Simultaneous pharmacokinetic assessment of cefadroxil and clavulanic acid in human plasma by LC–MS and its application to bioequivalence studies

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Abstract A simple, rapid and selective liquid chromatography–atmospheric pressure chemical ionization–mass spectrometry (LC–APCI–MS) assay method has been developed and fully validated for the simultaneous quantification of cefadroxil (CF) and clavulanic acid (CA) in human plasma. Analytes and internal standard (IS) were extracted from human plasma by solid-phase extraction (SPE) technique using Sam prep (3 mL, 100 mg) extraction cartridge. The extracted samples were chromatographed on a reverse phase C18 column using a 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. Quantification of the analytes and IS were carried out using single quadrupole LC–APCI–MS through selected-ion monitoring (SIM) at m/z 362 and m/z 198, for CF and CA, respectively. Method validation was performed as per the FDA guidelines and the results met the acceptance criteria. Plasma concentration of CF and CA followed by the oral administration of CF/CA (500/125 mg) pill to healthy male volunteers (n=12) was measured. Area under plasma concentration–time curve from 0 to 12 h (AUC0–12 h) and 0 h extrapolated to infinity (AUC0–∞) were calculated. The ratio of AUC0–12 h/AUC0–∞ was found to be >85% for all the subjects, as recommended by the FDA guidelines.

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1. Introduction

Cefadroxil (CF) (8-[2-amino-2-(4-hydroxyphenyl)-acetyl]amino-4-methyl-7-oxo-2thia-6-azabicyclo[4.2.0]oct-4-ene-5-carboxylic acid, C16H17N3O5S, MW 363.3), is a broad-spectrum first generation cephalosporin, used in the treatment of urinary tract infections. 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, C_9H_9NO_5, MW 199.2), is a beta-lactamase inhibitor. Plasmid mediated beta-lactamases are responsible for transferred drug resistance to beta-lactam antibiotics, such as penicillin and cephalosporin’s. CA covalently binds to a serine residue in the active site of the beta-lactamase, and inhibits them. This inhibition restores the antimicrobial activity of beta-lactam antibiotics against lactamase-secretting resistant bacteria.

A number of analytical methods have been reported for quantification of CF [1–17] and CA [18–22] individually or with other drugs in biological samples. To the best of our information, only one method has been reported for the simultaneous determination of CF and CA [23]. To date, no liquid chromatography mass spectrometry (LC–MS) method has been reported for the simultaneous determination of CF and CA in human plasma. The present work describes a simple, selective and sensitive method, which employs a simple solid-phase extraction (SPE) technique for sample preparation and LC–MS for the simultaneous quantitation of CF and CA in human plasma. The application of this assay method to a clinical pharmacokinetic study in healthy male volunteers following oral single pill administration of CF and CA is described.

2. Experimental

2.1. Chemicals and reagents

Working standards of CF (99.9% pure), potassium clavulanate (99.9% pure), chloramphenicol (99.9% pure) (Internal standard, IS) and CF/CA (500/125 mg) pills were obtained from Macleods Pharmaceuticals Pvt. Ltd. (Mumbai, 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). Sam prep (3 mL, 100 mg) extraction cartridge was purchased from Ranbaxy fine chemicals Ltd. (New Delhi, India). Pooled plasma was collected from healthy, drug-free volunteers and stored at −20 ± 2 °C until analysis.

2.2. Preparation of calibration standards and quality control samples

Calibration samples were prepared by spiking 950 μL of control human plasma with the appropriate working standard solution of the each analyte (25 μL dilution of CF and 25 μL of CA). Calibration curve standards consisting of a set of eight non-zero standards were plotted and solvents of analytical grade were purchased from Merck (Mumbai, India). Sam prep (3 mL, 100 mg) extraction cartridge was purchased from Ranbaxy fine chemicals Ltd. (New Delhi, India). Pooled plasma was collected from healthy, drug-free volunteers and stored at −20 ± 2 °C until analysis.

2.3. 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 Sam prep (3 mL, 100 mg) extraction cartridge that was pre-conditioned with 1.0 mL of methanol followed by 1.0 mL water. The extraction cartridge was washed with 2.0 mL of water. CF, CA and IS were eluted with 0.5 mL of mobile phase. Aliquot of 20 μL of the extract was injected into the LC–MS.

2.4. LC–MS analysis

Shimadzu LCMS-2010A (Shimadzu Corporation, Kyoto, Japan) consisting of Phenomenex C18 column (150 mm × 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, 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 CF, CA and IS was performed on the single quadrupole mass spectrometer in the negative ion mode. The ion source temperature, desolvation temperature, and the capillary voltage were set at 400 °C, 250 °C, and 1.3 kV, respectively. Detection of the ions was carried out in the selected-ion monitoring (SIM) mode, at m/z 362 for CF, m/z 198 for CA, and m/z 322 for the IS. The analysis data obtained were processed by LC–MS solutions data station.

2.5. Method validation

Method validation of CF and CA in human plasma was performed as per the US FDA guidelines [26]. The assay was validated for specificity, linearity, sensitivity, accuracy, precision, extraction recovery, matrix effect and stability.

Specificity of the method was determined by analyzing six different lots of human plasma samples. The responses of the interfering substances or background noises at the retention time of CF and CA are acceptable if they are less than 20% of the response of the lowest standard curve point or lower limit of quantification (LLOQ).

Linearity was tested for CF and CA in the concentration range of 5.0–30000.0 and 2.0–12000.0 ng/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 criterion, otherwise the calibration curve was rejected. Six replicate analyses were performed on each calibration standard. Sensitivity was established from the background noise or response from six spiked LLOQ samples. The six replicates should have a precision of ±20% and an accuracy of ±20%.

Intra-, inter-day 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).

Recoveries of CF, 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 \(\frac{A}{B \times 100}\)% was defined as the extraction efficiency. The extraction recovery of CF and CA were determined at concentrations of 15.0 and 5.0 (LQC), 15000.0 and 5000.0 (MQC), and 25000.0 and 9000.0 ng/mL (HQC), respectively, where as for IS was determined at a concentration of 100.0 μg/mL.

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.

Stability of CF and CA in 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 (3 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 ± 1 °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.

2.6. Application

Validated method was applied to measure the plasma concentration of CF and CA in human plasma after oral administration of CF/CA (500/125 mg) pill to healthy male volunteers \(n = 12\).

2.7. Clinical design

A clinical study was performed in healthy male subjects \(n = 12\). The institutional review board of JSS College of Pharmacy, Udhagamandalam, India, approved the protocol and the volunteers provided with informed written consent. The study was conducted in compliance with the ethical guidelines for biomedical research on human participants (2006) published by Indian council of medical research (ICMR), New Delhi. Twelve healthy male subjects were randomized into two groups, consisted of six in each group. A randomized open label crossover study was conducted with two treatment phases, separated by a two-week washout period. Blood samples (5 mL) were collected following oral administration of 500/125 mg fixed dose combination tablet of CF/CA at pre-dose 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.

2.8. Pharmacokinetic analysis

The plasma concentration–time profile obtained was fed into pharmacokinetic solutions, Add-on program to Microsoft excel, to determine the pharmacokinetic parameters. The maximum concentration \(c_{\text{max}}\) and the corresponding peak time \(t_{\text{max}}\) was determined by the inspection of the individual drug plasma concentration–time profiles. The elimination rate constant \(K_{\text{el}}\) was obtained from the least-square fitted terminal log-linear portion of the plasma concentration–time profile. The elimination half-life \(t_{1/2}\) was calculated as \(0.693/K_{\text{el}}\). The area under the concentration–time curve from 0 to 12 h \(\text{AUC}_{0–12 \text{ h}}\) was calculated by the linear trapezoidal rule. The area under the curve from 0 h extrapolated to infinity \(\text{AUC}_{0–\infty}\) was calculated as \(\text{AUC}_{0–12 \text{ h}} + C_{t}/K_{\text{el}}\) where \(C_{t}\) is the last concentration.

3. Results and discussion

3.1. 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. Fig. 1(A) and (B) shows Fig. 1 Chemical structure and mass spectrum of CF (A) and CA (B).
the deprotonated molecular ions of CF (m/z 362 ([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 CF and CA were carried out through SIM mode at m/z 362 and 198. 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 the 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. The retention time of CF, CA and the IS were 3.5, 2.5 and 4.9 min, respectively.

3.2. Specificity and selectivity

The degree of interference by endogenous plasma constituents with the analytes and the IS was assessed by inspection of chromatograms derived from processed blank plasma sample. As shown in Fig. 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.
3.3. Extraction recovery and matrix effect

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. Mean ($n=6$) percent recovery values of CF and CA were 92.6–94.6% and 93.9–95.7%, respectively (Table 1).

Assessment of matrix effect constitutes an important and integral part of validation for quantitative LC–MS for supporting pharmacokinetics studies. In the study of matrix effects, concentrations of CF and CA were 93.2, 95.8 (LQC), 94.2, 94.6 (MQC) and 92.7, 94.3% (HQC) of nominal concentrations, indicating that no significant signal suppression or enhancement occurred in the ionization of CF and CA.

3.4. Linearity and sensitivity

Linear calibration curves were obtained over the concentration range of 5.0–30000.0 ng/mL and 2.0–12000.0 ng/mL for CF and CA, respectively. A typical regression equation for the calibration curve was found to be $y = \frac{2.9x}{C_0}$ for CF and $y = 0.2x + 0.1$ for CA. The regression coefficient ($r^2$) for both the analytes was found to be $\geq 0.99$.

The lowest limit of reliable quantification for the analytes was set at the concentration of the LLOQ and it was found to be 5.0 ng/mL for CF with 93.5% accuracy and 4.6% precision, while CA showed an LLOQ of 2.0 ng/mL with 92.7% accuracy and 4.2% precision. The present method was proved to be sensitive than the former method [23].

3.5. Accuracy and precision

Accuracy and precision data for intra- and inter-day plasma samples for CF and CA are presented in Table 1. The assay values on both the occasions (intra- and inter-day) were found to be within the accepted variable limits.

3.6. Stability

Stability of CF and CA was tested by analysis of QCs ($n=3$) under the following conditions: freeze–thaw stability (3 cycles) at $-20 \pm 2 ^\circ$C, short-term stability at 25 $^\circ$C, long-term stability at $-20 \pm 2 ^\circ$C for 3 months and stock solution stability at 25 $^\circ$C, the mean% nominal values of the analytes were found to be within $\pm 15\%$ of the predicted concentrations for the analytes at their three QCs levels (Table 2). The results were found to be within the acceptable limits during the entire validation.

3.7. Application

The validated LC–MS method was successfully applied to measure the concentration of CF and CA in plasma samples

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**Table 1** Recovery, accuracy and precision studies of cefadroxil and clavulanic acid in human plasma ($n=6$).

| Analyte          | QCs (ng/mL) | Concentration found (Mean ± SD; ng/mL) | Mean recovery (%) | Intra-day | Inter-day |
|------------------|-------------|----------------------------------------|-------------------|-----------|-----------|
|                  |             | Concentration found (Mean ± SD; ng/mL) |                   | Precision (%) | Accuracy (%) | Precision (%) | Accuracy (%) |
| Cefadroxil       | 15          | 14.1 ± 0.3                             | 93.8              | 4.5       | 93.5      | 3.9          | 82.7         |
|                  | 15,000      | 14187.0 ± 459.7                        | 94.6              | 3.6       | 94.8      | 4.4          | 84.6         |
|                  | 25,000      | 23155.1 ± 664.5                        | 92.6              | 2.8       | 93.5      | 4.8          | 84.3         |
| Clavulanic acid  | 10          | 9.5 ± 0.3                              | 94.8              | 3.9       | 94.4      | 4.0          | 82.7         |
|                  | 5000        | 4787.0 ± 159.9                         | 95.7              | 2.3       | 95.1      | 3.8          | 82.9         |
|                  | 9000        | 8456.4 ± 209.7                         | 93.9              | 1.7       | 95.5      | 3.2          | 84.7         |

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**Table 2** Stability studies of cefadroxil and clavulanic acid in human plasma ($n=6$).

| Stability test         | Cefadroxil | Clavulanic acid |
|------------------------|------------|----------------|
|                        | QCs (ng/mL) | Mean ± SD (ng/mL) | Accuracy (%) | Precision (%) | Mean ± SD (ng/mL) | Accuracy (%) | Precision (%) |
| Freeze–thaw (3 cycles at $-20 \pm 2 ^\circ$C) | 15         | 13.6 ± 0.5 | 90.7 | 3.8 | 10 | 9.3 ± 0.3 | 93.0 | 3.3 |
|                        | 15,000     | 13650.0 ± 886.9 | 91.0 | 4.3 | 5000 | 4577.0 ± 183.1 | 91.5 | 4.0 |
|                        | 25,000     | 23576.0 ± 1155.2 | 94.3 | 4.9 | 9000 | 8294.0 ± 132.7 | 92.2 | 1.6 |
| Short-term (at 25 $^\circ$C for 8 h) | 15         | 13.5 ± 0.6 | 90.0 | 4.2 | 10 | 8.9 ± 0.3 | 89.0 | 3.3 |
|                        | 15,000     | 13363.0 ± 601.3 | 89.1 | 4.5 | 5000 | 4283.0 ± 141.3 | 85.7 | 3.3 |
|                        | 25,000     | 22882.0 ± 892.4 | 91.5 | 3.9 | 9000 | 7868.0 ± 39.3 | 87.4 | 0.5 |
| Long-term (at $-20 \pm 2 ^\circ$C for 3 months) | 15         | 12.8 ± 0.6 | 85.3 | 4.8 | 10 | 7.9 ± 0.3 | 79.0 | 3.3 |
|                        | 15,000     | 12376.0 ± 470.3 | 82.5 | 3.8 | 5000 | 3795.0 ± 98.7 | 75.9 | 2.6 |
|                        | 25,000     | 20554.0 ± 760.5 | 82.2 | 3.7 | 9000 | 7182.0 ± 64.6 | 79.8 | 0.9 |
| Stock solution (at 25 $^\circ$C for 8 h) | 15         | 14.4 ± 0.5 | 96.0 | 3.2 | 10 | 9.4 ± 0.3 | 94.0 | 3.3 |
|                        | 15,000     | 13962.0 ± 349.1 | 93.1 | 2.5 | 5000 | 4834.0 ± 77.3 | 96.7 | 1.6 |
|                        | 25,000     | 23844.0 ± 691.5 | 95.4 | 2.9 | 9000 | 8726.0 ± 165.8 | 96.9 | 1.9 |
obtained from the bioequivalence study. The mean plasma concentration ($n=12$) vs time profile of CF and CA is shown in Fig. 3(A) and (B), respectively and the pharmacokinetic data are listed in Table 3. Bioequivalence study protocols generally recommend plasma sample collection for a time period corresponding to three to four times the drug plasma elimination half-life [26], which brings terminal concentrations values of about 6% of the peak concentration value. Mean peak plasma concentration of CF and CA is about 15500.0 and 5300.0 ng/mL respectively and their plasma concentrations after four half-lives were 970.0 and 330.0 ng/mL, respectively. Since the method LLOQ was 5.0 ng/mL and 2.0 ng/mL for CF and CA, respectively, its sensitivity is adequate for the bioavailability studies. AUC $0-12$ h and AUC $0-N$ were calculated. The ratio of AUC $0-12$ h/AUC $0-\infty$ was found to be $>85$% for all subjects, as recommended by FDA guidelines [24–26].

### Table 3  Pharmacokinetic parameters of cefadroxil and clavulanic acid ($n=12$, Mean $\pm$ SD).

| Parameters | Cefadroxil | Clavulanic acid |
|-----------|------------|-----------------|
| $c_{\text{max}}$ (ng/mL) | $15460.0 \pm 0.1$ | $5270.0 \pm 0.8$ |
| $t_{\text{max}}$ (h) | $1.5 \pm 0.0$ | $2.0 \pm 0.0$ |
| $K_d$ (1/h) | $0.4 \pm 0.3$ | $0.2 \pm 0.2$ |
| $t_{1/2}$ (h) | $1.8 \pm 0.2$ | $3.0 \pm 0.5$ |
| AUC $0-12$ h (ng h/mL) | $31040.1 \pm 0.6$ | $18720.0 \pm 1.3$ |
| AUC $0-\infty$ (ng h/mL) | $32740.4 \pm 0.3$ | $20540.0 \pm 0.9$ |

## 4. Conclusion

A simple, specific, precise and accurate LC–MS method was developed for the simultaneous quantification of CF and CA in human plasma and was fully validated as per the FDA guidelines. Processing of the plasma sample using SPE technique was proved to be rugged and provided cleanest samples with good recoveries. The present method showed suitability for pharmacokinetic studies in humans and it was successfully applied to bioequivalence study of two drug products containing combination of CF/CA. The sensitivity and simplicity of the method makes it suitable for bioavailability and bioequivalence studies and routine therapeutic drug monitoring.

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