A Liquid Chromatography–Mass Spectrometry/Mass Spectrometry Method for the Quantification of Cefixime in Human Plasma

R Arunkumar1*, A Olaganthan2, Nageswara Rao2, V Sankar2, and S Gunasakaran2

1Department of Pharmacology, Chettinad Hospital and Research Institute, Kelambakkam, Tamil Nadu – 603103, India.
2Azidus Laboratories Ltd., School road, Rathnamangalam, Vandalur, Chennai – 600048, Tamil Nadu, India.

Article

ABSTRACT

Cefixime is a third generation cephalosporin effective against gram–positive and gram–negative organisms. It is available in solid and liquid oral dosages of 100 mg, 200 mg and 400 mg. It is used to treat respiratory and urinary tract infections. In this study, a liquid chromatography–Mass spectrometric method to quantify cefixime in human plasma was developed. The quantification range for the method was 114.5033 to 9374.2050 ng/ml and the method was validated as per US FDA standards for pharmaceutical development. Cephalexin was used as the internal standard. Chromatographic and mass spectrometric conditions and extraction procedures were optimized to quantify the levels of cefixime in human plasma accurately. 100 µL of K3EDTA human plasma was required for sample processing. Extraction of cefixime and cephalexin was done by Liquid–Liquid extraction and separation was achieved by reverse phase liquid chromatography. Specificity, selectivity, matrix effect, calibration curve, precision, accuracy, ruggedness, recovery, stability and dilution integrity were established for cefixime in human plasma. The method met acceptance criteria for all the validation parameters and can be successfully applied to human pharmacokinetic and bioequivalence studies of cefixime. The bioanalytical method was highly sensitive and selective for estimation of cefixime in human plasma samples containing the drug.

INTRODUCTION

Cefixime is a semisynthetic, third generation oral cephalosporin having antibacterial activity. Chemically, it is (6R,7R)-7-[2-(2-Amino-4-thiazolyl)glyoxylamido]-8-oxo-3–vinyl-5-thia-1-azabicyclo [4.2.0] oct-2–ene-2-carboxylic acid, 72-(Z)-(O-(carboxy methyl) oxime] trihydrate. Its molecular weight is 507.50 as the trihydrate form and the chemical formula is C16H15N5O7S2.3H2O.

The structural formula of cefixime is:
Antibacterial activity of cefixime is due to the inhibition of bacterial cell-wall synthesis. It is active against the gram positive and negative bacteria such as Streptococcus pneumonia, Streptococcus pyogenes, Haemophilus influenza, Moraxella catarrhalis, Escherichia coli, Proteus mirabilis and Neisseria gonorrhoeae.

Cefixime is indicated for the treatment of uncomplicated urinary tract infections, otitis media, pharyngitis, tonsillitis, acute exacerbations of chronic bronchitis and uncomplicated gonorrhea. The adult dose is 400 mg/day and pediatric dose is 8mg/kg/day administered in one or two divided doses.

Upon oral administration, maximum serum concentrations occur between 2 and 6 hours for a single 200 mg tablet, single 400 mg tablet or 400 mg of cefixime suspension. Maximum serum concentrations occur between 2 and 5 hours following a single administration of 200 mg of suspension and between 3 and 8 hours following oral administration of a single 400 mg capsule. Cefixime is not metabolized in the body and eliminated unchanged in the urine. The serum half-life in healthy subjects is independent of dosage form and averages 3 to 4 hours [1].

Objective

The objective of the present work was to develop and validate a specific Liquid Chromatography - Mass spectrometry/Mass spectrometry (LC-MS/MS) procedure for the determination of cefixime in Human K$_3$EDTA (Tripotassium salt of ethylene diamine tetra acetic acid) plasma to support bioequivalence /bioavailability and /or pharmacokinetic studies involving formulations of cefixime.

MATERIALS AND METHODS

• Analyte

  Cefixime, molecular weight of 453.45$^{[2]}$

• Internal standard (IS)

  Cephalexin, molecular weight of 347.389$^{[3]}$

• Biological matrices

  Normal K$_3$EDTA plasma, lipemic and hemolysed K$_3$EDTA plasma

• Quantification range of the analyte

  114.5033 to 9374.2050 ng/ml

• Instrumentation

  HPLC with autosampler and column oven was used with mass spectrometry as the detector.

• Sample preparation method

  Liquid–liquid extraction with 100 µl of plasma

• Sample processing

  100 µL of spiked plasma samples were transferred to the labeled tubes. 25 µL of internal standard (cephalexin 10000.000 ng/mL in Acetonitrile: Water / 1:1 (v/v) was added to all the tubes except for tubes labeled Blank. After vortexing, 100 µL of 20mM Ammonium Formate was added. Then 2 mL of Ethyl acetate was added, vortexed for 30 minutes at 2000 rpm. The tubes were centrifuged at 4000rpm at 4°C for 20 minutes. The organic layer was transferred into appropriately labeled tubes and evaporated at 40° C at 15 psi by using Low Volume evaporator. The samples were reconstituted with 1 mL mobile phase (Acetonitrile : 10mM Ammonium Formate pH 4.15 [65:35 (v/v)]. Reconstituted samples were vortexed for 1 minute and the samples were transferred into 350 µL inserts in appropriately labeled auto sampler vials.

• Chromatographic conditions

  The following chromatographic conditions were applied.

  ✓ Column – Hypurity C18, 50 x 4.6mm, 5µ
  ✓ Mobile Phase – Acetonitrile : 10mM Ammonium Formate pH 4.15 [65:35 (v/v)]
Flow Rate – 0.4 mL/minute
Autosampler Temperature – 5°C
Column oven Temperature – 30°C
Injection Volume – 15.0 µL
Run Time – 3.5 min
Retention time of analyte was 2.2 minutes and IS was 1.7 minutes.

Mass spectrometry (MS) conditions
- Ion Source – ESI+ Jet Stream
- Polarity – Positive
- Gas Temperature (°C) – 300
- Gas Flow (1/min) – 5
- Nebulizer (psi) – 50
- Sheath Gas Temp (°C) – 300
- Sheath Gas Flow (1/min) – 10
- Capillary (V) – 3000
- Nozzle Voltage (v) – 10

Calibration curve standards
The calibration curve was constructed with 8 standards (std) with the following concentrations of cefixime in ng/ml.

Quality control standards
Four levels of quality control (QC) samples consisting cefixime were used in the analysis.
- Lower limit of quantification QC (LOQQC) – 117.4614 ng/ml
- Low level QC (LQC) – 355.9435 ng/ml
- Mid level QC (MQC) – 3786.6331 ng/ml
- High level QC (HQC) – 7498.2834 ng/ml

Method validation results
The method was validated as per the guidelines recommended by USFDA for bioanalytical method validation[4].

Chromatography
Representative chromatograms of aqueous standard solution (analyte with internal standard), standard blank plasma, standard zero (blank with internal standard), LOQQC, LQC, MQC and HQC and calibration curve of cefixime are shown in Figure 1 to 8.

Specificity and Selectivity
Specificity and selectivity were evaluated by analyzing a total of six lots of plasma (four lots of blank Human K3EDTA plasma and each one lot of Lipemic & Haemolyzed K3EDTA plasma) obtained from independent sources. No significant interferences were observed at the retention times of analyte and internal Standard of the lots evaluated. The interference was 6.22% for analyte and 1.44% for IS in aqueous samples. It was 1.87% for analyte and 3.59% for IS in spiked plasma samples. The results were within the acceptable range.

Carry Over Test
Carry over was calculated as the percentage peak area observed in processed blank plasma injected immediately after a processed ULOQ (Upper limit of quantification) calibration standard. No carry over was observed for analyte and internal Standard. The percentage of carry over for analyte and IS was 0.

Matrix Effect
The potential for co–extracted matrix component to influence the detector response of analyte and IS was evaluated in four independent lots of blank Human K3EDTA plasma and one lot of Lipemic and Haemolyzed Plasma.
Aqueous standard equivalent to LQC and HQC level concentration along with intended concentration of internal standard was spiked to the post extracted blank matrix.

The percentage matrix effect of each lot of analyte at LQC concentration was 1.70, 8.29, -3.04, -1.78, -4.24, and -1.04. The percentage matrix effect of each lot of IS at LQC concentration was -1.09, 6.13, -2.54, -2.68, -3.14, and -1.08. The percentage CV of matrix factor for analyte & internal standard of LQC was found to be 4.54 & 3.54 respectively.

The percentage matrix effect of each lot of analyte at HQC concentration was 2.66, 7.78, 0.50, -0.52, 0.85, and -0.32. The percentage matrix effect of each lot of IS at HQC concentration was 5.47, 6.69, 4.67, 0.69, 2.94, and 0.47. The percentage CV of matrix factor for analyte & internal standard of HQC was found to be 3.08 & 2.47 respectively.

The results were within the acceptable limits of matrix effect.

- **Linearity**

Linearity was established by preparing an eight-point standard calibration curve in Human K$_2$EDTA plasma covering the cefixime concentration range from 114.5033 to 9374.2050 ng/ml using cephalixin as internal standard. Three precision and accuracy (P&A) batches were analyzed in this range and they produced acceptable results. Calibration curves were calculated by least square linear regression analysis of the response ratios (analyte/internal standard) in the calibration standards using a weighting factor of 1/X$^2$. Back calculated concentrations of cefixime in calibration standards were determined using the best-fit regression curve calculated for each batch. The calibration line was shown to be linear for cefixime as shown in Figure 8. The correlation coefficient ($r^2$) was consistently greater than 0.99 during the course of validation for all the P&A batches.

- **Weighting Factor of Regression Method**

To determine whether to fit the data for the calibration curves by weighted or unweighted linear regression, the functional dependence of the natural logarithm of standard deviation of the analyte/internal standard area ratio on natural logarithm of sample concentration was evaluated. The weighting factor used was 1/X$^2$ since the slope (m) of the regression line equals 0.783.

- **Lower Limit of Quantification, LLOQ**

The LLOQ for analyte in Human K$_2$EDTA plasma was determined based on the analysis of LLOQ in the precision and accuracy validation batches. LLOQ of 114.5033 ng/ml was determined for this method. The accuracy and precision from the three P&A batches for cefixime were 102.86 and 2.60%, respectively that were within the acceptable limits.

- **Detection limit**

Detection Limit was established by the analysis of solution of known and decreasing concentrations of the drug up to the lowest detectable level. Detection limit of cefixime was 7.1565 ng/ml in this method.

- **Calibration Curves of Precision and Accuracy analysis**

Inter–batch calibration standard accuracy for cefixime ranged from 92.74 to 106.70% with inter–day precision value of 0.73 to 2.38% during the course of validation demonstrating acceptable assay linearity. Correlation coefficient ($r^2$) was consistently greater than 0.99. A representative calibration curve for cefixime in Human K$_2$EDTA plasma is shown in Figure 08. The details are presented in table 1.

<table>
<thead>
<tr>
<th>Standard ID</th>
<th>Std 1</th>
<th>Std 2</th>
<th>Std 3</th>
<th>Std 4</th>
<th>Std 5</th>
<th>Std 6</th>
<th>Std 7</th>
<th>Std 8</th>
<th>Slope</th>
<th>Intercept</th>
<th>$r^2$</th>
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<td></td>
<td></td>
<td></td>
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<td>% Nominal</td>
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<td>104.09</td>
<td>106.70</td>
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</table>
Precision and Accuracy (P&A)

Assay precision and accuracy (inter batch and intra batch) values were determined across three precision and accuracy batches by analyzing six replicates each of LOQQC, LQC, MQC and HQC samples in each batch. A different analyst performed one of the precision and accuracy batches with different column to ensure ruggedness of the method.

Accuracy (Table 2 and Table 3)

The accuracy of the assay is defined as the absolute value of the ratio of the calculated mean values of quality control samples to their respective nominal values expressed as percentage.

### Table 2: Intra Batch Precision and Accuracy of cefixime

<table>
<thead>
<tr>
<th>QC ID</th>
<th>LOQQC</th>
<th>LQC</th>
<th>MQC</th>
<th>HQC</th>
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<td>Actual Concentration (ng/ml)</td>
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<tr>
<td>117.4614</td>
<td>355.9435</td>
<td>3786.6331</td>
<td>7498.2834</td>
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<tr>
<td>124.2370</td>
<td>349.6634</td>
<td>4292.3229</td>
<td>7573.5625</td>
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<tr>
<td>118.0013</td>
<td>352.7869</td>
<td>4023.5233</td>
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<tr>
<td>Mean</td>
<td>122.7628</td>
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<tr>
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<tr>
<td>%CV</td>
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<td>3.32</td>
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<tr>
<td>% Nominal</td>
<td>104.51</td>
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<table>
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<td>LQC</td>
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<td>MQC</td>
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<td>HQC</td>
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<td>98.81</td>
<td>106.57</td>
<td>100.64</td>
</tr>
</tbody>
</table>

**Intra Batch Accuracy**

The intra batch accuracy of cefixime for LOQQC ranged from 93.92 to 104.51%, LQC from 92.42 to 98.81%, MQC from 100.09 to 106.57 % and HQC from 92.05 to 100.64%.

**Inter Batch or Total Accuracy**

The inter batch accuracy of cefixime for LOQQC was found to be 99.70% and LQC, 96.39%. For MQC and HQC, it was 102.89 & 96.34%, respectively.

Precision (Table 2 and Table 3)

The precision of the assay was measured by the percentage co-efficient of variation over the concentration range of LOQQC, LQC, MQC and HQC samples of cefixime during the course of validation.
Table 3: Inter–Batch or Total Precision and Accuracy of cefixime

<table>
<thead>
<tr>
<th>QC ID</th>
<th>LOQQC (ng/ml)</th>
<th>LQC (ng/ml)</th>
<th>MQC (ng/ml)</th>
<th>HQC (ng/ml)</th>
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<tbody>
<tr>
<td>Actual Concentration (ng/ml)</td>
<td>117.4614</td>
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<td>% Nominal</td>
<td>99.70</td>
<td>96.39</td>
<td>102.89</td>
<td>96.34</td>
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</table>

✓ Intra Batch Precision

The within batch precision of cefixime for LOQQC ranged from 2.05 to 4.70%, LQC from 1.01 to 2.11%, MQC from 1.43 to 3.32% and HQC from 1.19 to 1.81%.

✓ Inter Batch or Total Precision

The total precision of cefixime for LOQQC was found to be 5.70% and LQC, 3.46%. For MQC, it was 3.56% and HQC 4.00%.

• Recovery

The recovery of cefixime was determined by comparing the detector response of analyte of three distinct levels of extracted low, medium and high quality control samples with detector response obtained from unextracted aqueous quality control samples. The average recovery of cefixime was 66.66%.

The recovery of IS was determined by the average detector response of IS in extracted low, medium and high quality control samples with average detector response obtained from unextracted aqueous quality control samples. The mean recovery of internal standard was 80.14%. The coefficient of variation (CV) of internal standard for aqueous and extracted was 1.70% and 2.06%, respectively.

• Stability

✓ Freeze–Thaw (FT) Stability

Six replicates of cefixime samples at LQC and HQC concentration in Human K$_2$EDTA plasma were analyzed after three freeze–thaw cycles. The samples were stored and tested at two temperatures, one at below -70ºC and the other at below -20ºC. The stability was determined by calculating the percentage nominal of LQC and HQC samples against freshly prepared calibration curve standards and compared with bulk spiked comparison samples.

The mean percentage nominal of stability samples calculated against freshly prepared CC at LQC and HQC concentrations for both below -70ºC and below -20ºC were 93.47 & 93.50% and 92.11 & 90.34%, respectively. The mean percentage nominal of stability samples when compared with comparison samples at LQC and HQC levels for below -70ºC and below -20ºC were 98.18 & 101.47% and 96.75 & 98.04%, respectively. The results were within the acceptable limits for three freeze–thaw cycles.
Bench top stability of cefixime in Human K3 EDTA plasma was evaluated at room temperature. Six replicates of LQC and HQC samples were processed after keeping the samples on bench for about 06Hrs 31Minutes. Bench top stability was assessed by calculating percent nominal at LQC and HQC levels against freshly prepared calibration curve and compared with the bulk spiked comparison samples.

The mean percentage nominal of bench top stability samples calculated against freshly prepared CC at LQC and HQC levels were 92.88 and 92.51%, respectively. The mean percentage nominal of stability samples when compared with comparison samples at LQC and HQC levels were 97.56% and 100.40%, respectively, demonstrating acceptable bench–top stability for at least 06Hrs 31Minutes at room temperature.

Auto Sampler Stability for cefixime

Six replicates of LQC and HQC samples processed for one of the P&A batches were retained after completing the analysis and kept stored in auto sampler at 08°C for 11Hrs 32Minutes. The auto sampler stability was determined against freshly prepared calibration curve standards and compared against bulk spiked comparison samples at LQC and HQC levels.

The mean percentage nominal of auto sampler stability samples calculated against freshly prepared CC at LQC and HQC levels after 11Hrs 32Minutes at 08°C were 94.34 and 95.21%, respectively. The mean percentage nominal of stability samples when compared with comparison samples at LQC and HQC levels were 99.10 and 103.33%, respectively demonstrating acceptable auto sampler stability for at least 11Hrs 32Minutes minutes at 08°C.

Wet Extract Stability at Room Temperature

Six replicates of LQC and HQC were processed and reconstituted. They were transferred to injector vials and placed for 06Hrs 52Minutes in room temperature. Samples were injected after 06Hrs 52Minutes and analyzed against freshly prepared calibration curve standards and comparison samples.

The mean percentage nominal of wet extract stability samples calculated against freshly prepared CC at LQC and HQC after 06Hrs 52Minutes are 96.38 and 96.57%, respectively. The mean percentage nominal of stability samples when compared with comparison samples at LQC and HQC levels were found to be 101.24 and 104.81%, respectively demonstrating acceptable wet extract stability for at least 06Hrs 52Minutes in room temperature.

Wet Extract Stability at 2–8°C

Six replicates of LQC and HQC were processed and reconstituted. The samples were transferred to injector vials and placed for 23Hrs 07Minutes in 2–8°C. Samples were injected after 23Hrs 07Minutes and analyzed against freshly prepared calibration curve standards and comparison samples.

The mean percentage nominal of wet extract stability samples calculated against freshly prepared CC at LQC and HQC after 23Hrs 07Minutes are 96.81 and 93.46%, respectively. The mean percentage nominal of stability samples when compared with comparison samples at LQC and HQC levels were found to be 101.69 and 101.43%, respectively demonstrating acceptable wet extract stability at 23Hrs 07Minutes in room temperature.

Short term Stock Solution Stability

Stability of the analyte and IS were evaluated by exposing the stock solution for a desired period. After completion of the desired storage period, samples were analysed and the results of stability samples and freshly prepared solutions were compared.

Percentage stability of stored stock of analyte after 06hrs 21Mins was 100.31%
Percentage stability of stored stock of IS after 06hrs 21Mins was 99.19 %

Reinjection Reproducibility

CC, LQC and HQC samples of one of the P&A batches were re–injected and analysed for reproducibility. Percentage nominal for LQC, MQC and HQC for cefixime was 96.54, 104.42 and 99.63%, respectively. The percentage CV for LQC, MQC and HQC for cefixime was 2.97, 1.17 and 1.22 respectively.

Ruggedness

Ruggedness was evaluated from the Precision and Accuracy batch processed by different analyst consisting of 6 replicates of QC samples at LOQQC, LQC, MQC and HQC levels.
Mean Accuracy of LOQQC, LQC, MMQC, MQC and HQC were 111.68%, 91.20%, 94.37%, 107.14% and 102.27%, respectively. Precision for LOQQC, LQC, MQC and HQC were 11.44%, 2.70%, 5.73%, 1.68% and 11.17%, respectively.

- **Dilution Integrity**

  Cefixime samples were prepared in Human K3EDTA plasma at 2 times concentrations of higher quality control samples and diluted 2 times and 4 times with Human K3EDTA plasma. The percentage nominal of cefixime for 2 times and 4 times dilutions were 92.46 and 99.43%, respectively.

**Figure 1**: Representative Chromatogram of an aqueous Sample

**Figure 2**: Representative Chromatogram of a Blank Matrix Sample

**Figure 3**: Representative Chromatogram of a Blank matrix Sample with Internal Standard (zero sample)

**Figure 4**: Representative Chromatogram of LOQQC Sample
CONCLUSION

The bio-analytical method to quantify cefixime in human plasma for the range of 114.5033 to 9374.2050 ng/ml was successfully validated. This method is suitable for sample analysis to support Bio-equivalence/bioavailability (BA/BE) and/or pharmacokinetic studies involving formulations of Cefixime. The method was highly specific, selective, precise, accurate, rugged and reproducible for the estimation of cefixime in human plasma.

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