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

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Article

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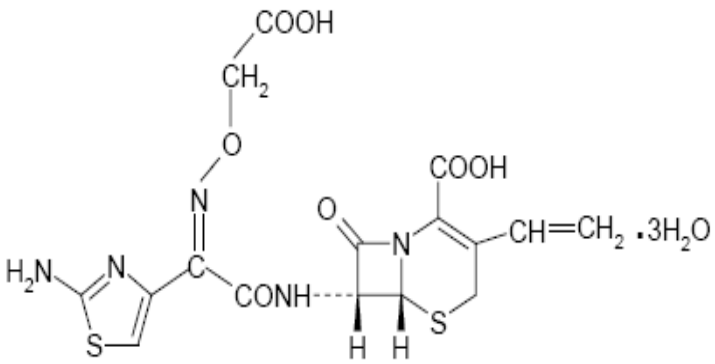
ABSTRACT

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 K_3EDTA 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 $C_{16}H_{15}N_5O_7S_2.3H_2O$.

The structural formula of cefixime is:



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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_3EDTA (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_3EDTA plasma, lipemic and hemolysed K_3EDTA 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)]

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- ✓ 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 (l/min) – 5
- ✓ Nebulizer (psi) – 50
- ✓ Sheath Gas Temp (°C) – 300
- ✓ Sheath Gas Flow (l/min) – 10
- ✓ Capillary (V) – 3000
- ✓ Nozzle Voltage (v) – 1000

- **Calibration curve standards**

The calibration curve was constructed with 8 standards (std) with the following concentrations of cefixime in ng/ml. Std 1– 114.5033, Std 2 –229.0066, Std 3 – 572.5164, Std 4 – 1431.2911, Std 5 – 3766.5556, Std 6 – 5380.7937, Std 7 – 7686.8481 and Std 8 – 9374.2050

- **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 K_3EDTA plasma and each one lot of Lipemic & Haemolyzed K_3EDTA 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 K_3EDTA plasma and one lot of Lipemic and Haemolyzed Plasma.

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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 5.47, 6.69, 4.67, 0.69, 2.94, and 0.47. The percentage CV of matrix factor for analyte & internal standard of LQC 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_3EDTA plasma covering the cefixime concentration range from 114.5033 to 9374.2050 ng/ml using cephalexin 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/X2. 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 (r2) 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/X2 since the slope (m) of the regression line equals 0.783.

- **Lower Limit of Quantification, LLOQ**

The LLOQ for analyte in Human K_3EDTA 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 (r2) was consistently greater than 0.99. A representative calibration curve for cefixime in Human K_3EDTA plasma is shown in Figure 08. The details are presented in table 1.

Table 1: Back calculated calibration curve concentrations for analyte and internal standard

Standard ID	Std 1	Std 2	Std 3	Std 4	Std 5	Std 6	Std 7	Std 8	Slope	Intercept	r^2
Concentration (ng/ml)	114.5033	229.0066	572.5164	1431.2911	3766.5556	5380.7937	7686.8481	9374.2050			
PA-01	118.4826	220.9270	533.5073	1338.3908	3708.0971	5489.2631	8189.5011	9969.8564	0.00014	-0.00419	0.9962
PA-02	119.2709	214.7755	554.5106	1315.7343	3792.6036	5603.3962	7862.5229	9951.2085	0.00015	-0.00371	0.9964
PA-03	117.2767	225.1837	538.3841	1327.9514	3803.2056	5403.2123	7951.8614	10085.8730	0.00014	-0.00226	0.9969
Mean	118.3434	220.2954	542.1340	1327.3588	3767.9688	5498.6239	8001.2951	10002.3126			
SD	1.00436	5.23277	10.99231	11.33987	52.12066	100.41970	169.00135	72.96360			
%CV	0.85	2.38	2.03	0.85	1.38	1.83	2.11	0.73			
% Nominal	103.35	96.20	94.69	92.74	100.04	102.19	104.09	106.70			

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- **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

QC ID	LOQQC	LQC	MQC	HQC
Actual Concentration (ng/ml)	117.4614	355.9435	3786.6331	7498.2834
Calculated Concentration (ng/ml) PA- 01	114.5901	343.0118	4047.1836	7555.2989
	124.2370	349.6634	4292.3229	7573.5625
	118.0013	345.0131	3918.2164	7600.2443
	123.3065	362.6156	3969.6045	7652.0296
	130.9795	357.1256	3962.0082	7506.3721
	125.4625	352.7869	4023.5233	7391.4873
Mean	122.7628	351.7027	4035.4765	7546.4991
SD	5.77471	7.40427	133.96765	89.97103
%CV	4.70	2.11	3.32	1.19
% Nominal	104.51	98.81	106.57	100.64
Calculated Concentration (ng/ml) PA- 02	115.4274	341.3852	3932.7736	7337.8934
	124.7603	350.3128	3926.0377	7153.3989
	114.2509	344.7425	3902.9037	7248.2787
	119.0426	355.8494	3742.2276	7291.9842
	121.2541	357.0270	3885.3387	7246.4261
	114.7556	342.6079	3786.2963	7054.3231
Mean	118.2485	348.6541	3862.5963	7222.0507
SD	4.20018	6.77234	79.24883	102.40890
%CV	3.55	1.94	2.05	1.42
% Nominal	100.67	97.95	102.01	96.32
Calculated Concentration (ng/ml) PA- 03	109.7928	324.8050	3801.2141	6856.6459
	109.7756	330.8447	3687.5893	7054.2956
	106.4565	327.4433	3807.3773	6931.8998
	112.8183	333.3280	3815.3993	6947.7345
	112.2187	326.1402	3845.5330	6682.1837
	110.8241	331.1153	3782.7090	6939.5990
Mean	110.3143	328.9461	3789.9703	6902.0598
SD	2.26280	3.31033	54.21784	124.87550
%CV	2.05	1.01	1.43	1.81
% Nominal	93.92	92.42	100.09	92.05

- ✓ **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.

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Table 3: Inter-Batch or Total Precision and Accuracy of cefixime

QC ID	LOQQC	LQC	MQC	HQC
Actual Concentration (ng/ml)	117.4614	355.9435	3786.6331	7498.2834
Calculated Concentration (ng/ml) PA- 01	114.5901	343.0118	4047.1836	7555.2989
	124.2370	349.6634	4292.3229	7573.5625
	118.0013	345.0131	3918.2164	7600.2443
	123.3065	362.6156	3969.6045	7652.0296
	130.9795	357.1256	3962.0082	7506.3721
	125.4625	352.7869	4023.5233	7391.4873
	115.4274	341.3852	3932.7736	7337.8934
Calculated Concentration (ng/ml) PA- 02	124.7603	350.3128	3926.0377	7153.3989
	114.2509	344.7425	3902.9037	7248.2787
	119.0426	355.8494	3742.2276	7291.9842
	121.2541	357.0270	3885.3387	7246.4261
	114.7556	342.6079	3786.2963	7054.3231
	109.7928	324.8050	3801.2141	6856.6459
Calculated Concentration (ng/ml) PA- 03	109.7756	330.8447	3687.5893	7054.2956
	106.4565	327.4433	3807.3773	6931.8998
	112.8183	333.3280	3815.3993	6947.7345
	112.2187	326.1402	3845.5330	6682.1837
	110.8241	331.1153	3782.7090	6939.5990
Mean	117.1085	343.1010	3896.0144	7223.5365
SD	6.67363	11.85541	138.62924	288.68948
%CV	5.70	3.46	3.56	4.00
% Nominal	99.70	96.39	102.89	96.34

✓ **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_3EDTA plasma were analyzed after three freeze-thaw cycles. The samples were stored and tested at two temperatures, one at below -70°C and the other at below -20°C. 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 -70°C and below -20°C 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 -70°C and below -20°C were 98.18 & 101.47% and 96.75 & 98.04%, respectively. The results were within the acceptable limits for three freeze-thaw cycles.

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✓ **Bench-Top Stability**

Bench top stability of cefixime in Human K_3EDTA 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.

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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 K_3EDTA 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

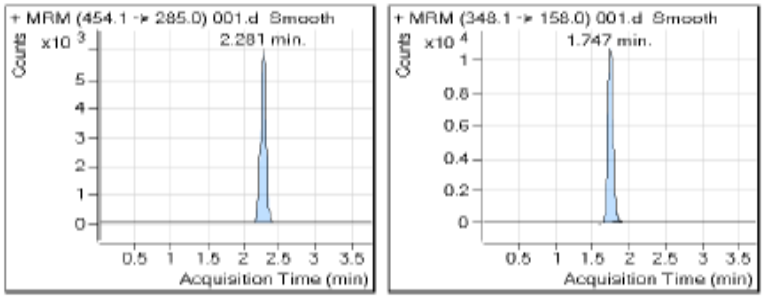

Figure 2: Representative Chromatogram of a Blank Matrix Sample

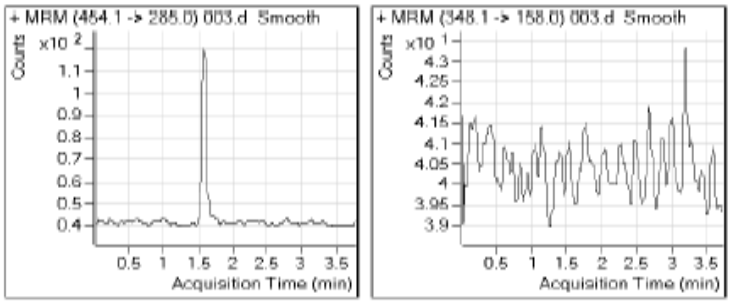

Figure 3: Representative Chromatogram of a Blank matrix Sample with Internal Standard (zero sample)

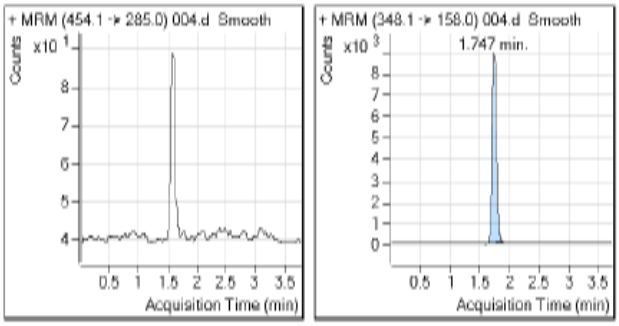

Figure 4: Representative Chromatogram of LOQQC Sample

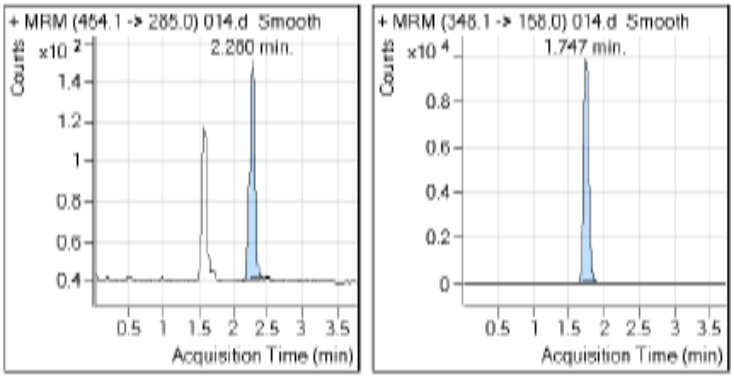

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Figure 5: Representative Chromatogram of LQC Sample

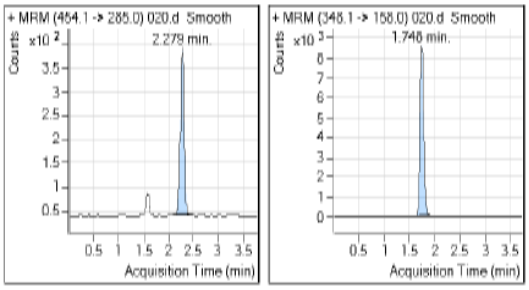


Figure 6: Representative Chromatogram of MQC Sample

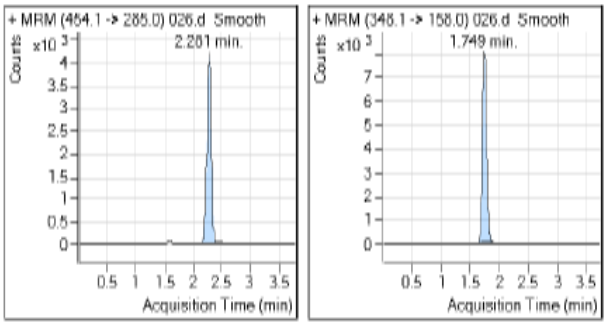


Figure 7: Representative Chromatogram of HQC Sample

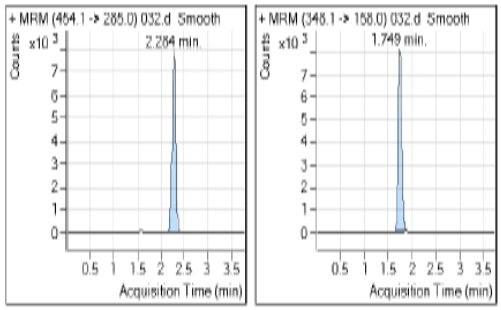
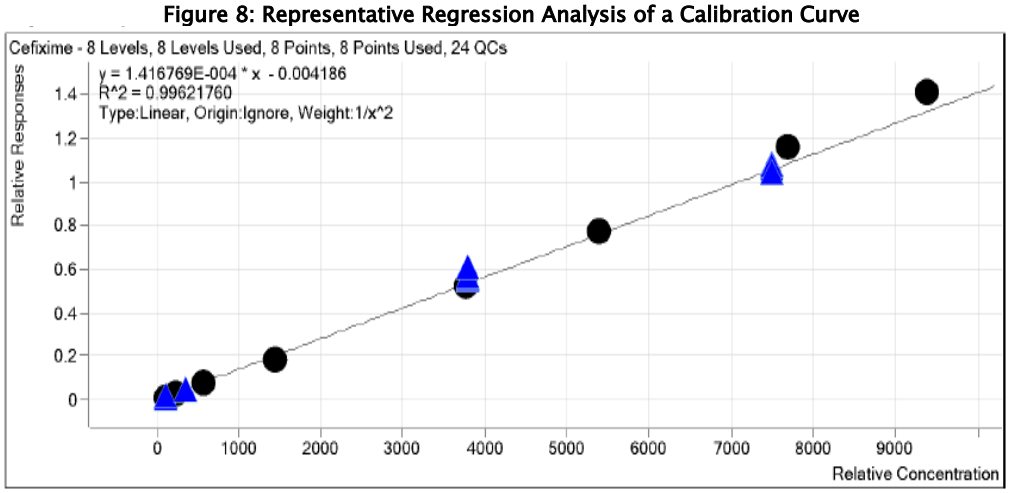


Figure 8: Representative Regression Analysis of a Calibration Curve



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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