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A quick and easy high performance liquid chromatography method for evaluation of cefixime in human plasma

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Abstract

Cefixime is essential member of orally energetic third generation cephalosporin and has tremendous activity aligned with many pathogens. The virtual bioavailability of a newly industrial dispersible tablet as compared with a recognized identified formulation. A simple and available reversed-phase HPLC method with UV detection has been urbanized and validate for cefixime evaluate in human plasma using a C18 analytical column and a mobile phase of tetrabutylammonium hydroxide (pH 6.5)-acetonitrile (3:1 v/v). The detection wavelength was 280 nm. To method observed major linear response-concentration association all through the cefixime concentration range of 15-100 ng/ml, with the average accuracy within-run and between-run values of 97.29% and 99.27%. The average drug recovery from plasma was 98.2% throughout the linear concentration range. The limits of detection (LOD) and quantitation (LOQ) of the method were 5 and 15 ng/ml, respectively. The method is quick, easy, very steady and precise for the partition, assignment, evaluation of cefixime in human plasma.

Keywords: Cefixime, human plasma, reversed-phase HPLC

Introduction

Cefixime (Fig. 1) [(6R, 7R, E)-7-(2-(2-amino thiazol-4-yl)-2- carboxy methoxy imino) acetamido)-8-oxo-3-vinyl-5-thia-1-azabicyclo [4.2.0] oct-2-ene-2-carboxylic acid] is belong to third generation of cephalsporin. The cefixime exists in off white crystals, melts over 220-250 $^\circ$C and soluble in alcohol (1). An orally active cefixime has outstanding activity against pathogens such as, *Anaerobes, Entero bacteria ceae*, gram negative species such as *Escherichia coli, Klebsiella, Haemophilus influenzae, Branhamella Catarrhalis, Neisseria gonorrhoeae, Serratiamarcescens, Providencia,*

Haemophilus, and *Meningococcus* including *b-lactamase* producing strains (1-3). Along with its broad spectrum antimicrobial activity and stability, cefixime was used as most suitable in proper dosage for adults as well as pediatrics and widely arranged among cephalosporin family in Iran. in spite of its poor lipophilicity and ionization at physiological pH, cefixime is significantly absorbed unaffected after oral administration (4). The absolute oral bioavailability of cefixime is in the range of 22-54%. Absorption is not appreciably customized by the presence of food. Cefixime may therefore be
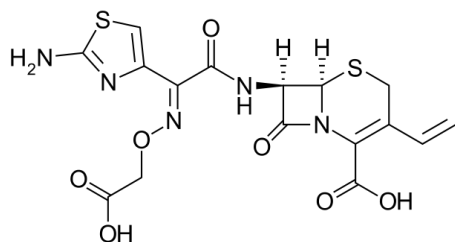
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Figure 1 Chemical structure of cefixime

offered without observe to meals. Following oral dosing, cefixime attain peak serum levels in approximately 4 h (5). From in vitro studies, serum or urine concentrations of 1 μg/ml or greater were measured to be adequate for most general pathogens beside which cefixime is active. The half-life is about 3 to 4 h and is not dose needy. Cefixime is excreted by renal and biliary mechanisms. About 50% of the absorbed dose is excreted unchanged in the urine within 24 h. There is no verification of metabolism of cefixime in vivo. Serum protein binding is well characterized for human and animal sera; cefixime is almost completely bound to the albumin fraction, the mean free fraction being approximately 30%. Protein binding of cefixime is concentration dependent in human serum only at very high concentrations which are not seen following clinical dosing (4). The area under the time versus concentration curve is superior by about 26.4% and the Cmax is bigger by around 20.7% with the oral suspension when compared to the tablet after doses of 400 mg. This enlarged absorption should be taken into consideration, if the oral suspension is to substitute the tablet. There are several investigations relating to the evaluation of cefixime alone and in combination with other drugs in

pharmaceutical planning and plasma by UV, HPLC, LC-MS, HPTLC methods (6-13). These methods engage spectrophotometric methods (14-15), voltammetric method (16), capillary electrophoresis (17). Though, HPLC is much more complicated technique as compared to in the past reported methods as it provides gangrenous information for all analytes along with consequent UV/ Vis spectra concurrently, which is very useful tool for the analysis of unknown mechanism of a mixture. The pervious our works was determination of drugs by LC–MS method in human plasma (18-22). In this way, a simple HPLC-UV method is residential and validated for cefixime analyze in human plasma.

Matterials and methods

Materials
Cefixime test tablets (Exir), Cefixime reference tablets (Wyeth) and cefixime reference standard (99.9% purity) were abounding and branded by Wyeth. Other chemicals and solvents were from chemical lab or HPLC purity grades, whenever needed, and were purchased. Drug-free human plasma was provided by Iranian Blood Transfusion Organization after routine safety evaluations.

Instrument and HPLC method
The HPLC system consisted of a double-reciprocating pump (Waters, model 600, MA, USA), a variable wavelength UV detector (Waters, model 2478, MA, USA) used at a wavelength of 280 nm with the outputs recorded and analysed using a compatible software (Millennium®, Waters, MA, USA). The drug separation was performed using a C_{18} analytical column

(250 mm×4.6 mm, particle size 5 μm; Perfectsill, MZ-Analysentechnik, Germany) arranged by a guard column of the same packing. The mobile phase was composed of tetrabutylammonium hydroxide (pH 6.5)-acetonitrile (3:1) with a flow rate of 1 ml/min. Sample injection to system (50 μL) was made by a loop injector (Rheodyne®7725i, Cotati, CA, USA).

Standard preparation
A stock solution of 1000 ng/ml cefixime in phosphate buffer (pH 7.4) was geared up, from which the concentrations of 15, 25, 50, 75 and 100 ng/ml were prepared by serially diluting this solution with the proper amount of phosphate buffer (pH 7.4). A series of spiked plasma samples with cefixime concentrations of 15, 25, 50, 75 and 100 ng/ml were prepared by 1:10 dilution of the described solutions with drug-free human plasma.

Assay procedure
To 150 μL calibration standards, QC samples, or plasma samples, 12 μL perchloric acid ($HClO_4$ 75% aqueous solution) and 400 μL methanol and 100 μL acetonitrile were added. The mixtures were vortex mixed for 20 s. After centrifugation at 15000×g in an eppendorf micro centrifuge tubes for 20 min, 50 μL of the supernatant was injected directly onto the analytical column for immediate HPLC analysis.

System suitability tests
The following parameters were calculated as system suitability indices of the developed method:
Number of theoretical plates $(N) = 16 (t_R/w)^2$
Peak symmetry = w/2f
Retain ability $(K') = (t_R/t_a) -1$

Where, t_R is the retention time of the analyte, w is the width of the analyte peak at its 0.05 height, f is the front half-width of the analyte peak at its 0.05 height and t_a is the retention time of non-retained analyte (solvent front).

Analysis validation tests
Standard curve (linear range)
The plasma samples with a series of known concentrations, prepared as described, were analyzed in three separate runs and, in each case, the linear regression analysis was carried out on known concentrations of cefixime against the corresponding peak heights and, then, the regression coefficient (r), slope, and y-intercept of the resulting calibration curves were determined.

Within-run variations
In one run, three samples with concentrations of 15, 50, and 100 ng/ml (from low, middle, and high regions of the standard curve) were prepared in triplicate and analyzed by developed HPLC method. Then, the coefficient of variations %(CV) of the corresponding determined concentrations were calculated in each case.

Between-run variations
On three different runs, samples from upper, intermediate, and lower concentration regions used for construction of standard curve (the same as within-run variations test) were prepared and analyzed by HPLC method. Then, the corresponding %CV values were calculated.

Absolute recovery (accuracy)
For each sample tested for within- and between-run variations, the absolute recovery of the method was determined

as the percent ratio of the measured concentration (determined using standard curve) to the corresponding nominal added concentration.

Relative recovery (matrix effect)
Three samples with concentrations of 15, 50, and 100 ng/ml (from high, middle, and low regions of the standard curve) were prepared in triplicate and analyzed by developed HPLC method. Then, the ratio of the recorded peak heights to the peak heights resulted from the direct injection of the aqueous solutions of cefixime with the same concentrations were determined as percentage in each case.

Limits of detection and quantitation
Limit of detection (LOD) of the method was determined as the lowest cefixime concentration producing a signal-to-noise (S/N) ratio of about 3. Limit of quantitation (LOQ) was determined as the lowest cefixime concentration capable of being quantities with enough accuracy and precision.

Repeatability test
For determination repeatability of method, concentration of 50 ng/ml of six independent spiked plasma samples were prepared as described. For the repeatability of the method a single injection of each preparation was made to HPLC and the %RSD between the results was determined.

Intermediate precision
The study of repeatability on a different day, a second analyst executed analysis of a further six samples prepared as described in repeatability test procedure. The analysis was performed by using

fresh reagents and a different HPLC column. The %RSD between six measurements was determined along with the %RSD between the total of 12 measurements from the repeatability and intermediate precision tests.

Reproducibility
For assay of reproducibility mean results for the same sample analysis between our laboratory and two different test facilities were obtained and the percentage of difference between content measurements was calculated using the equation:
[(highest value - lowest value) /mean value] × 100.

Stability:
Freeze and thaw stability
For determination of stability in freezer storing the three concentration levels of QC plasma samples temperature (-20 °C) for 24 h and thawed unassisted at room temperature. After complication of thawed, the samples were refrozen for 24 h under the same conditions. Twice repeated the freeze-thaw cycle, then the samples were tested after three freeze (-20 °C)-thaw (room temperature) cycles.

Short-term temperature stability
For determination of stability to kept at room temperature three concentration levels of QC plasma samples for a period that exceeded the routine preparation time of samples (around 6 h).

Long-term stability
For determination of stability the QC plasma samples of three concentration

levels at low temperature (-20 °C) were studied for a period of 4 weeks.

Standard curve and quality control sample in each batch
To calculate the concentration of cefixime in the unknown samples in the run was using of the standard curve in each analytical run. It was prepared at the same time as the unknown samples in the same batch and analyzed in the middle of the run. For analyzing of the QC samples in five duplicates at three concentrations (15, 50.0 and 100.0 ng/ml) were prepared with processed test samples at intervals per batch.

Results
Method development
In response to lack of an accessible, consistent, and simple to use analysis method for cefixime assay in plasma as an vital part of pharmacokinetic and bioequivalence estimate projects on the drug we urbanized a simple and offered HPLC method with UV detection based on the available equipments found in most pharmaceutical laboratories. To this end, initially a series of isocratic as well as gradient conditions using different usual mobile phase compositions, polarities, ionic strengths, and pH values were tested in order to determine the best condition for the analyte separation.

System suitability tests
The number of theoretical plates (N), peak symmetry, and retain ability (K') of the method for cefixime were 1296, 1.143, and 2.75, respectively. These data show that the developed method is of appropriate separation efficiency and peak shape, both of which are important factors in estimate of the

chromatographic method outputs.
Typical chromatograms produced from the developed method are shown in figure 2. Figure 2.A shows the spiked plasma sample with cefixime concentration of 100ng mL^{-1}, the retention times of cefixime was 6.67 min and the total HPLC analysis time was 7 min per sample. Figure 2.B shows a representative chromatogram of a plasma sample obtained at 7 h from a subject who received a single oral dose (400 mg), no interferences of the analyte were observed of the method(Fig. 2.C LOD, Fig. 2.D LOQ). The HPLC chromatogram for a blank plasma sample indicating no endogenous peaks at the retention positions of cefixime was shown in Figure 2.E.

Method validation tests:
Linearity
The method produced linear responses throughout the cefixime concentration range of 15-100 ng/ml, which is suitable for intended purposes. A typical linear regression equation of the method was: $y = 8.5564 \, x + 0.0654$, with x and y representing cefixime concentration (in mcg/ml) and peak height (in arbitrary units), respectively, and the regression coefficient (r^2) of 0.9916.

Within-run variations and accuracy
The within-run variations of the developed HPLC method as well as the corresponding absolute recoveries are shown in table1.

Between-run variations and accuracy
The between-run variations of the developed HPLC method as well as the corresponding absolute recoveries are shown in table 2.

(A)

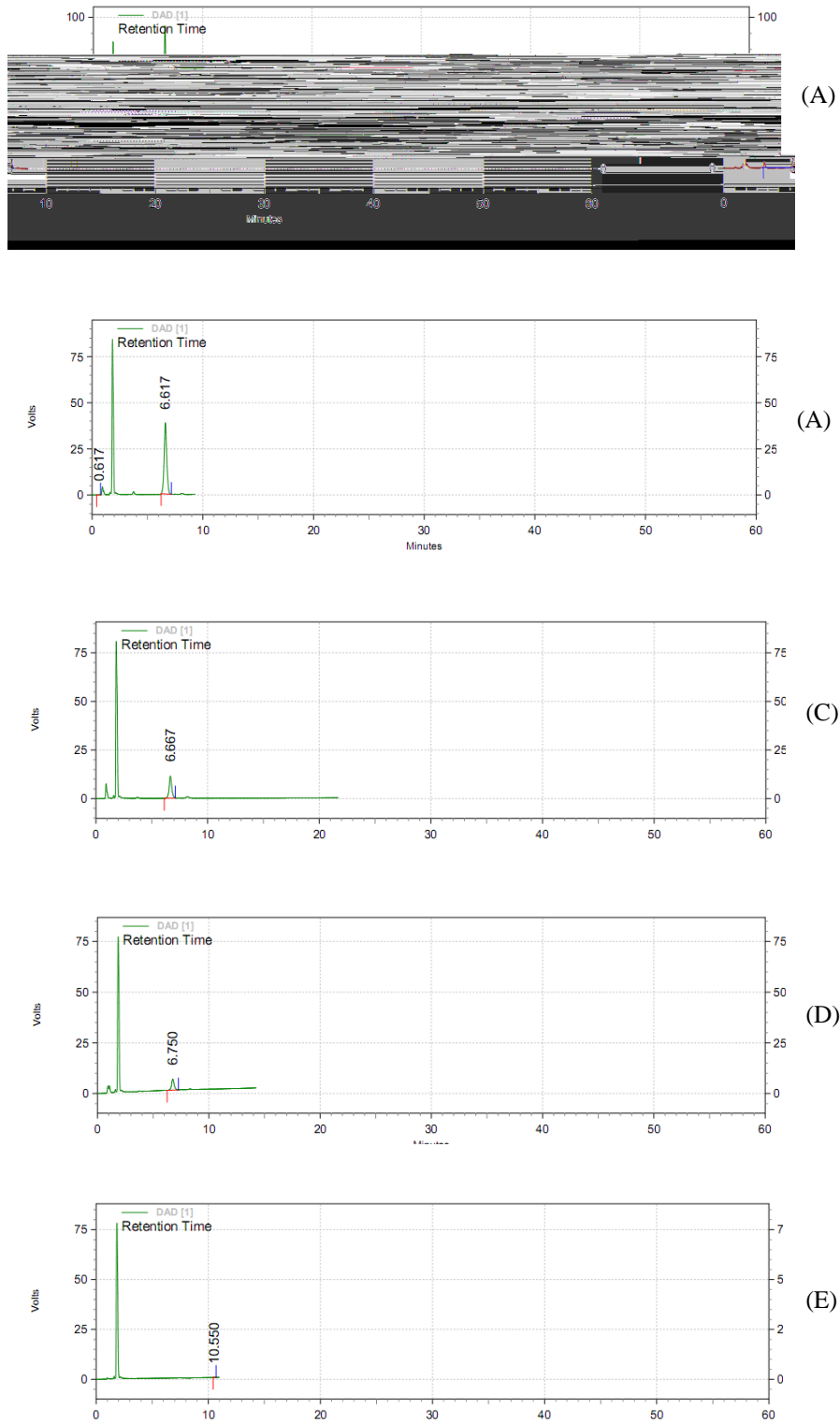(A)

(C)

(D)

(E)

Figure 2 Typical chromatograms of the HPLC method developed for cefixime assay in human plasma: Fig. 2 A) human plasma spiked to a 100 ng/ml drug concentration; Fig. 2.B chromatogram of a plasma sample obtained at 7 h from a subject who received a single oral dose (400 mg); Fig. 2.C LOD, Fig. 2.D LOQ of the method; Fig. 2.E the HPLC chromatogram for a blank plasma.

Table 1 Within–run variations and accuracy of the HPLC method for quantitation of cefixime (n = 3) .

Nominal added concentration (ng/ml)	Sample number	Measured concentration (ng/ml)	Mean ± SD	CV%	Accuracy	Mean ± SD
15	1	15.22	15.01 ± 0.18	1.22	95.23	95.54 ± 1.37
	2	14.95			94.36	
	3	14.87			97.05	
50	1	49.51	50.22 ± 1.7	3.39	99.24	97.12 ± 1.84
	2	52.17			95.89	
	3	48.99			96.23	
100	1	99.46	100.33 ± 0.93	0.93	100.02	99.23 ± 0.70
	2	101.32			99.02	
	3	100.23			98.65	

Table 2 Between–run variations and accuracy of the HPLC method for quantitation of cefixime (n=3).

Nominal added concentration (ng/ml)	Run number	Measured concentration (ng/ml)	Mean ± SD	CV%	Accuracy	Mean ± SD
15	1	15.01	14.78 ± 0.36	2.42	97.90	99.23 ± 1.62
	2	14.36			98.78	
	3	14.99			101.02	
50	1	50.32	49.68 ± 0.75	1.51	96.55	98.79 ± 2.34
	2	49.87			101.23	
	3	48.85			98.59	
100	1	100.16	99.86 ± 0.3	0.3	101.02	99.63 ± 1.25
	2	99.87			99.32	
	3	99.56			98.56	

Relative recovery

The relative recovery of cefixime using the developed assay method is shown in table 3.

Table 3 Relative recovery of cefixime by the HPLC method (n = 3)

Nominal added concentration (ng/ml)	Sample number	Recovery (%)	Mean± SD
15	1	95.21	98.77±
	2	102.01	3.41
	3	99.11	
50	1	96.18	96.62±
	2	101.14	4.30)
	3	92.56	
100	1	101.25	99.21±
	2	102.04	4.22
	3	94.36	

Limit tests

The limits of detection (LOD) and quantitation (LOQ) of the method were 5 and 15 ng/ml, respectively.

In general, the results of the validation tests indicated that the developed method has a remarkable degree of accuracy, repeatability, reproducibility, and recovery with application limits being in the desired range for routine applications.

Repeatability test

A remarkable repeatability for the drug assay in plasma for this development method by results as shown in Table 4.

Intermediate precision

As indicated, of the results the developed method shows in Table 5 anacceptable intermediate precision for cefixime assay of the intermediate precision.

Reproducibility

The highest test result of the spiked plasma with 50 ng mL^{-1}cefixime was 123654 and the lowest value was 122867 with the mean value of 123188. Therefore, the percentage of difference was 0.63% which means a high reproducibility for the method.

Stability

The results are shown in table 6 summarizes the freeze and thaw stability, short term stability, long-term stability and post-preparative stability data of cefixime. The stability behavior during these tests and there were no stability related problems during the samples routine analysis for the pharmacokinetic, bioavailability or bioequivalence studies. Based on the results obtained, the working solutions were stable within 6 h by tested stability of working solutions at room temperature.

Discussion

Cefixime is essential member of orally energetic third generation cephalosporin and has tremendous activity aligned with many pathogens. The virtual bioavailability of a newly industrial dispersible tablet as compared with a recognized identified formulation. There are several investigations relating to the evaluation of cefixime alone and in combination with other drugs in pharmaceutical planning and plasma by UV, HPLC, LC-MS, HPTLC methods (6-13). These methods engage spec-trophotometric methods (14-15), voltammetry method (16), capillary electrophoresis (17). Though, HPLC is much more complicated technique as compared to in the past reported methods as it provides gangrenous information for all analytes along with

Table 4 Repeatability of the test results for spiked plasma containing 50 ng mL^{-1}.

Sample	Peak area	Mean ± SD	CV%	Retention time (min)	Mean ± SD	CV%
1	123546	123188.2 ± 326.98	0.26	6.61	6.6 5 ± 0.05	0.88
2	123054			6.63		
3	122987			6.68		
4	123654			6.75		
5	122867			6.59		
6	123021			6.62		

Table 5 Intermediate precision of the test results for spiked plasma containing 50 ng mL^{-1} cefixime

Sample	Peak area	Mean ± SD	CV%	Retention time (min)	Mean ± SD	CV%
1	122956	123190.8 ± 351.77	0.28	6.61	6.64 ± 0.07	1.12
2	122863			6.55		
3	123065			6.63		
4	123620			6.71		
5	122987			6.75		
6	123654			6.60		

Table 6 Data showing stability of cefixime in human plasma at different QC levels (n = 5)

Stability	15 (ng/ml)	50 (ng/ml)	100 (ng/ml)
Short-term stability	95.45	94.32	91.01
Freeze and thaw stability	97.85	96.54	98.12
Long-term stability	96.32	98.21	96.32
Post-preparative stability	98.12	92.30	94.12

consequent UV/ Vis spectra concurrently, which is very useful tool for the analysis of unknown mechanism of a mixture. To achieve this goal, it is highly desired to have a single, simple and inexpensive analytical method. In this study, we established a HPLC-UV method for simultaneously detecting cefixime in human plasma. The outcomes showed that our HPLC-UV method fully satisfied these conditions as mentioned above. For this method, the plasma preparation for analysis consist of a protein precipitation method. Protein precipitation was necessary and important because this technique can not only purify but also concentrate the sample. Methanol, percholeric acid and acetonitrile were all attempted and acetonitrile: percholeric acid; methanol (75:5:20 v/v/v) was finally adopted because of its high extraction efficiency and less interference. Precipitation with and without adding 0.1 M NaOH (100 µL) were both tried, and obvious differences were not observed, so the precipitation using acetonitrile without adding 0.1 M NaOH was used at last. The validation tests on the developed method showed acceptable degree of linearity, sensitivity, precision, accuracy and recovery for the method.

Conclusion
An easy HPLC method was urbanized and validated for cefixime evaluate in plasma. Protein precipitation was necessary and important because this technique can not only purify but also concentrate the sample. The validation tests on the developed method indicated acceptable degree of linearity, sensitivity, precision, accuracy and recovery for the method. The method was used successfully for quantization of cefixime in human plasma. System appropriateness tests showed that the residential method is of suitable separation competence and peak shape.

Conflict of Interest
The authors declared no conflict of interest.

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