Journal of
Pharmacology & Clinical Research
ISSN: 2473-5574

Juniper Publishers
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Research Article
Volume 8 Issue 5 - November 2022
DOI: 10.19080/JPCR.2022.08.555750

J of Pharmacol & Clin Res

LC-MS/MS Application for Bioequivalence of Cefixime in Human Plasma

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Submission: November 19, 2022; **Published:** November 28, 2022

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Abstract

A new bioanalytical method was developed and validated for determination of cefixime in human plasma using LC-MS/MS technique, then successfully applied to bioequivalence study for evaluation of test product against the reference product of Cefixoral® 400 mg film-coated tablet, following comparative randomized, single dose, two-period cross over design, in thirty healthy male subjects, under fasting conditions. Cefixime with labeled internal standard (Cefixime [$^{13}C,^{15}N_2$]) was extracted from plasma by protein direct precipitation in single extracting step, and separated on ACE C_{18} column (4.6 x 50 mm, 5 µm) by mobile phase of 50% methanol in 10.0 mM ammonium formate and delivered isocratically at flow rate 0.7 ml/min. The established method was fully validated over dynamic range of 0.07-7.0 µg/ml according to the European guideline for bioanalytical methods validation, where all the test's results were within the acceptance criteria. The investigated pharmacokinetic parameters of C_{max} and AUC_{0-24} were 3.824 ug/ml and 28.268 hr.µg/ml, respectively, while the corresponding values for test product were 2.868 ug/ml and 20.958 hr.µg/ml, respectively. In conclusion, the test product was not bio comparable to the reference product accordingly.

Keywords: Cefixime; Bioequivalence; LC-MS/MS; Human plasma; Plasma precipitation

Abbreviations: PK: Pharmacokinetic; IS: Internal Standard; QC: Quality Control; JCPR: Jordan Center for Pharmaceutical Research

Introduction

Cefixoral® (Cefixime C16H15N5O7S2.3H2O); (Figure 1) is a semisynthetic, cephalosporin antibacterial for oral administration. 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, 7^2-(Z)- [O- (carboxy methyl) oxime] trihydrate. Cefixime as antibiotic is indicated in the treatment of the infections caused by susceptible strains of the microorganisms that cause acute uncomplicated cystitis and urethritis in urinary tract, otitis media, pharyngitis and tonsillitis and acute exacerbations of chronic bronchitis uncomplicated cervical or urethral gonorrhea [1]. The pharmacokinetic (pk) parameters for cefixime post oral administration of film-coated tablet 400 mg under fasting conditions have evaluated in youth and elderly male subject, where C_{max} was around 3.88 µg/ml, T_{max} 3.7 hr and AUC0-24 28.2 hr.µg/ml in youth [2], and the corresponding pk parameters were higher in elderly [3].

Cefixime has long been evaluated in human plasma by HPLC [4,7], while a very limited studies investigated cefixime by LC-MS/MS [8,9], where such technique provides more sensitive and selective results, but in less availability among research facilities. Herein, we designed in current study to use LC-MS/MS technique in the evaluation of bioequivalence of cefixime using newly developed and fully validated method in order to provide a supporting pk date that agree with the previous studies [2] which applied the corresponding cefixime dose on healthy adult and youth subject, where our pk finding data were a little lower (around 20%) than what are reported pk data in elderly subjects [3]. Furthermore, in current conclusion we provide a recommendation for the non-bio comparable test product, in order to enhance the bioavailability with comparative to the reference product.

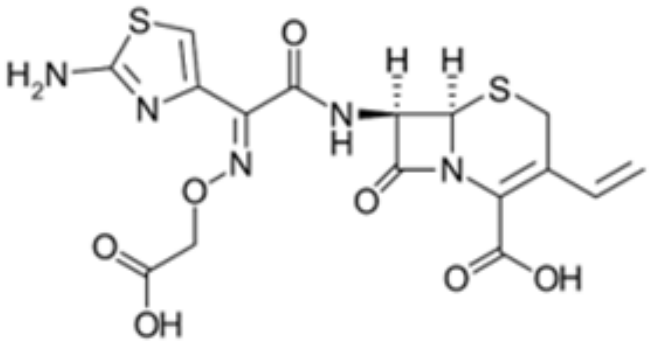

Figure 1: Cefixime chemical structure.

Experimental

Chemicals and reagents

Cefixime reference standard used in study (purity = 88%) and internal standard (IS) Cefixime [^{13}C,$^{15}N_2$] (purity = 90%) were obtained from TRC Inc. (Toronto, Canada). The blood blank samples were harvested from donors and collected in the clinical site, then plasma separated immediately for using in validation.

LC/MS-quality deionized water, methanol, acetonitrile, ammonium formate and ethyl acetate were purchased from Fisher, Germany, and the other chemicals were all of analytical grade.

Instrumentation

The Mass spectrometer was API 4000, Applied Biosystems, MDS SCIEX, coupled to LC from Agilent 1200 series. Computer System of Windows 7 SP1, and Analyst 1.6.3 software for data management system.

LC Conditions

The chromatographic conditions were consisted of mobile phase of 1.0 mM Ammonium formate: methanol (1:1 %, v/v), pumped isocratically through column of ACE C18 (50 x 4.6) mm, 5 µm, at constant flow rate of 0.7 ml/min under fixed temperature of 40 °C for column oven and samples tray temperature fixed at 15 °C where the injection volume was 10 µl and total run time of 1 min.

Mass spectrometric conditions

The optimized mass spectrometric conditions for MRM were DP 50, EP 5.3, CE23.8 and CXP 16. The ion source conditions were curtain gas = 12, CAD = gas 10, gas1 = 55, gas1 = 55, gas2 = 40, evaporation temperature = 600 °C and the ion source voltage = 5500 V under positive scan mode.

Standard solution

A stock (Master) solution of Cefixime (1.0 mg/ml) was prepared by weighing accurately equivalent to 10 mg of Cefixime in 10 mL V.F, added about 7 ml of MeOH, vortexed until dissolved, completed to volume with MeOH. A stock (Master) solution of Cefixime [^{13}C,$^{15}N_2$] (1.0 mg/ml) was prepared by dissolving with equivalent volume of MeOH and got a concentration (1.0 mg cefixime [^{13}C,$^{15}N_2$]/ml). A 1000 µl of cefixime master solution (1.0 mg/ml) diluted into 10 ml of diluent. The resultant concentration was (100.0 µg cefixime/ml working solution). A 500 µl of cefixime [^{13}C,$15N_2$] master solution (1.0 mg/ml) diluted into 50 ml of diluent. The resultant concentration was (10.0 µg cefixime [^{13}C,$^{15}N_2$] /ml working solution).

Calibrators and QCs' Aliquots:

Calibrators and quality control samples (QC) used during study samples analysis were prepared using human heparinized blood and spiked with prepared working solutions. The aliquots were tested for interference and stored in freezer under controlled temperature of -20 °C.

Standard calibration curves and quality control samples: Standard calibrators and quality control samples for cefixime in human plasma (analyte-free pooled plasma) were prepared by spiking 50 µl of working solution into 450 µl of plasma to prepare the calibrators of 0.07, 0.15, 0.30, 0.60, 1.20, 2.40, 4.80 and 7.00 µg/ml. Four QC samples of each four concentrations (0.210, 0.840, 2.800 and 5.600) µg/ml gathered in one set and processed among the batch samples. The proposed dynamic range of the calibration curve was following the European [10] and US FDA [11] guidelines for bioequivalence studies and upon maximum concentration of around 4 ug/ml cefixime in human blood [2].

Sample preparation

A 200 µL volume of plasma was transferred to a 1.5 ml polypropylene tube, where 200 µL of 5% w/v trichloroacetic acid contains I.S was added. The mixture was vortexed for 30 seconds using a Vibrax Type VX-Z, VXR Basic Vortexer (IKA-Werke GmBH & Co. Staufen, Germany) and centrifuged using Multitude Sigma1-15 (Sigma, Germany) for 5 min at 14000 rpm. The supernatant was transferred to an auto sampler micro vial and 2 µL was injected into the analytical column.

How to cite this article: Ahmed A-a, Khaled W O, Basel A, Eyad M, Mona B, et al. LC-MS/MS Application for Bioequivalence of Cefixime in Human Plasma. J of Pharmacol & Clin Res. 2022; 8(5): 555750. DOI:10.19080/JPCR.2022.08.555750

Bioanalytical method validations

The developed method for investigation of cefixime in human plasma was fully validated in concordance with the European [12] and US FDA [13] guidelines for bioanalytical methods validation. The method was validated in terms of specificity, LLOQ, carryover, sensitivity, response linearity, accuracy, precision, dilution integrity, matrix effect, recovery and stability.

Clinical design

This study was conducted in compliance with declaration of Helsinki according to GCP and GLP guidelines [14]. The protocol was approved by the local institutional review board. A written informed consent and consent form were obtained from all participant volunteers before proceeding study. This study was designed an open label, randomized, single oral dose, two periods, two sequences, two treatments, laboratory-blinded, crossover study, under fasting conditions. The dosage of test and reference products were not related to analytical shortages, but to the necessity to authorize the 400 mg dosage form according to international regulations. Reference: Cefixoral® 400 mg film coated tablet. Each film coated tablet contains 400 mg Cefixime, manufactured by A Menarini Industrie Farmaceutiche Riunite s.r.l., Italy. 30 subjects were randomized, enrolled, completed and analyzed. In each study period, a (2 x 8 ml) blood samples were collected pre-drug administration and a series of 20 x 7 ml blood samples were collected at the following times: 0.5, 1, 1.5, 2, 2.5, 2.75, 3, 3.25, 3.5, 3.75, 4, 4.5, 5, 5.5, 6, 7, 8, 10, 12 and 24 hours post drug administration.

Result and Discussion

LC–MS/MS analysis

The optimized tandem MS's parameters exhibited a high quantitative detection efficiency, where the molecular ion for cefixime was detected with its daughter fragment at m/z 453.9/126.1 and 456.9 /129.1 for IS.

Method validation results

The used extraction method from human plasma was specific and accurate enough to quantitate cefixime over IS properly, and no endogenous peaks observed through validation and routine analysis (Table 1). Summarizes the results obtained from the achieved validation sections, and (Table 2) shows the stability test results.

Table 1: Validation sections results for cefixime in human plasma.

Sections	Result
Selectivity	There are interfering peaks with acceptance criteria found at the analytes or internal standards retention time.
Carry Over	3.49 % for analyte, within acceptable limits.
Lower Limit of Quantitation	0.070 µg/ml, CV% 3.73, Accuracy 84.29 %
Linearity and Calibration Range	R^2 = 0.9963 for 11 curves, range: 0.070 – 7.000 µg/ml
Within Run Accuracy	Day-1: Between 101.43 and 109.50 % and LLOQ: 84.29 %
	Day-2: Between 98.81 and 102.64 % and LLOQ: 104.29 %
	Day-3: Between 95.71 and 97.36 % and LLOQ: 92.86 %
Between Runs Accuracy	Between 99.05 and 102.32 % and LLOQ: 94.29 %
Within Run Precision	Day-1:CV% between 2.74 and 13.55 % and LLOQ: 3.73 %
	Day-2:CV% between 2.46 and 4.64 % and LLOQ: 10.55 %
	Day-3:CV% between 2.22 and 4.28 % and LLOQ: 8.00 %
Between Runs Precision	CV% between 4.45 and 9.25 % and LLOQ: 11.82 %
Reinjection Reproducibility Accuracy (Evaluated using original Cal. Curve)	Between 99.61 and 101.43 % and LLOQ: 101.43 %
Reinjection Reproducibility Accuracy (Evaluated using fresh Cal. Curve)	Between 98.10 and 103.18 % and LLOQ: 84.29 %
Reinjection Reproducibility Precision (Evaluated using original Cal. Curve)	CV% between 2.31 and 5.08 % and LLOQ: 2.96 %

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Reinjection Reproducibility Precision	CV% between 2.32 and 5.17 % and LLOQ: 3.56 %
(Evaluated using fresh Cal. Curve)	
Dilution Integrity Accuracy	Double: 96.06 % and quadruple: 97.50 %
Dilution Integrity Precision	CV% for double 6.04 % and 7.82 % for quadruple
Matrix Effect	No considered effect for the matrix on the drug and I.S
I.S-Normalized Matrix Factor	CV% between 3.90 and 4.37 %
Cefixime Absolute Recovery	146.75 %,130.56%, 131.38 %, and 125.03 % QC_{low}, QC_{med-1}, QC_{med-2} and QC_{high} respectively with 6.98 CV%
Average I.S Absolute Recovery	91.48 % with 6.50 CV%

Table 2: Stability validation section results for cefixime in human plasma.

Cefixime Stock Solution Stability at 2-8 °C for 5 days	Between 89.20 and 96.05 %
I.S Stock Solution Stability at 2-8 °C for 5 days	Between 103.19 and 111.27 %
Cefixime Stock Solution Stability at R.T for 30 hours	Between 93.55 and 96.25 %
I.S Stock Solution Stability R.T for 30 hours	Between 102.88 and 106.18 %
Cefixime Working Solution Stability at 2-8 °C for 5 days	Between 90.77 and 95.99 %
I.S Working Solution Stability at 2-8 °C for 5 days	Between 103.16 and 109.42 %
Cefixime Working Solution Stability at R.T for 30 hours	Between 92.72 and 95.70 %
I.S Working Solution Stability at R.T for 30 hours	Between 103.70 and 107.10 %
Short Term Stability at R.T for 14:26 Hours: minutes	Between 103.00 and 104.29 %
4^{th} Cycle Freeze Thaw Stability	Between 94.54 and 95.24 %
Injection phase stability at ambient temperature for 55:41 hr:min	Between 95.24 and 100.45 % for Cefixime
	Between 99.35 and 101.93 % for Cefixime [^{13}C,$^{15}N_2$]
I.S Injection phase stability at R.T for 55:41 hr:min	PSS Total stability: 55:41 hr:min
Cefixime Long Term Stability at -20°C for 90 Days	Between 94.29 and 100.09 %

Clinical application

During clinical application, there were no clinically relevant abnormalities at physical examination, and all findings were normal for all volunteers, and there were no safety concerns during the course of the study. Table 3 summarizes the final statistical analysis for all volunteers' samples upon the described method. The illustration of concentration-time profile for analysis of cefixime in human plasma post oral administration of 400 mg under fasting conditions is presented in (Figure 2). for participant volunteers treated either by test or reference product.

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Table 3: Summarizes the final statistical analysis for all volunteers' samples upon the described method.

Parameter		Test	Reference	Ratio of Geometric Mean	90% Confidence Interval (%)
Cmax (µg/ml)	Geometric Mean	2.868	3.824	75	69.53 – 80.91
	Arithmetic Mean	2.992	3.984		
	(CV %)	28.97	29.45		
	SD	0.8669	1.1734		
	N	30	30		
AUC0-t (hr.µg/ml)	Geometric Mean	20.958	28.268	74.14	67.02 – 82.02
	Arithmetic Mean	23.04	30.784		
	(CV %)	45.5	45.67		
	SD	10.482	14.0588		
	N	30	30		
AUC0-inf (hr.µg/ml)	Geometric Mean	23.956	31.233	76.7	70.53 – 83.42
	Arithmetic Mean	25.84	33.521		
	(CV %)	38.97	41.04		
	SD	10.069	13.7567		
	N	30	30		
Tmax (hr)	Median	4.5	4.5		
	Arithmetic Mean	4.71	4.14		
	(CV %)	18.76	23.23		
	SD	0.883	0.962		
	N	30	30		
T½ (hr)	Geometric Mean	3.28	3.13		
	Arithmetic Mean	3.34	3.19		
	(CV %)	19.62	21.15		
	SD	0.655	0.675		
	N	30	30		

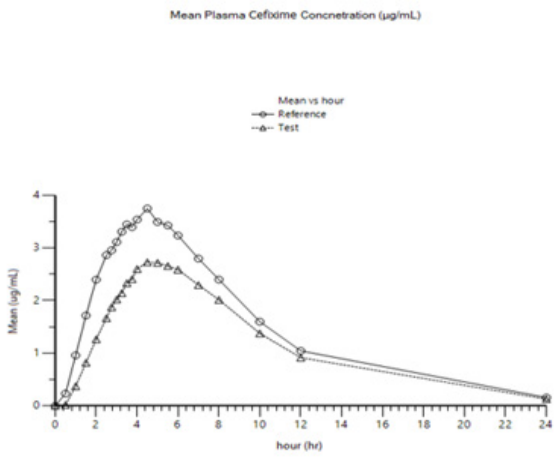

Figure 2: The concentration-time profile for analysis of cefixime in human plasma post oral administration of 400 mg under fasting conditions for both of reference and test products.

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Conclusion

The described method for determination of Cefixime in human plasma by tandem MS was successfully validated and used to estimate a clinical bioequivalence of 400 mg Cefixime on healthy adult and youth male subjects under fasting conditions. The test product of 400 mg capsule and Cefixoral® 400 mg film coated tablet, each dose contains 400 mg Cefixime are not bioequivalent with regard to C_{max} and AUC0-t. Herein, the recommendation generic manufacturer is to decrease the particle size of the used raw powder material.

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