A Method for Analysis of Wilfordmine in Human Plasma by Liquid Chromatography Coupled with Ion Trap Mass Spectrometry

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A simple and rapid liquid chromatography-ion trap mass spectrometric (LC-IT/MS) method has been developed and validated for quantification of wilfordmine in human plasma. After the protein precipitation was carried out by acetonitrile and the solution was cleaned by solid-phase extraction, the chromatographic separation was performed on a Zorbax Plus RRHD C18 column by using a mixture of acetonitrile and 10.0 mmol/L ammonium acetate solution (70:30, v/v) as the mobile phase at a flow rate of 0.7 mL/min. Detection was performed on an atmospheric-pressure chemical ionization source in the positive multiple reaction monitoring mode using aconitine as an internal standard (IS) with transitions of m/z 806→710 for wilfordmine, and 646→586 for IS, respectively. The obtained calibration curve was linear (r = 0.9992) over the concentration range of 0.5–100.0 µg/L with a lower limit of quantification of 0.5 µg/L in plasma. The intra- and inter-day relative standard deviations were <7.0 and 12.3%, respectively. The recoveries were between 86.0 and 97.0%. The proposed method was found to be applicable to clinical studies.

Introduction

Wilfordmine (Figure 1) is one of the main biologically active alkaloids from Tripterium wilfordii Hook f. (TW) (1–3), which has been used as traditional Chinese medicine to treat cancer, rheumatoid arthritis, autoimmune diseases, skin disorders and in male-fertility control for hundreds of years (4). Wilfordmine exhibits marked depressant effects on humoral-mediated immunity using hemolytic reactions as indices (5) and demonstrates good anti-feedant activity against the lepidopteran Spodoptera littoralis (6), whereas there are no other literatures for the pharmacology of this potential bioactive natural compound. In our previous work, we have reported the studies for the analysis of four TW alkaloids (e.g., wilfortrine, wilfordine, wilforgine and wilforine) in the TW extract and biological samples (7–9). So far, no methods are available for the analysis of wilfordmine in biological samples or other matrix. Recently, mass spectrometry has become one of the most important analytical tools employed in the analysis of pharmaceuticals (10–16). As a continuation of our studies on the bioanalytical analysis of the bioactive compounds from TW, we describe herein a sensitive analytical assay for wilfordmine from the TW extract in plasma samples.

Experimental

Reagents and materials

Wilfordmine (purity >98.0%, as determined by HPLC and characterized by 1H NMR, 13C NMR and HPLC–MS) was isolated from the TW crude extract by chromatographic techniques in our laboratory (17). Aconitine (purity >99.5%, see structure in Figure 1) was obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China) and used as the internal standard (IS). HPLC-grade acetonitrile and acetic acid were obtained from Merck (Darmstadt, German). HPLC-grade ammonium acetate was purchased from Sigma (Steinheim, Germany), whereas Oasis® MCX solid-phase extraction (SPE) cartridge (Oasis® mixed-mode cation-exchange, 50 mg/3 mL) used for sample preparation was procured from Waters (Milford, MA, USA). Ultrapure water (18.2 MΩ cm) was generated using a Milli-Q water purification system (Millipore, Molsheim, France). Blank plasma samples were obtained from six healthy volunteers ranging from 18 to 25 years old, who come from Zhejiang Gongshang University (Hangzhou, Zhejiang, China).

Equipment

Samples were analyzed on an Agilent LC-MSD Trap SL system (Agilent Technologies, Germany) equipped with an Agilent 1100 HPLC system, an atmospheric-pressure chemical ionization (APCI) source and an electrospray (ESI) source (Agilent Technologies, Wilmington, DE, USA). The LC-MSD Trap Software 4.2 (Bruker Daltonics, Bremen, Germany) was used to control the HPLC and mass spectrometer and to capture the mass spectrometer data, perform linear regression analysis and calculate sample concentrations.

Chromatographic conditions

The separation of wilfordmine from plasma components was achieved on an analytical Zorbax Plus RRHD C18 column (30×2.1 mm, 3.5 µm) (Agilent Technologies, Wilmington, DE, USA). The column oven was maintained at a constant temperature of 35°C. The injection volume was 20 µL. The mobile phase was a mixture of acetonitrile and 10.0 mmol/L ammonium acetate solution (70:30, v/v) at a flow rate of 0.7 mL/min.

Mass spectrometry analysis

The APCI interface was used as the ion source operating in positive ion mode. Acquisition was performed by an ion trap mass spectrometry (IT/MS) in multiple reaction monitoring (MRM) mode using the transitions m/z 806→710 and m/z 806→748 and m/z 616→586 and m/z 616→526 for the identification of...
Preparation of stock solution and quality controls

The standard stock solutions of wilfordmine and aconitine (IS) were dissolved in acetonitrile at a concentration of 1.0 g/L. Appropriate serial dilutions of the wilfordmine stock solution were made in acetonitrile/water (70:30, v/v) in 100 mL volumetric flasks to obtain a standard working solution (10.0 mg/L) for spiking blank biometrics. Internal standard working solution (10.0 mg/L) was prepared by diluting internal standard stock solution with acetonitrile. All solutions were stored at 4 °C until use. Calibration standards at seven concentrations (0.5, 1.0, 2.0, 10.0, 20.0, 50.0 and 100.0 μg/L) were prepared by spiking appropriate volume of wilfordmine working standard solution (10.0 mg/L) into human plasma blanks, wherein the concentration of IS was 10.0 μg/L. The standards were prepared according to the sample preparation procedure given below. Three levels of them (0.5, 2.0 and 50.0 μg/L) were considered as quality control (QC) samples.

Sample preparation

Each collected blood sample was immediately centrifuged at 4,000 rpm for 5 min and plasma was transferred into a clean Eppendorf tube. All plasma samples were stored at −20 °C until analyses. Accurately 200 μL aliquot of plasma sample spiked with 20 μL of internal standard working solution (10.0 mg/L) was vortex-mixed with Vortex-Genie-2 mixer for 5 min and then deproteinized with 1.0 mL of acetonitrile. The precipitate was removed by centrifugation at 7,800 rpm at 4 °C for 10 min (Micromax RF, Thermo Electron Corporation, San Jose, USA). The upper organic layer was transferred to a disposable glass tube and acidified with 2.0 mL of 0.2% (v/v) acetic acid, and then loaded on a MCX cartridge, which was preconditioned with 1.0 mL of acetonitrile and 1.0 mL of water. The cartridge was washed with 2.0 mL of water and followed by 1.0 mL of acetonitrile/water (25:75, v/v). Alkaloids and IS were eluted with 1.0 mL of 10.0 g/L of aqueous ammonia in acetonitrile. The eluate was evaporated to dryness under a stream of nitrogen and dissolved in 200 μL of mobile phase, and 20 μL of the solution was then injected into the liquid chromatography (LC)-IT/MS system.

### Table I

<table>
<thead>
<tr>
<th>Item</th>
<th>Condition</th>
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<tbody>
<tr>
<td>Source voltage</td>
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<tr>
<td>Capillary voltage</td>
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</tr>
<tr>
<td>Dry temperature</td>
<td>350 °C</td>
</tr>
<tr>
<td>Vaporizer temperature</td>
<td>450 °C</td>
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<tr>
<td>Dry gas</td>
<td>Nitrogen</td>
</tr>
<tr>
<td>Dry gas purity</td>
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</tr>
<tr>
<td>Dry gas flow rate</td>
<td>8.0 L/min</td>
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<tr>
<td>Nebulizer pressure</td>
<td>60.0 psi</td>
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<tr>
<td>Corona current</td>
<td>4.0 μA</td>
</tr>
</tbody>
</table>

### Table II

<table>
<thead>
<tr>
<th>Compound</th>
<th>QC concentration (μg/L)</th>
<th>Found* (μg/L)</th>
<th>Recovery (%)</th>
<th>RSD (%)</th>
<th>Intraday</th>
<th>Interday</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wilfordmine</td>
<td>0.5</td>
<td>0.43 ± 0.03</td>
<td>86.0</td>
<td>7.0</td>
<td>12.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>1.94 ± 0.08</td>
<td>97.0</td>
<td>4.1</td>
<td>8.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>50.0</td>
<td>45.86 ± 1.35</td>
<td>91.7</td>
<td>2.9</td>
<td>5.9</td>
<td></td>
</tr>
</tbody>
</table>

*a = 3 replicates per day × 5 days within a week period, expressed as mean ± SD.

Method validation

Method validation was performed following the FDA’s Guidance for Industry Bioanalytical Method Validation (18). The following factors were used to assess the assay performance: selectivity, linearity, recovery, relative standard deviation (RSD%), matrix effect and stability.

Selectivity was assessed by comparing the chromatograms of six different batches of blank plasma obtained from six different sources. The chromatographic peak was verified by comparing the retention time (tR) and the mass fragment ions with that of reference standard.

The linearity was assessed using the internal standard method and up to seven calibrators with the concentrations from 0.5 to 100.0 μg/L. The calibration curve was constructed by plotting wilfordmine to aconitine (IS) area ratio (y) against the nominal concentration (x) of wilfordmine in the plasma by weighted (1/x²) least square linear regression. The limit of quantification (LOQ) for wilfordmine was established on the basis of a signal-to-noise (S/N) ratio of 10.

The recovery was determined by comparing the peak area obtained from blank plasma samples spiked with analyte at three concentrations (0.5, 2.0 and 50.0 μg/L) before extraction with those after extraction. Intraday RSD (n = 6) for wilfordmine
was assessed by the testing results in the same day. Interday RSD was assessed by analysis of the QC samples over five consecutive days within a week period.

In this paper, the absolute matrix effect with SPE procedure was determined by comparing the mean peak areas of QC samples (0.5, 2.0 and 50.0 μg/L) spiked after SPE extraction (B) with those of the neat solutions (A). While the absolute matrix effect without SPE procedure was determined by comparing the mean peak areas of QC samples (0.5, 2.0 and 50.0 μg/L) spiked after acetonitrile precipitation without SPE clean-up with those of the neat solutions. The values obtained in this study can be calculated as follows: absolute matrix effect (%) = B/A × 100 (19). The matrix effect of internal standard was also evaluated using the same method.

### Stability

The freeze–thaw stability was determined by six replicates of each QC concentrations over three freeze–thaw cycles. These samples were frozen at −20 °C, thawed at room temperature, and, when completely thawed, refrozen for 24 h under the same conditions with at least a 12-h interval between each cycle. Long- and short-term stabilities were measured by leaving QC samples kept at −20 °C or at ambient temperature for a certain time period (20 days or 24 h, respectively), before extraction. All the amounts measured were then compared with the QC samples added freshly.

### Sample analysis

The developed method was applied to the analysis of clinical samples obtained after administration of a single oral dose of TW tablets (10 mg TW active compounds per tablet, and ~0.1 mg winfordmine per piece). Seven patient volunteers who were accepted treatment in Hangzhou Traditional Chinese Medical Hospital (Hangzhou, Zhejiang, China) for the disease of rheumatoid arthritis were considered participating in this clinical study. A physical examination for all volunteers was performed, and the written informed consent forms were obtained before initiation of the TW alkaloids analysis study. The clinical doses and dosing schedules were administered by the clinical doctors. The informed consent forms and study protocol were authorized by the ethics committee of Ningbo Municipal Center for Disease Control and Prevention, China (No. 2013-003). The blood samples (3 mL aliquots, A–E) were collected from five patient volunteers, who took the TW tablets three pieces per day for >5 days. The pharmacokinetic blood samples were collected from another two volunteers, a series of ~0.5 mL venous blood samples were collected in 1.5 mL heparinized tubes at 0, 30 min, 1.0, 1.5, 2.0, 3.0, 4.0, 6.0, 8.0, 12.0, 24.0 h, after a single oral dose of three TW tablets. Along with the clinical samples, the QC samples were also assayed in replicates. For the determination of wilfordmine, the plasma samples, which was aliquoted to polypropylene tubes and stored at −20 °C until analysis, were spiked with the IS and processed as the extraction procedure described above, and then subjected to LC–IT/MS/MRM analyses.

### Results

#### Matrix effects

The absolute matrix effects with SPE procedure were 88.3, 93.1 and 95.6% for wilfordmine, and 89.5, 94.3 and 96.5% for aconitine (IS) at three concentration levels of 0.5, 2.0 and 50.0 μg/L, respectively. While the absolute matrix effects without SPE procedure were 58.4, 63.7 and 65.5% for wilfordmine, and 74.5, 64.7 and 72.3% for aconitine (IS) at the same concentrations, respectively. The results indicated that an insignificant matrix effect in the different sources occurred in this method with SPE procedure, which was in agreement with the requirement of the guidance for industry and bioanalytical method validation by the food and drug administration (18), while it brought an obvious matrix effect without SPE procedure.

#### Calibration curves, linear range and LOQ

The calibration curves, obtained by injecting six different concentration levels of wilfordmine standard solutions and analyzed...
in triplicate, showed a good linearity in the range of 0.50–100.0 mg/L with a correlation coefficient of 0.9992. The regression equation was $y = 10.8827x + 0.0312$. The LOD was determined by the analysis of samples of known concentrations and found to be 0.15 mg/L and LOQ found to be 0.5 mg/L for wilfordmine in plasma.

**Recovery and RSD**

Good results were obtained by testing QC samples at three concentration levels (0.5, 2.0 and 50.0 mg/L). The intra- and interday RSD% values were 2.9–7.0 and 5.9–12.3%, respectively, as shown in Table II. The recoveries were 86.0, 97.0 and 91.7% for wilfordmine, respectively. These results indicate that the RSD% of the assay is within the acceptance limits (the guidance of FDA) of ±20% at LOQ and ±15% at the other concentration levels.

**Stabilities**

The summary of the stability tests using the analyte is shown in Table III. The results obtained revealed that wilfordmine in plasma at three concentrations was found to be stable at room temperature for 24 h (between 92.7 and 99.6%), at the –20°C for 20 days (between 94.3 and 102.1%), at freeze and thaw stability (between 92.3 and 104.4%). The results indicate that wilfordmine was stable under the investigated conditions as the measured concentrations were within acceptable limits of ±15%, the analytical method was applicable for clinical analysis.

**Sample analysis**

In the present study, the established LC-IT/MS–MRM method has been successfully applied to determine wilfordmine in plasma samples after an oral administration of TW tablets. The results show that the concentrations for wilfordmine were 5.2, 7.4, 0.9, 9.6 and 15.7 mg/L for samples A–E, respectively. The initial plasma concentration–time profile of wilfordmine after an oral administration of three TW tablets was shown in Figure 2; however, pharmacokinetic parameters were not summarized because of the absence of enough pharmacokinetic data. Based on the above analyses, it shows that this assay can be used for further studies on the pharmacokinetics evaluation of wilfordmine in human plasma.

**Discussion**

**Sample preparation and IS selection**

Three precipitants (acetonitrile, methanol and ethyl acetate) were evaluated during the method development. We found that the sample preparation with a protein precipitation had higher recoveries (86.0–97.0%) by using acetonitrile, while

<table>
<thead>
<tr>
<th>Mobile phase</th>
<th>Compound</th>
<th>APCI</th>
<th>ESI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>In positive mode*</td>
<td>In negative mode*</td>
</tr>
<tr>
<td>Acetonitrile/water (70:30, v/v)</td>
<td>Wilfordmine</td>
<td>$1.2 \times 10^{5}$</td>
<td>$3.6 \times 10^{3}$</td>
</tr>
<tr>
<td></td>
<td>Aconitine (IS)</td>
<td>$7.9 \times 10^{5}$</td>
<td>$1.2 \times 10^{3}$</td>
</tr>
<tr>
<td>Acetonitrile/10.0 mmol/L ammonium acetate solution (70:30, v/v)</td>
<td>Wilfordmine</td>
<td>$3.0 \times 10^{5}$</td>
<td>$6.4 \times 10^{3}$</td>
</tr>
<tr>
<td></td>
<td>Aconitine (IS)</td>
<td>$2.5 \times 10^{5}$</td>
<td>$2.6 \times 10^{3}$</td>
</tr>
</tbody>
</table>

*aSIM at m/z 806 for wilfordmine and m/z 646 for aconitine (IS).*

*bSIM at m/z 804 for wilfordmine and m/z 644 for aconitine (IS).*
lower recoveries (58.2–85.4%) by methanol and ethyl acetate. Moreover, the protein precipitation was much simpler, better reproducible and less time-consuming and therefore utilized in the study.

Matrix effects like signal enhancement or suppression can severely compromise quantitative analysis of biological samples with LC–MS or LC–MS/MS. In order to reduce the matrix effect, a reliable SPE procedure was performed for removing the organic biological matrix. Wilfordmine, as an alkaloid from TW, can be cationized easily in the acidic solution and separated conveniently from the non-alkaline compounds with a cation-exchange cartridge, such as Waters MCX cartridges. Therefore, the extract was acidified with 2.0 mL of 0.2% (v/v) acetic acid before loading on cartridges, and then wilfordmine was cationized and cation-exchanged on MCX cartridges. As a result, in comparison with the matrix effect without SPE procedure, the matrix effect with SPE procedure decreased greatly. It shows that the SPE procedure for this method is necessary; otherwise, the matrix interference would occur seriously in the analyses.

During the experiment, several internal standards were tested under present experimental conditions such as aconitine, strychnine and salsoline. However, strychnine and salsoline could not give satisfactory retention times under the optimized experimental conditions. Aconitine had a moderate retention time and could give molecular ions [M+H]+ as the most intensive precursor ions in positive ion scan mode. Its chromatographic and mass spectrometric behaviors were similar to wilfordmine, and finally it was selected as the IS in this work.

**Chromatographic separation and MRM conditions optimization**

The selection of mobile phase was focused on good peak shape and mass spectral response, as well as a short run time. In this paper, a comparison between the mobile phase of acetonitrile with different volume of water and acetonitrile with different volume of 10.0 mmol/L ammonium acetate solution was performed. The obtained mass intensities were shown in Table IV. The results show that acetonitrile with a mixture of acetonitrile and 10.0 mmol/L ammonium acetate solution (70:30, v/v) could achieve an excellent peak shape, a short run time and better ionization efficiency. Therefore, these were finally used as mobile phase at a flow rate of 0.7 mL/min which could achieve a satisfactory separation and suitable retention time.
To get the richest relative abundance of precursor ions and product ions of wilfordmine and IS, their standard solutions (100.0 μg/L) were directly infused into the mass spectrometer at a flow rate of 10 μL/min. The parameters, e.g., fragment energies and collision energies, for both APCI and ESI with acquisition in the positive MRM mode were optimized. The obtained results show that under the optimized conditions, the intensities for both compounds in positive APCI mode were higher than that in positive ESI mode. Figure 3 shows the spectra of full scan product ions of precursor ions of wilfordmine and aconitine (IS). Therefore, the positive APCI mode was chosen for the next study, the transitions of m/z 806→710 for wilfordmine and m/z 646→586 for aconitine (IS) were selected for MRM quantification.

Selectivity
The degree of interference by endogenous plasma constituents with wilfordmine and IS was assessed by inspection of chromatograms derived from processed blank plasma sample. Representative chromatograms obtained from blank plasma, blank plasma spiked with wilfordmine and aconitine (IS), and a plasma sample from patients after treatment with wilfordmine are shown in Figure 4. No interferences from endogenous substances with analyte or aconitine (IS) were detected in drug-free plasma at the retention time of the analytes.

Conclusions
In the present work, a sensitive, accurate, precise and efficient LC–IT/MS–MRM method was developed for the analysis of wilfordmine in human plasma. According to these results, the method was proved to be linear in the concentration range as well as accurate, precise and selective. Moreover, the precision and accuracy tests were within 15% for the intra- and interday. The method is suitable for quantitative assay and should be useful for routine monitoring of wilfordmine concentration in human plasma for pharmacokinetic, bioavailability and bioequivalent studies.

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