Determination of Cefaclor by UPLC–MS-MS for a Chinese Pharmacokinetic Study

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A novel method has been developed for the determination of cefaclor in human plasma by ultra-performance liquid chromatography combined with tandem mass spectrometry (UPLC–MS-MS). The plasma was treated by a single step of protein precipitation with acetonitrile. The chromatographic separation was performed on a Waters Acquity UPLC BEH C18 (2.1 x 100 mm, 1.7 μm) with a gradient mobile phase consisting of 0.1% formic acid and acetonitrile at a flow rate of 0.4 mL/min. The analyses were conducted by multiple reaction monitoring using the precursor-to-product combinations of m/z 367.5 → 173.8 (cefaclor) and m/z 454.1 → 160.3 (internal standard). Validation results indicated that the lower limit of quantification was 2 ng/mL and the assay exhibited a linear range of 2–10,000 ng/mL. Quality control samples (5, 200 and 5,000 ng/mL) in five replicates from three different runs of analysis demonstrated a relative standard deviation of 3.7–10.7%, an intra-assay precision of 5.8–8.9%, and an overall accuracy of <15%. A sensitive and specific method for quantifying cefaclor in human plasma has been devised and successfully applied to a pharmacokinetic study.

Introduction

Cefaclor, a second generation semi-synthetic orally administered cephalosporin antibiotic, exhibits a broad antibacterial spectrum against various gram-positive and gram-negative bacteria.

A few analysis methods for cefaclor have been reported that use high-performance liquid chromatography (HPLC) methods (2–6); the lowest lower limit of quantification (LLOQ) was 60 ng/mL, which satisfied the needs for a pharmacokinetic study of the conventional immediate release formulation of cefaclor. One HPLC–tandem mass spectrometry (MS-MS) method (1) was reported for the study of the sustained-release formulation of cefaclor, which required 0.5 mL of plasma and produced an LLOQ of 2 ng/mL. However, in this method, the plasma samples were treated with two sample preparation procedures: protein precipitation (PPT) with an LLOQ of 100 ng/mL, and solid-phase extraction (SPE) with an LLOQ of 2 ng/mL.

In this paper, a simple, rapid and sensitive method was developed and validated by UPLC–MS-MS for determination of the released tablet of cefaclor with a single step of PPT and with an LLOQ of 2 ng/mL; the method was successfully applied to a clinical pharmacokinetic study in Chinese people.

Experimental

Instrumentation and reagents

Cefaclor (purity 93.2%) and flucloxacinil [internal standard (IS), purity 91.6%] were purchased from the National Institute for Control of Pharmaceutical and Biological Products (Beijing, PR China). HPLC grade acetonitrile was obtained from SK Chemicals (Republic of Korea). HPLC grade formic acid was purchased from Dikma (Richmond Hill, NY). HPLC grade water was produced by a Milli-Q Reagent Water System (Millipore, MA). Other chemicals and solvents were analytical grade.

Ultra-performance liquid chromatography (UPLC) was performed on a Waters Acquity UPLC system, which was equipped with a binary solvent delivery manager and a sample manager. MS was performed on a Waters Micromass Quattro Premier tandem quadrupole mass spectrometer. The LC–MS-MS system was controlled by Masslynx 4.1 with QuanLynx Application Manager.

The UPLC separation was performed on a Waters Acquity ethylene-bridged (BEH) C18 column (100 x 2.1 mm, 1.7 μm) at 40°C with a flow rate of 0.4 mL/min. A gradient elution mode was adopted, using two mobile phases: acetonitrile (A) and 0.1% formic acid (B). The gradient elution program is shown in Table I. The autosampler was conditioned at 4°C and the injection volume was 7.5 μL using partial loop mode for sample injection. The total UPLC run time was 7.5 min.

All MS optimization experiments were performed in MS scan and product scan modes. All quantifications were performed in multiple reaction monitoring (MRM) mode. The tune page parameters and conditions for each of MRM transition were optimized by infusing the individual neat standard solution into the MS at 10 μg/mL. To ensure that the tune page parameters were compatible with the LC flow during the tuning, an LC flow of 0.1 mL/min at 50% acetonitrile was introduced into the MS at the same time by utilizing a T-unit (Upchurch Scientific, Oak Harbor, WA).

The electrospray ionization (ESI) source was operated in positive ionization mode. Quantification was performed by MRM of the transitions of 367.5 → 173.8 (cefaclor) and 454.1 → 160.3 (IS), with a scan time of 0.2 s per transition. The optimal MS parameters were as follows: capillary voltage of 4.5 kV, cone voltage of 20 V, source temperature of 120°C, desolvation gas flow of 650 L/h and cone gas flow of 50 L/h.

Methods

Preparation of standards

A stock solution of cefaclor was prepared by dissolving the accurately weighed reference compound in 10% acetic acid to produce a final concentration of 1 mg/mL, which was stored at 4°C. The solution was serially diluted with 50% acetonitrile to achieve standard working solutions at concentrations of 20, 50, 100, 200, 500, 1,000, 2,000, 5,000, 10,000, 20,000, 50,000 and

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100,000 ng/mL for cefaclor. A 20 μg/mL IS working solution was prepared by diluting the 1 mg/mL stock solution of flucloxacillin with 50% acetonitrile.

Calibration curves and quality control samples
Calibrators were prepared by adding the standard working solutions (40 μL) to 400 μL of plasma. The resulting calibration curve consisted of 11 concentration levels (2, 5, 10, 20, 50, 100, 200, 500, 1,000, 2,000, 5,000 and 10,000 ng/mL) for cefaclor. Next, 20 μL of 10% acetic acid was added and the calibrators were extracted in the same manner as the unknown samples. Peak area ratios were plotted against concentrations and linear regression analysis was performed by using QuanLynx 4.1.

For quality control (QC), samples were prepared by plasma at cefaclor concentrations of 5 ng/mL (low), 200 ng/mL (medium) and 5,000 ng/mL (high).

Sample preparation
Calibration standards, QC samples and clinical plasma samples were treated by a single step of PPT with acetonitrile. Aliquots (420 μL) of human plasma, including 20 μL of 10% acetic acid and 400 μL of plasma, spiked with 50% acetonitrile (40 μL) and IS working solution (25 μL), were vortex-mixed for 30 s and precipitated with acetonitrile (0.4 mL) for 1 min by using a vortex mixer (Scientific Industries, Inc., Bohemia, NY). After centrifugation at 14,000 × g for 10 min at 4 °C, the supernatant was transferred and 7.5 μL was used for analysis.

Method validation
The specificity of the method was tested by screening six different batches of blank human plasma. Each blank sample was tested for interferences in selected ion monitoring (SIM) channels by using the proposed preparation procedure and chromatographic/mass spectrometric conditions, and the results were compared with those obtained for an aqueous solution of the analyte at a concentration near to the LLOQ.

Accuracy was calculated as the mean deviation of each concentration from the theoretical value.

Extraction recovery and matrix effect
The extraction recovery of cefaclor was calculated by comparing the peak areas of extracted standards to those prepared in mobile phase at corresponding concentrations. The matrix effect was evaluated by comparing the peak areas of post-extraction plasma spiked with analytes to those prepared in mobile phase at corresponding concentrations.

The stability was assessed at three concentration levels (5, 200 and 5,000 ng/mL). The freeze and thaw stability samples at three concentrations were frozen (~80 °C) and thawed (room temperature) three times before these samples were analyzed using the previously described method. The stability of cefaclor in plasma at ambient temperature was assessed by processing and analyzing plasma samples in triplicate after storage for 6 h on the laboratory bench.

To assess the stock solution stability of cefaclor and the IS, five aliquots were diluted separately from the stock solutions of cefaclor and the IS, which were kept at 4 °C for 1 month. The mean peak areas of cefaclor and the IS were compared with those from solutions at the same concentration from freshly prepared stocks.

Application of the method
Ethical permission for the study was obtained from the Health Authority Ethics Committee of the First Affiliated Hospital of Soochow University. Twenty adult male volunteers were included, aged between 21–31 years (mean 26 years, medium 26 years), weighing between 55 and 71 kg (mean 64 kg, median 63 kg) and with height between 1.58 and 1.75 m (mean 1.71 m, median 1.71 m). All volunteers provided written consent prior to their participation in the study.

The volunteers were hospitalized for at least 24 h before the investigation and maintained under standard conditions. Approximately 2 mL blood samples were collected into BD vacutainers with K2-EDTA before administration and 30 min, 1, 1.5, 2, 2.5, 3, 3.5, 4, 6, 8 and 12 h after the administration of 0.375 g of a cefaclor released tablet (Eli Lilly). The blood samples were centrifuged at 4,000 rpm for 5 min and plasma was separated and added to 10% acetic acid (20:1, v/v), which increases the stability of cefaclor (1); these were stored immediately at ~80 °C until analysis.

Statistical analysis of data
The pharmacokinetic (PK) parameters for cefaclor were evaluated with the non-compartment model by DAS 2.0. Results were presented as mean ± SD unless stated otherwise.

Results
LC–MS–MS method
The ESI produced optimum sensitivity for cefaclor in positive ionization mode. The product ion scans of the [M + H]⁺ ions of

<table>
<thead>
<tr>
<th>Table I</th>
<th>Gradient Elution Program for the Separation of Cefaclor*</th>
</tr>
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<tbody>
<tr>
<td>Time</td>
<td>Row</td>
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<tr>
<td>0</td>
<td>0.4</td>
</tr>
<tr>
<td>2.5</td>
<td>0.4</td>
</tr>
<tr>
<td>3</td>
<td>0.4</td>
</tr>
<tr>
<td>6</td>
<td>0.4</td>
</tr>
<tr>
<td>6.2</td>
<td>0.4</td>
</tr>
<tr>
<td>7.5</td>
<td>0.4</td>
</tr>
</tbody>
</table>

*Acetonitrile (A); 0.1% formic acid (B).
Cefaclor \((m/z\ 367.5)\) and flucloxacillin \((m/z\ 454.1)\) displayed clear and abundant product ions at \(m/z\ 173.8\) and 160.3, respectively (Figure 1).

Two channels were used for recording: Channel 1 for the cefaclor with a retention time of 3.6 min and Channel 2 for flucloxacillin with a retention time of 5.7 min. As shown in Figure 2, cefaclor and the IS were well separated with excellent peak shapes, and no interfering peaks were observed in the plasma or any samples.

**Method validation**

**Specificity**
The selectivity was assessed by comparing the chromatograms of six different batches of plasma samples with the corresponding spiked plasma. As shown in Figure 2, there was no interference from endogenous substances at the retention times of the analytes.

**Matrix effect**
The possibility of a matrix effect, caused by ionization competition between the analytes and co-eluents, exists when using MS-MS for analysis. To evaluate the matrix effect, the peak areas of plasma extracts spiked with the analyte after extraction \((A)\) were compared with those of the standard solutions dried directly and reconstituted with the mobile phase \((B)\). All ratios \((A/B \times 100\%)\) were between 85 and 115\%, which meant no significant matrix effects for cefaclor and the IS in this method. Thus, ion suppression or enhancement from the plasma filtrate matrix was negligible for this method.

**Figure 1.** Full scan and daughter scan product ion mass spectra of \([M + H]^+\) of: cefaclor (A); flucloxacillin (B).
Sensitivity and linearity

The linear regression of the peak area ratios versus concentrations was fitted over the concentration range of 2–10,000 ng/mL for cefaclor in human plasma. A typical equation for the calibration curve was $y = 4.4561x + 0.258352$ ($r = 0.9981$).

The LLOQ for cefaclor was 2 ng/mL with 7.5 μL injected onto the UPLC column; as shown in Figure 2B, the response was 48 times the blank response.

Accuracy and precision

The precision and accuracy data are presented in Tables II and III. The intra-batch precision values [relative standard deviations (RSDs)] for QC samples (5, 200 and 5,000 ng/mL) were 10.7, 3.7 and 8.0%, and those of inter-batch analyses were 8.9, 5.8 and 8.1% with an accuracy [relative error (RE)] within 85–115%. These data indicated the adequate precision and accuracy of the present method for the determination of cefaclor in human plasma.

Recovery and stability

The extraction recovery values of cefaclor at concentration levels of 5, 200 and 5,000 ng/mL from plasma were 73.52 ± 6.04%, 83.90 ± 2.57% and 82.02 ± 3.09%, respectively.

The results of stability experiments showed that no significant degradation occurred during the chromatography, extraction and sample storage of the plasma samples of cefaclor. QC samples of cefaclor obtained by extraction showed no significant degradation after at least 6 h at room temperature. The concentration variations found after three cycles of freezing and thawing were within ±15% of nominal concentrations, indicating no significant substance loss after three repeated freezing and thawing cycles. When processed samples were stored at 4°C in the autosampler, cefaclor showed very good stability and the responses varied no more than ±15.0% within 24 h of storage at the studied concentrations.

The stock solution stability of cefaclor and the IS showed no significant degradation after 1 month at 4°C.

### Table II

<table>
<thead>
<tr>
<th>Nominal concentration (ng/mL)</th>
<th>Determined concentration (mean ± SD, ng/mL)</th>
<th>Accuracy (%)</th>
<th>Precision (RSD, %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>4.93 ± 0.53</td>
<td>96.6</td>
<td>10.7</td>
</tr>
<tr>
<td>200</td>
<td>208.99 ± 7.62</td>
<td>104.5</td>
<td>3.7</td>
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<tr>
<td>5,000</td>
<td>5,320.04 ± 424.35</td>
<td>106.4</td>
<td>8.0</td>
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</table>

### Table III

<table>
<thead>
<tr>
<th>Nominal concentration (ng/mL)</th>
<th>Determined concentration (mean ± SD, ng/mL)</th>
<th>Accuracy (%)</th>
<th>Precision (RSD, %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>5.16 ± 0.46</td>
<td>103.2</td>
<td>8.9</td>
</tr>
<tr>
<td>200</td>
<td>203.74 ± 11.74</td>
<td>101.9</td>
<td>5.8</td>
</tr>
<tr>
<td>5,000</td>
<td>5,053.63 ± 408.73</td>
<td>101.1</td>
<td>8.1</td>
</tr>
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</table>

Figure 2. Representative MRM chromatograms of cefaclor (peak 1, channel 1), flucloxacillin (peak 2, channel 2) in plasma: a blank plasma sample (A); a blank plasma spiked with cefaclor at the LLOQ of 2 ng/mL and IS (B); plasma sample from a volunteer after administration of cefaclor (C). The retention times for cefaclor and flucloxacillin were 3.6 and 5.7 min, respectively.
Results of PK study

The method was applied to the determination of cefaclor in Chinese healthy volunteers after administration of the dosage (0.375 g) of a released tablet of cefaclor. The mean plasma concentration–time profile for cefaclor is presented in Figure 3. The PK parameters are shown in Table IV.

Table IV

Areas under Curve, Terminal Half-Life, Maximum Concentration and Time to Reach Maximum Concentration* for Cefaclor in Human Plasma Following 0.375 g of Cefaclor

<table>
<thead>
<tr>
<th>PK parameters</th>
<th>t1/2 (h)</th>
<th>Cmax (µg/L)</th>
<th>Tmax (h)</th>
<th>AUC0−t (µg/L/h)</th>
<th>AUC0−∞ (µg/L/h)</th>
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</thead>
<tbody>
<tr>
<td>Mean</td>
<td>1.056</td>
<td>5,281.4</td>
<td>2.2</td>
<td>14,228.6</td>
<td>14,236.7</td>
</tr>
<tr>
<td>SD</td>
<td>0.244</td>
<td>1,351.8</td>
<td>0.7</td>
<td>3,105.9</td>
<td>3,108.0</td>
</tr>
<tr>
<td>RSD (%)</td>
<td>23.1</td>
<td>25.6</td>
<td>33.6</td>
<td>21.8</td>
<td>21.8</td>
</tr>
</tbody>
</table>

*PK parameters: area under curve from time zero to the last measurable concentration [AUC0−t], area under curve from time zero to infinity [AUC0−∞], terminal half-life [t1/2], maximum concentration [Cmax] and time after administration when maximum concentration was reached [Tmax].

Discussion

In previous papers, almost all of the reported methods for cefaclor only satisfied the needs of a PK study of the conventional immediate release formulation; these methods can only determine 6–8 h samples and cannot satisfy the needs of a study of the sustained-release formulation of cefaclor.

In this method, the very narrow chromatographic peaks produced by UPLC, in addition to the application of a gradient elution mode, resulted in an increase in the chromatographic efficiency and sensitivity and a decrease in the matrix effect. Therefore, the LLOQ for cefaclor was 2 ng/mL, which can be determined for 12 h samples and can totally satisfy the needs of a study of the sustained-release formulation of cefaclor. In addition, the total run time was 7.5 min per sample, which meets the requirements for a high sample throughout.

The method was validated for specificity, linearity, precision, accuracy, extraction recovery, matrix effect and stability according to Food and Drug Administration guidance for the validation of bioanalytical methods. The results of method validation all complied with this guidance.

Conclusion

A sensitive, simple and rapid HPLC–MS-MS method is described for the determination of cefaclor in human plasma. Compared with the published methods, one simple step of PPT and a gradient mobile phase provides higher sensitivity with an LLOQ of 2 ng/mL. The method was successfully applied to a PK study of the sustained-released cefaclor tablets in Chinese healthy volunteers.

References


Figure 3. Average concentration of cefaclor versus time in human plasma following 0.375 g of cefaclor.