02 ± 2.48</td>
<td>9.8</td>
<td>23.61 ± 2.46</td>
</tr>
<tr>
<td></td>
<td>0.82</td>
<td>0.74 ± 0.03</td>
<td>4.3</td>
<td>0.64 ± 0.05</td>
</tr>
<tr>
<td>Wogonin</td>
<td>112.5</td>
<td>110.35 ± 16.74</td>
<td>14.2</td>
<td>100.95 ± 15.74</td>
</tr>
<tr>
<td></td>
<td>11.3</td>
<td>9.59 ± 0.62</td>
<td>9.4</td>
<td>9.68 ± 0.44</td>
</tr>
<tr>
<td></td>
<td>0.36</td>
<td>0.22 ± 0.02</td>
<td>8.9</td>
<td>0.28 ± 0.02</td>
</tr>
</tbody>
</table>

### TABLE 3 Extraction recovery and matrix effect of the analytes and IS in rat plasma (n = 5)

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Extraction recovery (%)</th>
<th>Matrix effect (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Low concentration</td>
<td>Medium concentration</td>
</tr>
<tr>
<td>Baicalin</td>
<td>66.00 ± 5.14</td>
<td>95.82 ± 5.01</td>
</tr>
<tr>
<td>Baicalein</td>
<td>69.80 ± 4.22</td>
<td>85.31 ± 3.97</td>
</tr>
<tr>
<td>Wogonoside</td>
<td>68.38 ± 6.27</td>
<td>87.53 ± 2.07</td>
</tr>
<tr>
<td>Wogonin</td>
<td>70.24 ± 4.21</td>
<td>77.89 ± 6.12</td>
</tr>
</tbody>
</table>
and wogonoside) were similar and their absorption and elimination were quite slow.

4 | CONCLUSION

In this study, a sensitive, rapid and simple UPLC-MS/MS method was developed and validated for the simultaneous determination of baicalin, baicalein, wogonoside and wogonin in rat plasma. Furthermore, the developed method was successfully used to investigate the pharmacokinetic characteristics of the four components after oral administration of Radix Scutellariae extract. The pharmacokinetic results could provide useful information for mechanism and clinical application of Radix Scutellariae.

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