Simultaneous Liquid Chromatography–Mass Spectrometry Quantification of Cefixime and Clavulanic Acid in Human Plasma

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A simple and specific liquid chromatography–atmospheric pressure chemical ionization–mass spectrometry (LC–APCI–MS) assay method has been developed and fully validated for the simultaneous quantification of cefixime (CX) and clavulanic acid (CA) in human plasma. Analytes and internal standard were extracted from human plasma by a solid phase extraction technique using a Sam prep (3 mL, 100 mg) extraction cartridge. The extracted samples were chromatographed on a reverse phase C18 column using a mixture of methanol: acetoniitrite: 2 mM ammonium acetate (pH 3.5) (25:25:50, v/v/v) as the mobile phase at a flow rate of 0.8 mL/min. Quantification of the analytes were carried out using single quadrupole LC–APCI–MS through selected ion monitoring at m/z 452 and 198, respectively, for CX and CA. The assay was linear over the concentration range of 0.05–10.0 and 0.1–10.0 μg/mL, respectively, for CX and CA. The mean plasma extraction recoveries of the CX and CA were found to be 95.20–96.27% and 94.67–95.58%, respectively. The method was successfully applied for the determination of pharmacokinetics of CX and CA after oral administration of single dosage CX/CA (200/125 mg) pill to the humans (n = 12).

Introduction

Cefixime (CX) ((6R,7R)-7-[[2-(2-amino-1,3-thiazol-4-yi)-2-(carboxy methoxyimino)acetyl]amino]-3-ethenyl-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid) is a broad-spectrum third-generation cephalosporin. It has significant activity against both gram-positive and gram-negative bacteria. Clavulanic acid (CA) ((Z)-(2R,5R)-3-(2-hydroxyethylidene)-7-oxo-4-oxa-1-azabicyclo [3.2.0]heptane-2-carboxylate) is a β-lactamase inhibitor. Plasmid-mediated β-lactamases are responsible for transferred drug-resistance to β-lactam antibiotics, such as penicillin and cephalosporin. CA covalently binds to a serine residue in the active site of the β-lactamase and inhibits them. This inhibition restores the antimicrobial activity of β-lactam antibiotics against lactamase-secreting resistant bacteria.

A number of analytical methods have been reported for quantification of CX (1–10) and CA (11–21) individually or with other drugs in biological samples. Falkowski et al. (1) developed an isotropic high-performance liquid chromatography (HPLC) method for the determination of CX in human serum with the minimum quantifiable limit of 0.05 μg/mL. Liu et al. (2) described a double column and double pump HPLC switching system for the analysis of CX in human plasma with the detection limit of 0.05 μg/mL. Pisarev et al. (3) reported a method of HPLC for comparative study of CX in two different dosage forms in plasma. The reported lower limit of quantification (LLOQ) is 0.06 μg/mL. Dhoka et al. (4) and Khan et al. (5) developed a HPLC method for simultaneous determination of CX with dicloxacillin sodium and cefdinir, respectively.

Owing to its poor absorption in a UV region, early HPLC methods for the estimation of CA involved precolumn derivatization steps (11, 12), ion pair technology using the bathochromic shift of tetraethylammonium bromide (13, 14) and amperometric detection (15). Tsou et al. (16) developed a HPLC method for simultaneous determination of amoxicillin (amox) and CA using β-cyclodextrin stationary phase. It involves the use of tetracyethylammonium acetate as an additive reagent. Foroutan et al. (6) and Hoizey et al. (17) reported a HPLC method for simultaneous determination of amox and CA in human plasma. To the best of our information, only one method has been reported for the simultaneous determination of CX and CA. Khan et al. (22) reported a HPLC method for the simultaneous determination of CX and CA.

Liquid chromatography–mass spectrometry (LC–MS) methods provide high sensitivity and avoid tedious derivatization steps. Meng et al. (10) developed a sensitive LC–MS/MS method for CX with LLOQ of 0.05 μg/mL in human plasma. Yoon et al. (20) reported a LC–MS method for simultaneous determination of amox and CA in human plasma. The LLOQ is found to be 0.12 μg/mL for amox and 0.062 μg/mL for CA. Reins et al. (21) reported a method for the quantification of CA in calf plasma using LC–MS/MS in negative ionization mode with a quantification limit of 25 ng/mL.

To date, no LC–MS method has been reported for the simultaneous determination of CX and CA in human plasma. Our study describes a simple and selective method, which employs a simple solid phase extraction (SPE) technique for sample preparation and LC–MS for the simultaneous quantitation of CX and CA in human plasma. The method was optimized, validated and applied to analyze the plasma samples obtained after oral administration of 200/125 mg fixed-dose combination of CX/CA pill.

Experimental

Chemicals and reagents

Working standards of CX (99.9% pure), potassium clavulanate (99.9% pure), chloramphenicol (99.9% pure) internal standard (IS) and CX/CA (200/125 mg) pills were obtained from Twenty First Century Pharmaceuticals (Chennai, India). Water used for the LC–MS analysis was prepared from Milli Q water purification system procured from Millipore (Bangalore, India). HPLC grade methanol and acetonitrile were procured from Merck (Mumbai, India). All other chemicals and solvents of analytical grade were purchased from Merck (Mumbai, India). A Sam
Preparation of calibration standards and quality control samples
Primary stock solution of CX (1.0 mg/mL) and CA (1.0 mg/mL) was prepared in a mixture of acetonitrile : water (1:1). Secondary working stock solutions of CX and CA were prepared by diluting their primary stock solutions. The working IS solution was prepared in the same way, giving a concentration of 100.0 μg/mL. Calibration standards of CX and CA were prepared by serial dilution with blank human plasma, yielding final concentrations of 0.05, 0.5, 1.0, 2.0, 4.0, 6.0, 8.0, and 10.0 μg/mL and 0.1, 0.5, 1.0, 2.0, 4.0, 6.0, 8.0, and 10.0 μg/mL, respectively. Quality control samples (QCs) for CX (0.15, 5.0 and 9.0 μg/mL) and CA (0.3, 5.0 and 9.0 μg/mL) were freshly prepared by diluting appropriate amounts of working stock solutions into drug-free human plasma and used for method validation.

Sample processing
A 500 μL aliquot of human plasma sample was mixed with 500 μL of the IS working solution (100 μg/mL) and vortexed for 10 s. The sample mixture was loaded onto a 3 mL (100 mg) extraction cartridge that was preconditioned with 1 mL of methanol followed by 1 mL water. The extraction cartridge was washed with 2 mL of water. CX, CA and IS were eluted with 0.5 mL of mobile phase. An aliquot of 20 μL of the extract was injected into the LC–MS.

LC–MS analysis
Shimadzu LCMS-2010A (Shimadzu Corporation, Kyoto, Japan) consisting of a Phenomenex C18 column (150 × 4 mm i.d., 5 μm), LC-10 AD-Vp solvent delivery system (pump), SIL 10 AD-Vp auto injector, CTO 10 Vp column oven and DGU 14AM de gasser was used for the study. Aliquots of the processed samples (20 μL) were injected into the column, which was kept at 20°C. The isocratic mobile phase, a mixture of methanol : acetonitrile : 2 mM ammonium acetate (pH 3.5) (25 : 25 : 50, v/v/v), was delivered at 0.8 mL/min into the atmospheric pressure chemical ionization (APCI) chamber of the mass spectrometer. Quantitation of CX and CA was performed on the single quadrupole mass spectrometer in the selected ion monitoring (SIM) mode, at m/z 452, 198 and 322, respectively, for the CX, CA and IS. The analysis data obtained were processed by LC–MS solutions data station.

Method validation
Method validation of CX and CA in human plasma was performed as per the US FDA guidelines (23). The assay was validated for specificity, linearity, sensitivity, accuracy, precision, extraction recovery, matrix effect, ion suppression and stability.

Specificity
The specificity of the method was evaluated by comparing blank plasma samples collected from six different humans with the corresponding spiked plasma samples to investigate the potential interferences at the LC peak region for analytes and IS.

Linearity and sensitivity
The linearity was tested for CX and CA in the concentration range of 0.05–10.0 and 0.1–10.0 μg/mL, respectively. For the determination of linearity, standard calibration curves containing eight points (non-zero standards) were plotted and checked. The acceptance limit of accuracy for each of the back-calculated concentrations is ±15% except LLOQ, where it is ±20%. For a calibration run to be accepted at least 67% of the standards, the LLOQ and upper limit of quantification (ULOQ) are required to meet the acceptance criteria, otherwise the calibration curve was rejected. Six replicate analyses were performed on each calibration standard. The LLOQ was measured for CX and CAs as the lowest analyte concentration with signal-to-noise ratio >10 (S/N ≥ 10) that can be determined with an accuracy and precision < ±20%.

Accuracy and precision
Intra- and interday assay precision and accuracy were determined by analyzing six replicates at three different QC levels on three independent days. The acceptance criteria included accuracy within ±15% from the nominal values and a precision of ±15% coefficient of variation (CV).

Extraction recovery
The extraction recovery of CX and CA from the human plasma after the extraction procedure was assessed in three QCs (0.15, 5.0 and 9.0 μg/mL, for CX; 0.3, 5.0 and 9.0 μg/mL, for CA), whereas IS was determined at a concentration of 100.0 μg/mL. Recoveries of CX, CA and IS were determined by comparing the peak area of extracted analyte standard (A) with the peak area of non-extracted standard (B) at the same nominal concentrations. The ratio (A/B × 100) was defined as the extraction efficiency.

Matrix effect and ion suppression
Matrix effects were evaluated by comparing peak areas of post-extraction blank plasma spiked with QC solutions with peak areas of post-extraction aqueous blank samples spiked with QC solutions. The acceptance criteria included matrix effect should be within ±15% from the nominal values. The degree of ion suppression was checked by a postcolumn infusion experiment. A standard solution containing analytes and IS was infused through a syringe pump at 20 μL/min. Once the baseline stabilizes, an injection of extracted blank plasma was made and SIM chromatograms were acquired for analytes and IS.

Stability
The stability of CX and CA in the biological matrix was determined by the analysis of six replicates of QCs (n = 3) exposed...
to various storage conditions. For freeze–thaw stability studies, QCs were subjected to freeze–thaw cycles (three cycles). Each sample was stored at −20°C for 24 h and thawed at room temperature, after which the samples were refrozen for 12–24 h under the same conditions. At the end of each cycle, the samples were processed, analyzed and compared with the freshly prepared QCs. For the short-term and stock solution stability study, QCs were kept at 25°C for 6 h and samples were processed at different time points and were analyzed and compared with the freshly prepared QCs. To assess the long-term stability, QCs were stored at −20°C for 3 months, which exceeds the time between sample collection and sample analysis.

Pharmacokinetic study

The Institutional Ethics Committee approved the study protocol. Twelve healthy Indian male subjects with age group of 20–40 years and weight of ±15% from ideal weight for subjects’ height and elbow breadth were included in the study. The subjects were certified as healthy subjects by physician based on biochemical, hematological and vital physiological parameters. Subjects were excluded from the study, if one or more, following criteria that were present at the time of medical screening: allergic to antibiotic, history or clinical data of renal or liver disease, positive test for hepatitis B, HIV, history of alcohol, drug addiction or donated blood within 72 days prior to the study. Blood samples (5 mL) were collected following oral administration of single dosage 200/125 mg fixed-dose combination of CX/CA pill at predose and 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 5.0, 6.0, 8.0, 10.0 and 12.0 h in heparinized glass vials. The blood samples were immediately centrifuged at 4000 rpm for 10 min at room temperature. The separated plasma samples were stored at −20 ± 2°C till the analysis. Plasma samples were spiked with the IS and processed as per the extraction procedure described earlier. Clinical samples were assayed by the validated LC–MS method. Plasma concentration–time profile of CX and CA was calculated by using pharmacokinetic solutions software (Add-on program to Microsoft excel). An incurred sample re-analysis (ISR) was also conducted by selecting the 12 subject samples (two samples from each subject) near T_{max} and the elimination phase (24). The percentage difference included in the ISR should be with in ±20% from the nominal values. The variability (percentage difference) should be calculated using the mean of the original and repeat results as described by the following formula

\[
\text{Variability(\%)} = \frac{\text{Repeat} - \text{Original}}{\text{Mean}} \times 100
\]

Results

LC–MS analysis

LC–APCI–MS parameters were tuned in both positive and negative ionization modes for the analytes. Good response was achieved in negative ionization mode. Figure 1A and B shows the deprotonated molecular ions of CX (m/z 452 [(M–H)−]) and potassium salt of CA (m/z 198 [(M–K)−]), respectively, without any evidence of adduct formation. Owing to its high selectivity, quantitation of CX and CA were carried out through SIM mode at m/z 452 and 198, respectively. The chromatographic conditions, especially the composition of mobile phase, were optimized through a number of trials to achieve good resolution and symmetric peak shapes for the analytes and IS. Separation was attempted using various combinations of methanol, acetonitrile and buffer on a Phenomenex C18 column. It was found that a mixture of methanol:acetonitrile : 2 mM ammonium acetate (pH 3.5) (25 : 25 : 50, v/v/v) could achieve this purpose and was finally adopted as the mobile phase.

LC–MS method validation

Specificity

The degree of interference by endogenous plasma constituents with the analytes and the IS was assessed by inspection of chromatograms derived from the processed blank plasma sample. As shown in Figure 2, no significant direct interference in the blank plasma traces was observed from endogenous substances in drug-free plasma at the retention time of the analytes and IS.

Extraction recovery

A simple SPE with Sam prep, C18 (3 mL, 100 mg) extraction cartridge using mobile phase as an eluent was provided cleanest samples. The recoveries of analytes and the IS were good and
reproducible. The mean \( n = 6 \) percent recovery values of CX and CA were 95.20–96.27% and 94.67–95.58%, respectively (Table I).

Matrix effect and ion suppression
The assessment of matrix effect constitutes an important and integral part of validation for quantitative LC–MS for supporting the pharmacokinetics studies. In the study of matrix effect, the nominal concentration of CX and CA at three QC levels was ranged from 92.7 to 94.2% and 93.8 to 94.6%, respectively, and their precision (%CV) values were ranged from 4.5 to 5.4% and 4.3 to 5.2%, respectively, indicating that no significant signal suppression or enhancement occurred in the ionization of CX and CA.

Linearity and sensitivity
Linear calibration curves were obtained over the concentration range of 0.05–10.0 and 0.1–10.0 \( \mu \)g/mL for CX and CA.
respectively. A typical regression equation for the calibration curve for CX and CA was found to be $y = 0.45x - 0.048$ and $y = 0.23x + 0.013$, respectively. The regression coefficient ($r^2$) for both the analytes was found to be >0.999. The lowest limit of reliable quantification for the analytes was set at the concentration of the LLOQ and it was found to be 0.05 $\mu$g/mL for CX with 95.64% accuracy and 5.28% precision, whereas CA showed LLOQ of 0.1 $\mu$g/mL with 94.82% accuracy and 4.53% precision.

**Accuracy and precision**

Accuracy and precision data for intra- and interday plasma samples for CX and CA were presented in Table I. The assay values on both the occasions (intra- and interday) were found to be with in the accepted variable limits.

**Stability**

Stability of CX and CA was tested by analysis of QCs ($n = 3$) under the following conditions: freeze–thaw stability (three cycles) at $-20 \pm 2^\circ C$, short-term stability at $-25^\circ C$ for 6 h, long-term stability at $-20 \pm 2^\circ C$ for 3 months and stock solution stability at $-25^\circ C$ (Table II).

**Application of the method**

The validated method was applied for quantitative estimation of CX and CA in plasma samples obtained from the healthy male volunteers ($n = 12$) after oral administration of 200/125 mg fixed-dose combination of CX/CA pill. The mean plasma concentration–time profile for CX and CA is shown in Figure 3. The pharmacokinetic parameters such as $C_{\text{max}}$, $T_{\text{max}}$, $T_{1/2}$, $K_{\text{el}}$, $AUC_{0-12h}$, and $AUC_{0-\infty}$ for CX was $1.85 \pm 0.22 \mu$g/mL, 2.5 h, 3.58 $\pm$ 0.24 h, 0.19 $\pm$ 0.31 h$^{-1}$, 4.97 $\pm$ 0.52 and 6.58 $\pm$ 0.25 $\mu$g/mL, respectively, whereas for CA, 5.87 $\pm$ 0.77 $\mu$g/mL, 2 h, 2.67 $\pm$ 0.53 h, 0.26 $\pm$ 0.42 h$^{-1}$, 17.51 $\pm$ 1.61 and 18.78 $\pm$ 0.74 $\mu$g/mL, respectively.

**Incurred sample re-analysis**

The ISR was performed using two plasma samples from each subject and re-assayed in a separate batch run. The difference in concentration between the ISR and initial value for all the tested samples is summarized in Table III.
Simultaneous LC–MS Quantification of CX and CA in Human Plasma

Discussion

Previously described methods for estimation of CX and CA individually that are in combination with other drugs are cumbersome to execute bioavailability study and show less sample throughput with long sample run time (2, 4, 5, 17, 19). In the present method, CX, CA and IS were separated using a simple mixture of methanol : acetonitrile : 2 mM ammonium acetate (pH 3.5) (25 : 25 : 50, v/v/v) as the mobile phase at a flow rate of 0.8 mL/min and that results high sample throughput (total analytical run 5 min). To optimize APCI–MS conditions for detection of analytes based on monitoring of the product ions for both the positive and negative ion modes were investigated. Both the analytes showed maximum response in negative ionization mode. Plasma sample preparation is an important step, employed with an aim to remove interferences from biological samples using a simple procedure having suitable recovery. Liquid–liquid extraction, protein precipitation and SPE are the most widely used sample preparation techniques during the development. In this study, three methods were investigated and compared at different conditions individually. Even though the protein precipitation technique is simple, all the samples were processed with SPE, because of its less matrix effect and more recovery of the analytes when compared with other two methods. In the quantitative analysis of analytes in biological samples, an appropriate IS is needed. Several compounds were investigated to find a suitable IS. Chloramphenicol was chosen because of similar chromatographic behaviors and extraction characteristics. The results of method validation using chloramphenicol as the IS was acceptable in this study.

The present method is accurate and precise for both the analytes in plasma and as seen from the inter- and intraday precision and accuracy, it is reproducible. Day-to-day reproducibility is demonstrated by the intra- and inter-batch analysis of the QCs (n = 3). Intra- and interday precision values of CP and CA, expressed as % CV, ranged from 4.9 to 6.9% and 4.5 to 6.5%, respectively, for CX and 4.9–6.4% and 4.3–4.9%, respectively, for CA, whereas accuracy values expressed as % nominal, ranged from 94.9 to 96.2% and 89.9 to 90.9%, respectively, for CX and 93.3–94.2% and 86.7–89.8%, respectively, for CA. The linear response of the method in the range of the concentrations used was evaluated. A good determination coefficient (r² > 0.999) with the calibration curve was obtained for the both the analytes. Analytes were found to be stable under the different storage conditions tested. The results were found to be within the acceptable limits, i.e. mean % nominal values of the analytes were within ± 15% of the predicted concentrations at their three QC levels. The mean % nominal values for free—thaw (three) cycles were ranged from 91.3 to 92.6% and 92.5 to 93.4%, respectively, for CX and CA. The short-term stability of the extracted sample was found to be stable for at least 6 h, when stored at −25 °C. The long-term stability at −20 ± 2 °C was found to be stable in plasma for a period of 3 months with mean % nominal values ranged from 85.4 to 86.8% and 85.3 to 87.1%, respectively, for CX and CA.

Khan et al. (22) developed a HPLC for the simultaneous determination of CA and CX in the synthetic mixture form. The method exhibited linearity in the concentration ranges of 12.5–62.5 and 20–100 µg/mL for CA and CX, respectively. This sensitivity is adequate for the estimation of analytes in synthetic mixtures, but for the bioanalytical estimation high sensitivity is required. As per the US FDA guidelines, bioequivalence study protocols generally recommend plasma sample collection for a time period, corresponding to three to four times the drug plasma elimination half-life, which brings terminal concentration values of ~6% of the peak concentration value. The mean peak plasma concentration of CX and CA were 1.85 and 5.87 µg/mL, respectively, and

<table>
<thead>
<tr>
<th>Table III</th>
<th>Incurred Samples Analysis Data</th>
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<tbody>
<tr>
<td>Subject number</td>
<td>Cefoxime</td>
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<tr>
<td></td>
<td>Original concentration (µg/mL)</td>
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<tr>
<td>1 2.5 1.85</td>
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<tr>
<td>10 0.32</td>
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<tr>
<td>2 2.5 1.86</td>
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<td>0.30</td>
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<tr>
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<tr>
<td>10 0.36</td>
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</table>

*aExpressed as (repeat − original concentration)/ (mean concentration) × 100.
their plasma concentrations followed by four half-lives were 0.116 and 0.367 μg/mL, respectively. The present method LLOQ was 0.05 μg/mL for CX and 0.1 μg/mL for CA, which is adequate for determining the pharmacokinetics of CX and CA. The presence of double peaks in the plasma concentration–time profile was observed. This could be because of enterohepatic recirculation, selective and differential absorption from gastrointestinal tract, formation of a deposit on the intestinal wall and/or variation in gastrointestinal motility. The pharmacokinetic parameters such as C_{max}, T_{max}, T_{1/2}, K_{el}, AUC_{0–12 h} and AUC_{0–∞} were calculated and these values were in close proximity when compared with earlier reported values (25–30).

Assay reproducibility for incurred samples was the defining moment in establishing ISR as a mandatory exercise in demonstrating assay reproducibility using dosed subject samples. The difference in concentration between the ISR and initial value for all the tested samples were <20%, indicating good reproducibility of the present method.

Conclusion
A simple, specific and accurate LC–MS method for the simultaneous quantification of CX and CA in human plasma was developed and fully validated as per the FDA guidelines. Processing of the plasma sample using the SPE technique was proved to be rugged and provided cleanest samples with good recoveries. The present method showed suitability for clinical studies and it was successfully applied for analysis of CX and CA in plasma samples obtained after oral administration of CX/CA (200/125 mg) pill. The sensitivity and simplicity of the method make it suitable for bioavailability and bioequivalence studies.

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