Determination of Cefdinir in Human Plasma using HPLC Coupled with Tandem Mass Spectroscopy: Application to Bioequivalence Studies

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ABSTRACT

A sensitive and selective liquid chromatographic method coupled with tandem mass spectrometry has been developed and validated for the determination of cefdinir in human plasma. The analytes cefdinir and cephalexin (internal standard) were separated on a reversed phase column (Merck, Purospher RP-C18, 30 X 4.6 (mm), 3µm) using a mobile phase consisting of an aqueous solution of formic acid in water (0.10 %) and acetonitrile (85: 15 v/v (%)), flow rate 0.50 (mL/min.). Detection utilized a tandem MS/MS, the analytes were ionized using an ESI source in the positive ion mode prior to detection and analysis using Multiple Reaction Monitoring mode (MRM). The analytes were monitored at the following transitions (m/z) 396.10 → 226.90, and (m/z) 348.24 → 158.10 for cefdinir and cephalexin respectively. Cefdinir linearity was demonstrated over the concentrations ranging from 10 to 1200 (ng/ mL). The developed method was fully validated prior to its application on a bioequivalence study involving cefdinir (125 mg/5 ml) suspension in healthy volunteers (N= 26) under fasting conditions.

Keywords: Cefdinir, HPLC-MS/MS, plasma, ESI source, positive ion mode, MRM mode.

1. INTRODUCTION

Cefdinir (6R -[6α, 7β (z) -7) [[2-amino-4-4-thiazolyl) (hydroxyimino) acetyl] amino] -3-ethenyl-8-oxo-5-thia-1azabicyclo [4.2.0] oct -2-ene-2-carboxylic acid. (empirical formula ($C_{14}H_{13}N_5O_5S_2$) molar mass 395.42, is presented in the structure below. Cefdinir is a Cefixime analogue with a carboxy-methoxyimino moiety found in most orally active cephalosporins at the 7-position. This enhances the activity against gram positive bacteria[1,2]. Cefdinir therefore has a broad spectrum of antibacterial activity encompassing a number of gram-positive and gram-negative pathogens that are commonly causative in community-acquired respiratory and skin infections[3].

After single oral doses of 300 mg and 600 mg in adults, the observed mean maximum plasma cefdinir concentrations (C_{max}) were 1.6 and 2.87 (mg/l) respectively, with a T_{max} of about 3 hours. In paediatric patients, C_{max} values were 2.3 and 3.86 (mg/l), observed after dosing of 7 and 14 mg/kg cefdinir suspension, respectively, with a T_{max} of about 2 hours. Cefdinir demonstrated linear pharmacokinetic profile over a 200 – 400 mg dose range, but displayed nonlinear pharmacokinetics at higher than 600 mg doses[3]. In spite of the apparent benefits of cefdinir compared to other cephalosporins, few reports were published on BA/BE or PK studies (4). This may be attributed to the following: (A) difficulty in extracting cefdinir from biological matrices, because of the problems associated with its hydrophilicity and solubility, since it is insoluble in several solvents including: water, methanol, acetonitrile, ethanol, acetone, ethyl acetate and ether. (B) Lack of selectivity and sensitivity of the previously employed

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analytical methods particularly the nonspecific general UV detection, which dominated the literature prior to about 2006. (C) Analytical complications due to matrix effects, as well as carry over effects which resulted in unacceptable precision and accuracy, particularly at the lower limits of quantitation.[4-12].

Some reports aimed at developing stability indicating methods, or at determining of cefdinir in different dosage forms, or the use of expensive on-line solid phase extraction prior to UV detection in beagle dog plasma samples[1].

Few reports targeted BE/BA studies particularly after dosing with cefdinir 125 mg/5 ml suspension[13-15]. A more selective and sensitive method using LC-MS/MS detection was reported using positive Electrospray Ionization. (ESI) with Selected Ion Monitoring (SIM)[2]. In spite of the favourable merits of method performance, the reported method was not compliant with the current regulatory expectations for bioanalytical method validation([16,17]. Calculations of matrix factors and the use of the IS-Normalized matrix factors, in addition to evaluating reproducibility through performing Incurred Sample Re-analysis (ISR), and Incurred Sample Accuracy (ISA) were not evaluated[2].

The present study developed and validated a sensitive, selective, accurate and precise method which employed a positive ESI mode with a Multiple Reaction Monitoring (MRM) method, using a simple sample preparation technique and a highly sensitive triple quadruple MS/MS. The reported method is compliant with the global current regulatory guidance, and added further insight into adopting a highly proactive quality bioanalytical strategy, often neglected by regulatory guidance documents[16, 17]. The method was applied to a BE study of a test and a reference drug products, containing cefdinir (125 mg/5ml suspension) in healthy participants, (N=26) under fasting conditions.

2. Experimental

2.1 Chemicals and Reagents

Reference standards for cefdinir (98.7%) and cephalexin (100.23%) were provided by Pharma International (Amman, Jordan). HPLC grade methanol was from Merck, Germany, analysis grade formic acid and dimethyl sulfoxide (DMSO) analysis grade were from Merck, Germany. Ultra-pure water was prepared by PharmaquestJo bioanalytical lab using a sartorious water purification system. The prepared reagents were: (1) A formic Acid solution in water (0.10% v/v) made by diluting 1.0 ml of the acid in 1 Litre water. (2) A formic Acid solution in Acetonitrile (3.0 v/v%) was prepared by diluting 3.0 ml formic acid in a 100.00 ml Acetonitrile. Blank plasma samples were kindly donated by the Central Blood Bank (Amman, Jordan).

2.2 Instruments and Apparatus

An HPLC-MS/MS system was employed. The HPLC was an Agilant 1200 series (Agilant Technologies, Germany) equipped with a binary pump, a degasser, an autosampler, and a column oven. The mass spectrometer was an API 5000 (AB Sciex) which comprised a triple quadruple instrument purchased from Applied Biosystems. (Ontario, Canada) The mass spectrometer was operated using a positive Electro Spray Ionization (ESI) mode. Data acquisition was performed using Analyst 1.4.2 software (Applied Biosystems, Ontario, Canada). The apparatus used, among others included a 5 place analytical balance (Sartorious, Germany), a centrifuge (Eppendorf, Germany) and adjustable micro-pippettes (Hirshmann, Germany)

2.3 Preparation of Standard Solutions

2.3.1. Preparation of non-matrix based standard solutions

A cefdinir stock solution was prepared by dissolving an amount equivalent to 25.00 mg in a 100 ml volumetric flask. Cefdinir was dissolved in a (4%v/v) DMSO solution in methanol to make up a cefdinir stock solution containing (250.00 µg/ml). More dilute non-matrix based solutions containing 25.00 and 1.00 (µg/ml) cefdinir were also prepared. A stock solution of cephalexin, the internal standard was prepared by dissolving 20.0 mg cephalexin in 100.00 ml volumetric flask using a solution containing a 0.1% formic acid in water with acetonitrile (70:30

v/v%) to make up a cephalexin solution containing 200.0 (µg/ml).

2.3.2 Preparation of matrix based calibration curve standard solutions and quality control samples

Blank plasma aliquots were individually transferred to volumetric flasks (5.00ml capacity each). Ten matrix based calibration standards (calibrators) were prepared to cover the following concentrations: 10.00, 50.00, 75.00, 150.00, 325.00, 500.00, 675.00, 850.00, 1000.00 and 1200.00 (ng/ml).

Quality control samples were prepared at three concentration levels: (1) a low quality control sample (QC_L) containing 36.00 (ng/ml) (2) a medium quality control sample (CQ_M) containing 600.00 (ng/ml) and a high quality control sample (CQ_H) containing 900.00 (ng/ml) cefdinir.

Prior to extraction of 250.00 µl sample aliquots, a 200.00 ng aliquots of the internal standard cephalexin were individually spiked onto each sample.

2.4 Extraction of analytes from plasma samples

Onto each plasma aliquot (250 µL) in an eppendorf tube (3.0 mL capacity), a 1.00 mL aliquot of a mixture of formic acid in acetonitrile (3.0 (v/v) %) solution containing the IS (200ng/ mL) was added, samples were then vortexed (30s) and centrifuged (4000 rpm). A 200 µL aliquot of the supernatant was transferred to a 10 ml glass tube. The mixture was then diluted to a total volume of 4.00 mL. The solution was vortexed (30s) before transferring 200 µL to the well plate of the autosampler. A 20 µL volume was injected onto the equilibrated chromatographic system prior to MS/MS detection.

2.5 Chromatographic and MS/MS experimental conditions

2.5.1 Chromatographic experimental conditions

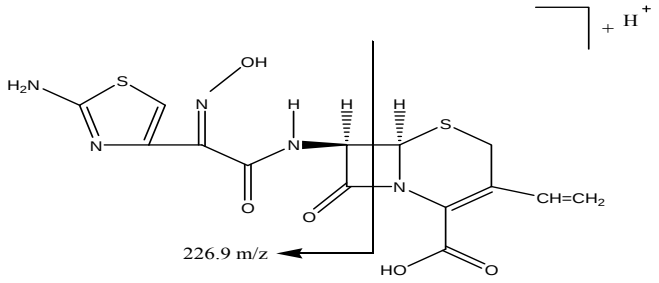
Chromatographic separations were performed using a reversed phase column, RP -18 (Purosphere: 30 mm length, 4.6 mm ID, 3µm particle size) (Merck, Germany). The optimized mobile phase was a mixture of an aqueous solution of formic acid with water (0.10%) and Acetonitrile (85: 15 (V/V) %). Separations were performed under isochratic conditions at a flow rate of 0.5 ml/min. An injection volume was set at 20 µl, with a column temperature set at 40º C.

Table1. Experimental setting for the tandem mass-spectrometer during the analysis of cefdinir and cephalexin (IS)

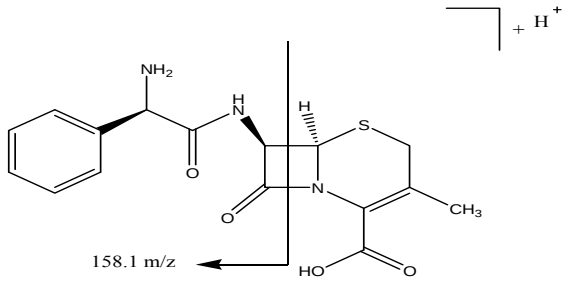
Parameter	Value (unit)
Source temperature	600.0°C
Nebulizer gas	25 psi
Turbolon gas	45 psi
Curtain gas	25 psi
Collision gas	12 psi
Ion spray voltage	5500 V
Dwell time per transition	400 ms
Entrance potential	5 V for Cefdinir and 10 V for Cephalexin
MRM transition (amu)	396.10 → 226.90 for Cefdinir and 348.24 → 158.10 for Cephalexin
Collision energy	20 V for Cefdinir and 17 V for Cephalexin
Declustering potential (DP)	100 V for Cefdinir and 91 V for Cephalexin
Collision cell exit potential	20 V for Cefdinir and 12 V for Cephalexin
Mode	+ ve mode for both compounds

2.5.2 Mass spectrometric experimental conditions

Mass spectrometric experimental conditions were optimised during the tuning procedures. Mass spectra of the eluting compounds, cefdinir and the cephalexin internal standard were recorded. Ionization was achieved using a positive Electro-Spray Ionization (ESI) method, where the sample is sprayed across a high potential difference from a needle into an orifice to produce ionized species in the gas phase. Following is a summary of the experimental conditions set for the HPLC and the triple quadruple MS/MS.



a.



b.

Figure 1: Fragmentation of (a) Cefdinir and (b) Cephalexin

During the LC-MS/MS analysis mass spectra of the eluting compounds were continuously recorded, a total ion chromatogram (TIC) was utilized to determine when the analytes were eluted from the column figure (1). On the other hand, the computer was set to look at ions corresponding to specific species, and to provide a re-constructed mass chromatogram. A Selected Ion Monitoring (SIM) or a Selected Reaction Monitoring (SRM) were initially employed. A Multiple Reaction Monitoring (MRM) was employed as the chosen analytical method for quantitation with MS/MS detector.

2.6 Method Development and Method Validation

2.6.1 Overview

Bioanalytical method validation includes all the experiments designed to verify that a bioanalytical method is suitable for the intended purpose, and is capable of providing accurate, precise, reliable, and valid analytical data. To achieve this; several experiments were performed to fully characterize the method during the development stage in terms of (1) selectivity (2) matrix effect and recovery (3) calibration model and linearity (4) limit of detection, lower and upper limits of quantitation and dilution integrity. (5) sensitivity and (6) stability. Accuracy, precision and reproducibility were investigated during the method validation phase.

2.6.2 Method Development Phase

2.6.2.1 Selectivity

The selectivity of a bioanalytical method is its ability

to measure unequivocally to differentiate the analyte(s) in the presence of other components, which may be expected to be present in the sample extract. Analytical signals were measured from six lots of plasma samples, and from samples at the lower limits of quantitation (LLOQ) containing 36.0 (µg/ml) cefdinir were recorded and illustrated in figure (2).

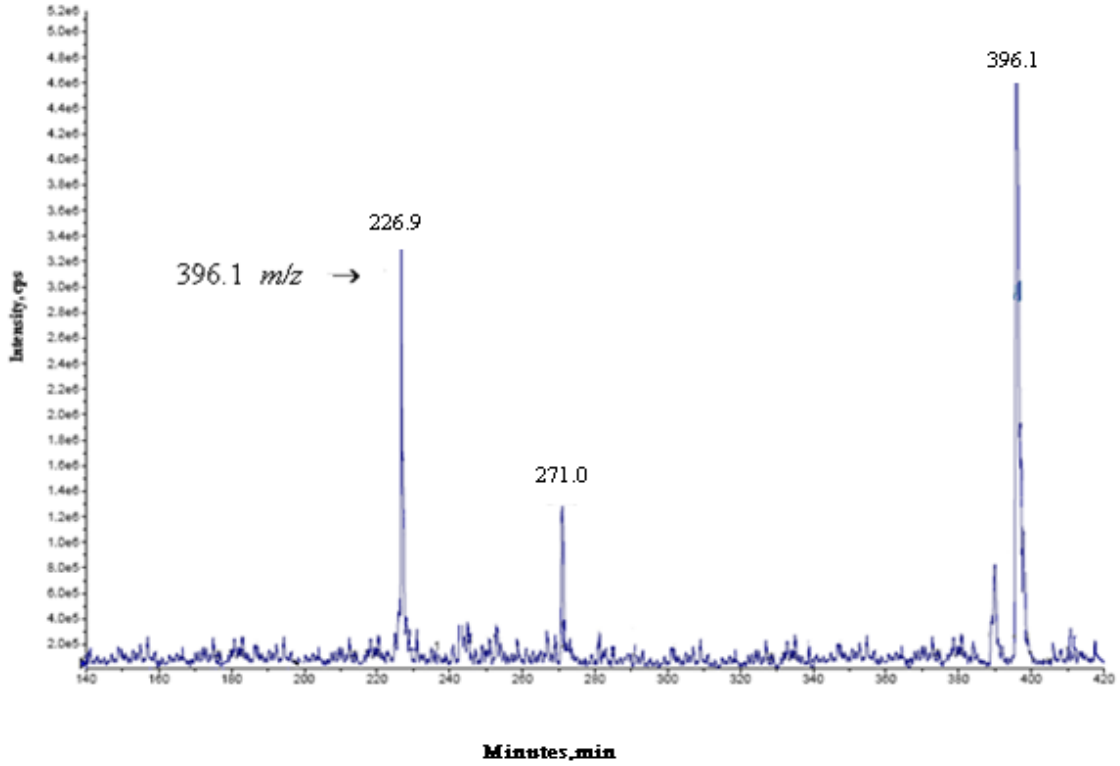


Figure 2: Full scan product ion spectrum of $[M+H]^+$ of cefdinir

2.6.2.2 Matrix effect and recovery

Matrix Effect is defined as the suppression or enhancement of ionization of the analyte(s) caused by the presence of interferants in the extracted sample. In mass spectrometry reduction or enhancement of the analytical response of the analyte, or the internal standard, are due to co-eluting endogenous or exogenous substances extracted from the matrix, using the optimized sample preparation and/or extraction technique.

In the present research, qualitative and quantitative experiments were conducted to ensure that matrix effects are accounted for. Qualitative evaluation was based on post column infusion technique, and was utilized to identify specific regions in the chromatogram were the analyte(s) response may be susceptible to matrix effects. Quantitatively matrix effect was evaluated both in the absolute and in the relative sense.

Absolute Matrix Factors (AMFs) were evaluated for cefdinir and cephalexin in accordance with the following equation:

(AMF) = Peak response in blank matrix extract spiked post extraction/Peak response in a neat solution (analyte in the mobile phase)

The Relative Matrix Factors (RMFs) were measured using the following equation:

IS Normalized Matrix Factor = AMF of analyte/ AMF of the IS

Similar to matrix effect, recovery was also evaluated both in the absolute and in the relative sense. Absolute recovery was evaluated by comparing the analytical MS/MS response measured from extracted test samples with those measured from neat solutions containing the same concentration levels. Table 2 summarizes the results of matrix effects and recovery experiments.

Table 2. A. Peak areas measured from absolute recovery experiment for Cefdinir

	QC_L		QC_M		QC_H		Internal standard	
	Area *plasma*	**Area *m.phase***	**Area *plasma***	**Area *m.phase***	**Area *plasma***	**Area *m.phase***	**Area *plasma***	**Area *m.phase***
1.	22530	30320	395460	651908	610654	1085678	685038	820004
2.	19350	29192	441288	658075	608056	1021821	622753	875011
3.	19452	30007	421408	651089	589451	1083091	597875	870495
Average	**20444**	**29840**	**419385**	**653691**	**602720**	**1063530**	**635222**	**855170**
Absolute recovery %	**68.51**		**64.16**		**56.67**		**74.28**	

Table 2 B: Measured concentrations for evaluating relative analytical recovery for Cefdinir

	conc. 1 measured	**conc. 2 measured**	**conc. 3 measured**	**Average recovered conc.**	**Relative recovery%**
QC_L	31.61	29.94	31.29	**30.95**	**103.16**
QC_M	616.03	667.12	633.73	**638.96**	**106.49**
QC_H	1023.98	995.27	1007.72	**1008.99**	**105.10**
Average					**104.92**

2.6.2.3 Calibration Model and Linearity

Prior to validation, six calibration growth curves were constructed. Each curve comprised ten none zero concentration levels. Several weighting factors were statistically evaluated, the least residual error model, using a statistical weight of $1/x^2$ was employed by plotting Cefdinir concenrations Vs peak area ratios (Cefdinir/Cepahlexin) covering the ranges: 10.00, 50.00, 75.00, 150.00, 325.00, 500.00, 675.00, 850.00, 1000.00, and 1200.00 (ng/ml cefdinir).

An average slope of 1.03 X 10^{-3} with an average intercept of 0.44 x 10^{-3} and an average product moment correlation coefficient (R) of 0.99755 were obtained. The standard equation was used to determine the concentrations of freshly prepared matrix based three quality control samples, having the following nominal concentrations: 36.00 (ng/ml) 600.00 (ng/ml) and 900.00 (ng/ml) cefdinir.

The concentration response curves were used to choose the optimum calibration model and to ensure that the developed method is fit for the purpose of analysis. Linearity however, was established by preparing six new sets of matrix based calibration standards. These utilized different concentrations from the calibrators used for constructing the calibration curves. Using the regression equation, linearity was established by plotting the nominal concentrations Vs the measured concentrations. Statistical evaluation has included the compatibility of the slope with 1.0 and the intercept with 0.0. Linearity was thus established.

Table 3 summarizes the calibration results

Table 3. Area ratio *vs.* nominal concentration values for Cefdinir calibrators

Sample	Nominal values (ng/mL)	Curve 1	Curve 2	Curve 3	Curve 4	Curve 5	Curve 6
1	**10.00**	0.010	0.012	0.011	0.011	0.010	0.011
2	**50.00**	0.042	0.048	0.050	0.049	0.048	0.047
3	**75.00**	0.065	0.071	0.074	0.074	0.072	0.072
4	**150.00**	0.156	0.163	0.171	0.160	0.159	0.157
5	**325.00**	0.285	0.336	0.329	0.324	0.332	0.327
6	**500.00**	0.466	0.525	0.540	0.537	0.519	0.534
7	**675.00**	0.704	0.738	0.799	0.766	0.755	0.719
8	**850.00**	0.851	0.854	0.928	0.920	0.893	0.937
9	**1000.00**	1.076	0.948	1.168	1.158	1.070	1.115
10	**1200.00**	1.103	1.124	1.235	1.166	1.150	1.195
	Slope	**0.000957**	**0.000997**	**0.00108**	**0.00105**	**0.00104**	**0.00103**
	Intercept	**0.00032**	**0.00205**	**-0.000154**	**-0.000151**	**-0.00099**	**0.000231**
	R	**0.9956**	**0.9977**	**0.9976**	**0.9977**	**0.9987**	**0.9980**

2.6.2.4 Limits of Detection, Upper and Lower Limits of Quantitation and Dilution Integrity

The Limit of Detection (LOD) defined as: the lowest cefdinir concentration that can well be detected but is not precisely quantified. The LOD was 3.30 (ng/ml).

The measured Lower Limit of Quantitation (LLOQ), which was the concentration recorded with acceptable precision, was 10.00 (ng/ ml), since a 9.09 CV% was obtained from six replicate measurements.

The Upper Limit of Quantitation (ULOQ) was 1200.00 (ng/ml), and demonstrated an acceptable CV% of 4.13.

Dilution Integrity Experiments were also carried out to validate concentrations higher than 1200.00 (ng/ml), in case such values were encountered in test samples. Matrix based concentrations 2x QC_H and 3x QC_H were prepared. Six replicates were measured, CV % of 1.4, and 3.37 were obtained for cefdinir having concentrations of 1800.00 and 2700.00 (ng/ml), respectively.

2.6.2.5 Sensitivity

Calibration sensitivity is equivalent to the slope of the calibration graph which averaged 1.03 X 10^{-3}, whereas the analytical sensitivities were calculated for the three quality control samples as slope/S_c (where S_c is the standard deviation for each concentration. Analytical sensitivities of 5.3 X 10^{-3} for the QC_L, and 0.03 X 10^{-3} for the QC_M, and 0.03 X 10^{-3} for the QC_H were obtained.

2.6.2.6 Stability

The stability of cefdinir and the internal standard cephalexin were evaluated under different experimental conditions that took into consideration the following: (1) stock solution and matrix based stability involving short term and long term stability studies stability including (2) freeze-thaw stability (3) post preparative auto-sampler stability at the optimized temperature (40 ºC).

Quality Control samples were used as surrogates for analyte(s) stability tests, which mimicked the anticipated experimental conditions of study samples including the type of anti-coagulant used, container material and sample preservation and handling.

The short term stock solution stability test lasted for 6 hours at room temperature, whereas the long term

stability studies lasted for 42 days under frozen conditions (-80ºC).

Freeze-thaw stability was evaluated for 5 cycles, initially frozen for 24 hours and 12 hours between thaws. Post preparative auto-sampler stability was evaluated for 65 hours using two concentration levels, QC_L and QC_H, with analysis against both the original calibration curve, and a freshly prepared set of calibrators and QC samples. Tables 4, 5, 6 and 7 summarize the results of the cefdinir stability studies.

Table 4. Cefdinir matrix based solution (Short-term temperature stability)

QC_L	0 hrs (RT)	6 hrs (RT)
1.	12335	19168
2.	20414	18485
3.	19075	18594
Average	**17275**	**18749**
Stability	**100.00**	**108.53**
QC_H	**0 hrs (RT)**	**6 hrs (RT)**
1.	668855	566600
2.	634999	583392
3.	660255	598091
Average	**654703**	**582694**
Stability	**100.00**	**89.00**

Table 5. Cefdinir matrix based Stability under prolonged storage conditions (-80°C)

Day	QC_L		QC_M		QC_H	
	Calculated Conc.	Rec.%	Calculated Conc.	Rec.%	Calculated Conc.	Rec.%
05/05/2009	27.17	90.57	567.40	94.57	925.51	96.41
24/08/2009	29.32	97.73	587.87	97.98	943.68	98.30
02/09/2009	34.07	113.57	602.56	100.43	973.46	101.40
13/10/2009	30.99	103.30	594.71	99.12	954.90	99.47
28/10/2009	28.41	94.70	569.67	94.95	906.03	94.38
19/11/2009	28.85	96.17	574.70	95.78	967.69	100.80
Mean	**29.80**	**99.34**	**582.82**	**97.14**	**945.21**	**98.46**
S(±)	**2.43**	**8.11**	**14.37**	**2.40**	**25.78**	**2.68**
C.V %	**8.15**	**8.16**	**2.47**	**2.47**	**2.73**	**2.72**

2.6.3 Method Validation Phase

2.6.3.1 Accuracy Precision and Reliability

The ultimate objective of method development procedures and evaluation of the aforementioned merits of method performance is to obtain reliable bioanalytical data, precise and accurate where systematic determinate errors, which impact accuracy, are known and accounted for, and random indeterminate errors, which impact precision, fall within the regulatory acceptance criteria[16,17].

To accomplish the above, accuracy and precision were investigated by replicate determinations of QC samples containing known amounts of cefdinir at three

different concentration levels: low, medium, and high.

Tables 8, 9 and 10 summarise the results obtained for (1) evaluating method accuracy, trueness and precision in addition to (2) intra-day accuracy and precision and (3) inter-day accuracy and precision.

Table 6. Results for concentration for stability experiment after five freeze thaw cycle

QC_L							
FTC	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	**Average**	**Recovery (%)**
Zero	24.69	29.78	27.79	27.46	28.79	**27.70**	**92.33**
One	28.27	26.79	28.08	28.42	27.30	**27.77**	**92.57**
Two	28.31	27.92	29.29	28.48	27.80	**28.36**	**94.53**
Three	29.75	28.27	30.35	28.55	29.13	**29.21**	**97.37**
Four	28.64	28.07	28.03	28.39	28.11	**28.25**	**94.17**
Five	32.58	31.48	32.06	31.36	31.01	**31.70**	**105.67**
QC_M							
FTC	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	**Average**	**Recovery (%)**
Zero	460.47	589.02	557.51	587.48	583.09	**555.51**	**92.59**
One	578.55	574.98	570.97	560.04	616.15	**580.14**	**96.69**
Two	569.89	553.01	569.52	585.69	534.33	**562.49**	**93.75**
Three	555.95	567.20	550.35	592.86	551.56	**563.58**	**93.93**
Four	553.53	553.02	532.07	560.25	559.43	**551.66**	**91.94**
Five	602.24	629.98	586.71	595.18	596.56	**602.13**	**100.36**
QC_H							
FTC	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	**Average**	**Recovery (%)**
Zero	914.18	917.94	947.14	945.84	992.89	**943.60**	**98.29**
One	943.14	943.50	981.40	916.18	973.08	**951.46**	**99.11**
Two	953.00	946.28	926.95	919.04	929.46	**934.95**	**97.39**
Three	954.66	1007.19	958.18	944.83	934.35	**959.84**	**99.98**
Four	930.15	912.47	921.52	903.47	914.11	**916.34**	**95.45**
Five	1046.71	1048.80	1062.50	1047.01	994.70	**1039.94**	**108.33**

Incurred Sample Re-analysis (ISR) is currently regarded as a fundamental concept in bioanalysis to assess reproducibility and reliability of the validated method conducted for PK, BA and BE studies. ISR, and Incurred Sample Accuracy (ISA) are the current tools for assessing that the validated method remained valid during the analysis of test samples, while reaffirming reproducibility and reliability of the validated bioanalytical method.

10% of the harvested samples were randomly selected

for reanalysis. The results demonstrated that 90% of the repeat samples were within 20% of the mean values. No determinate method errors were spotted.

Table 7. Results of concentration measured (ng/mL) during post preparative auto-sampler stability

QC_L	0 hrs (RT)	12 hrs (RT)	44 hrs (RT)	65 hrs (RT)
1.	29.33	30.65	30.30	29.34
2.	30.71	30.74	30.82	29.50
3.	28.97	31.20	30.44	29.76
Average	**29.67**	**30.86**	**30.52**	**29.53**
Stability	**100.00**	**104.01**	**102.86**	**99.53**
QC_H	**0 hrs (RT)**	**12 hrs (RT)**	**44 hrs (RT)**	**65 hrs (RT)**
1.	939.52	966.09	951.82	919.64
2.	939.68	987.74	931.8	922.04
3.	921.75	998.89	928.07	913.62
Average	**933.65**	**984.24**	**937.23**	**918.43**
Stability	**100.00**	**105.42**	**100.38**	**98.37**

Table 8. Cefdinir method accuracy, trueness, and precision

	Concentration (ng/mL)		
Sample	QC_L	QC_M	QC_H
1.	30.62	594.86	1004.89
2.	28.19	603.20	937.64
3.	29.54	583.53	1021.63
4.	29.97	587.42	968.35
5.	29.61	633.66	1003.70
6.	29.55	642.97	999.03
	Precision:		
Average	***29.58***	***607.61***	***989.21***
Std. Dev. (S)	***0.80***	***24.89***	***30.65***
CV (%)	***2.70***	***4.10***	***3.10***
	Accuracy		
Error	***-0.42***	***7.61***	***29.21***
Relative error %	***-1.40***	***1.27***	***3.04***

3. Method Application

The validated bioanalytical method was applied to evaluate the BE of a test and reference drug products, containing cefdinir (125mg/5mL) suspension in healthy volunteers (N=26) under fasting conditions. (Study Code: 09B015).

3.1 Study Design

The study design was an open label, single dose, randomized, two-treatment, two-period, two-sequence, crossover of cefdinir (125mg/5mL paediatric suspension) comparing equal doses (5mL) of cefdinir (Pharma International Company, Jordan) and OMNICEF® (125 mg/5 mL Suspension) (Manufactured by Jazeera Pharmaceutical Industries, Riyadh-Saudi Arabia (HIKMA Pharmaceuticals, Amman-Jordan) in healthy male participants. A washout period of one week between doses was observed.

Table 9. Summary of intra-day trueness and accuracy and precision of cefdinir

	Day One			Day Two			Day Three		
	QC_L	QC_M	QC_H	QC_L	QC_M	QC_H	QC_L	QC_M	QC_H
1	29.29	605.42	1007.03	29.41	626.68	983.43	30.88	673.39	1093.99
2	25.72	591.73	938.53	30.69	653.95	977.95	28.65	669.49	1075.38
3	29.96	583.24	879.77	29.99	626.44	1030.19	29.29	620.69	1088.02
4	31.71	593.45	968.82	29.89	626.63	1000.43	27.30	656.91	1066.41
5	33.27	590.68	919.36	31.84	626.13	1013.82	30.17	658.14	1072.92
6	29.44	568.45	965.65	30.65	653.58	1012.86	29.15	656.52	1079.35
Precision									
Average	**29.90**	**588.83**	**946.53**	**30.41**	**635.57**	**1003.11**	**29.24**	**655.86**	**1079.35**
Std dev. (S)	**2.56**	**12.29**	**44.23**	**0.85**	**14.10**	**19.85**	**1.24**	**18.64**	**10.14**
CV%	**8.56**	**2.09**	**4.67**	**2.8**	**2.22**	**1.98**	**4.24**	**2.84**	**0.94**
Accuracy									
Error	**-0.10**	**-11.17**	**-13.47**	**0.41**	**35.57**	**43.11**	**-0.76**	**55.86**	**119.35**
Rel. Error%	**-0.33**	**-1.86**	**-1.40**	**1.37**	**5.93**	**4.49**	**-2.53**	**9.31**	**12.43**

Table 10. Summary of inter-day accuracy, trueness and precision of cefdinir

		Concentration (*n*g/mL)		
Day	**Date**	QC_L	QC_M	QC_H
1	**19/11/2009**	31.26	613.41	1048.60
2	**21/11/2009**	30.25	578.11	976.25
3	**22/11/2009**	28.43	620.49	978.44
4	**23/11/2009**	26.80	538.24	951.00
5	**24/11/2009**	31.81	631.21	1004.79
6	**01/12/2009**	30.91	562.56	957.72
Average		**29.91**	**590.67**	**986.13**
Std Dev.		**1.92**	**36.73**	**35.91**
CV%		**6.42**	**6.22**	**3.64**
Error (Bias)		**-0.09**	**-9.33**	**26.13**
Rel. Error (%)		**-0.30**	**-1.56**	**2.72**

Table 11. Pharmacokinetic parameters of Cefdinir (5mL/125 mg) evaluated in 26 healthy volunteers under fasting conditions

Parameter (unit)	Test (cefdinir)		Reference (OMNICEF ®)	
	Geometric Mean	**Range**	**Geometric Mean**	**Range**
C_{max} (ng/mL)	812.783	347.410 - 1607.300	751.501	334.630 - 1197.230
$AUC_{0\rightarrow last}$ (ng.h/mL)	3944.18	1869.87 - 6867.97	3806.25	1605.49 - 6182.72
$AUC_{0\rightarrow inf}$ (ng.h/mL)	4010.55	1906.94 - 7023.67	3865.86	1634.59 - 6396.29
	Median	**Range**	**Median**	**Range**
t_{max} (h)	2.67	1.67 - 4.50	2.67	1.33 - 4.00
$t_{1/2}$ (h)	1.54	1.07 - 2.07	1.51	1.23 - 2.03

Table 12. Summary of Statistical data including confidence interval (1-2α)% for the ratio of geometric mean (µT/µR), power and observed intra-subject variability (CV%), (N=26)

Parameter	Point Estimate (Ratio of geometric mean%)	Lower limit %	Upper limit %	Confidence Level (1-2α)%	Power%	CV%
$AUC_{0\text{-}Last}$	103.997	93.422	115.770	90	87.89717	22.37820
$AUC_{0\rightarrow inf}$	104.111	93.490	115.938	90	87.39069	22.45431
C_{max}	108.533	98.161	120.001	90	75.62532	20.92827

Confidence level (1-2α)%.

3.2 Selection of Subjects

The subject population for the present BE study was based on an appropriate sample size (N=26) estimated statistically to provide enough power (at least 80%) to depict a 20% difference between the drug products. Twenty six healthy adult male Middle Eastern volunteers were selected after assessment of their health status including a thorough medical history, physical examination, ECG, in addition to haematology, biochemistry, electrolytes, and urinalysis testing and urine screening for drugs of abuse. The volunteers were free of cardiac, hepatic, renal, pulmonary, gastrointestinal, neurological impairment. The subject age averaged 28 years with a body mass index (BMI) averaging 27.0 (kg/m^2).

3.3 Study Conduct

The BE study conditions were standardized in order to minimize the variability for all factors involved, except that of the product under testing. Diet, fluid intake and exercise were standardized. Subjects fasted for 10 hours prior to drug administration with 240 ml water each. Breakfast was taken after 4 hours of drug dosing.

3.4 Sampling Times

Sampling time points were planned so that frequent sampling around C_{max} and T_{max} occurred to provide a reliable estimate of peak exposure and a reliable estimate of the extent of exposure. The following sampling time points were thus planned: a pre-dose sample (16 ml) and (8 ml) samples at 0.50, 1.00, 1.33, 1.67, 2.00, 2.33, 2.67, 3.00, 3.67, 4.00, 4.50, 5.00, 6.00, 8.00, 10.00, and 12.00 hours post dose (a total of 18 samples per patient per period were collected). The plasma samples were separated and stored at -80°C until further analysis.

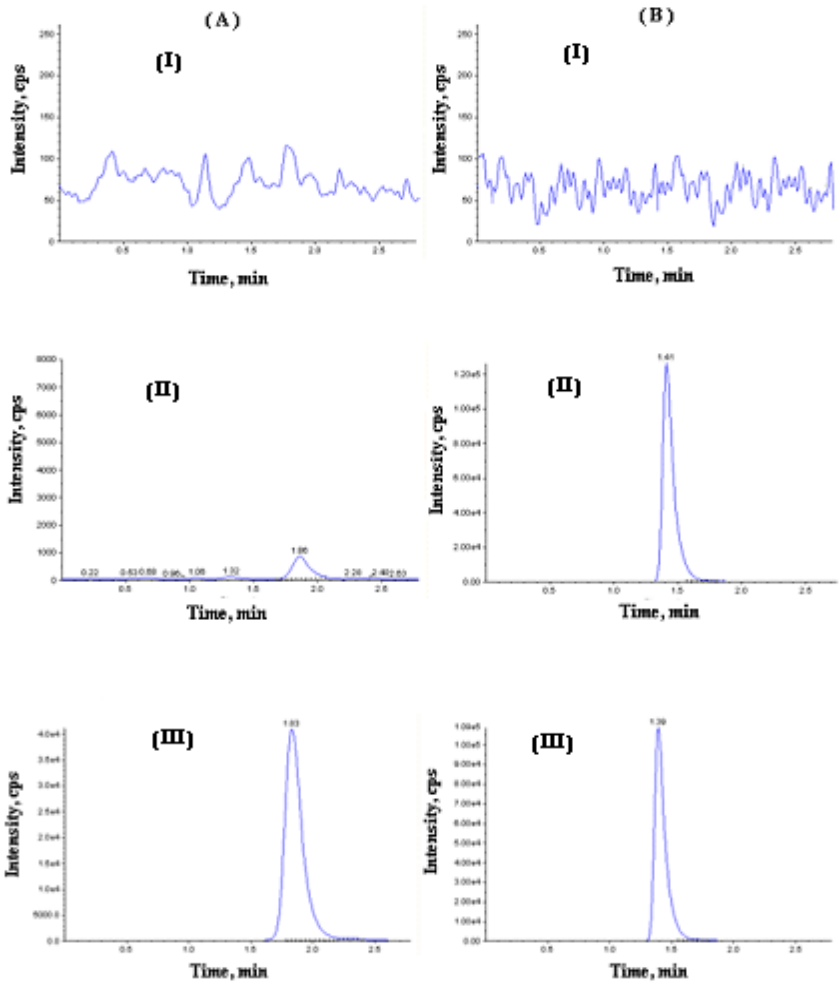


Figure 3. Representative chromatograms of extract human plasma for: (A) Cefdinir and (B) Cephalexin (IS). (I) blank plasma sample, (II) blank plasma sample spiked with Cefdinir at lower limit of Quantitation (10 ng/mL), and (III) plasma extract of a volunteer 3hour after administration (125 mg Cefdinir /5mL suspension).

3.5 Ethical Conduct of the Study

The present BE study was conducted in accordance with the international ethical guidelines for clinical studies in humans set out in the Declaration of Helsinki, as well as the ICH-GCP and the Jordan FDA requirements.

The study was conducted in compliance with the protocol that received prior favourable opinion by the Pharmaquestjo IRB/ Institutional Ethics Committee. The protocol was authorized by the Jordan Food and Drug Administration (Clinical Trials Committee), and the recruited volunteers signed an informed consent prior to the screening phase.

4. Pharmacokinetic and Statistical Results

Based on the concentration time profiles, the pharmacokinetic parameters of C_{max}, T_{max}, $t_{½}$, $AUC_{0\text{-}Last}$, and $AUC_{0\text{-}\infty}$ were evaluated prior to statistical assessment of bio-equivalency, the results of which are summarized in Tables 11 and 12. The mean concentration time profile

is illustrated in figure (4).

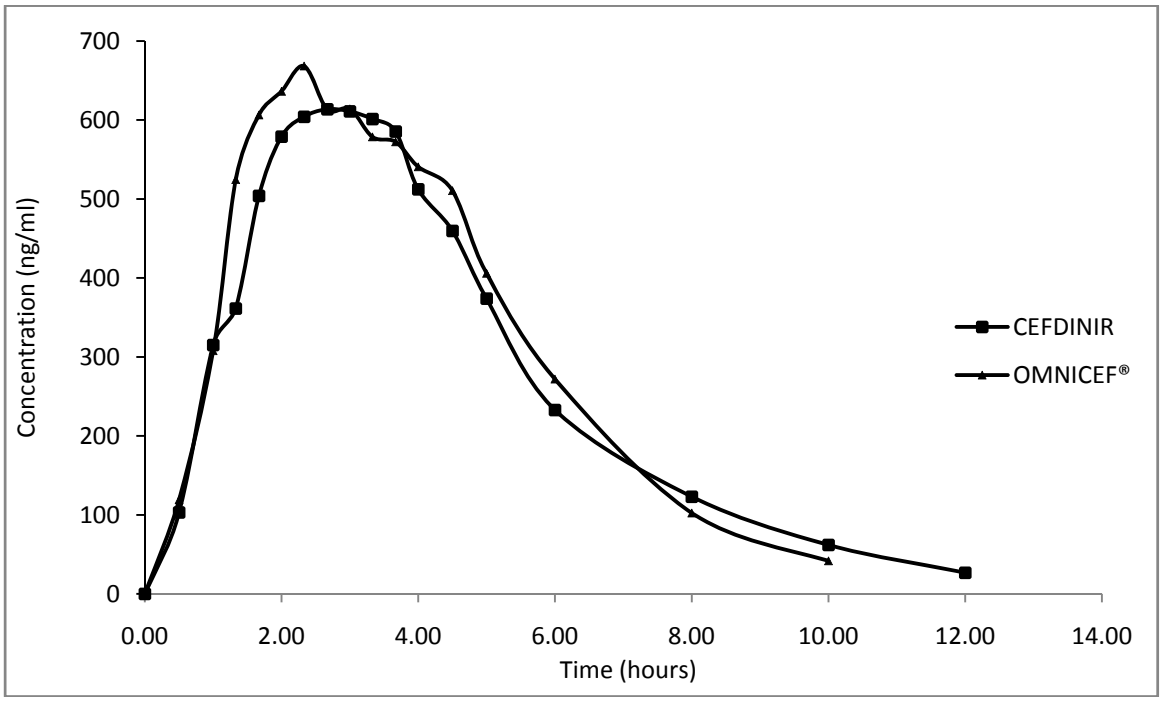


Figure 4. Mean Plasma Concentration (ng/ml) of cefdinir versus time (hours) curves following a single dose of 125 mg/5 ml Suspension cefdinir based on 26 participants

5. DISCUSSION

5.1 The Developed HPLC-MS/MS Method

The developed method was optimized by using an ESI, which is a soft ionization technique that produces high mass-to-charge $[M+H]^+$ parent ion, with minimal fragmentation of analytes. Cefdinir and cephalexin both produced protonated parent ions $[M+H]^+$ in the positive ESI mode. The major ion observed in the MS/MS spectra was at 226.9 m/z for cefdinir. Additional tuning of the ESI source including capillary temperature, flow of the auxiliary gas nitrogen and spray voltage resulted in transitions (m/z) of 396.1→ 226.9 for cefdinir and 348.24→158.18 for cephalexin. Using the MRM mode higher sensitivity and selectivity were obtained using the optimized chromatographic and mass spectrometric experimental conditions described earlier.

5.2 Validation Parameters Vs. Regulatory Acceptance Criteria

Similar to all analytical applications, bioanalysis is a systematic, relative, (comparative) technology. Test samples are harvested during a BE study and are of unknown concentrations that fall within a wide concentration range. The reported concentration levels are measured by comparing the MS/MS analytical signals, obtained from extracted test samples with those from calibration standards. To achieve a reliable bioanalytical method, bio-analysts face several challenges including (1) developing and validating a method using a surrogate matrix (plasma from a blood bank) and pretending that this matrix is identical to that of the harvested plasma matrix from participants. (2) dealing with the assumption that the plasma samples harvested from the same subject but at different time points, have

the same matrix even though the matrix compositions significantly vary depending on whether the sample was collected under fasting conditions (zero to 4 hours) after dosing, or after the subject receives a meal, or whether the subject is hospitalized, or gives samples after hospitalization. (3) Bio-analysts must face the daunting challenge of satisfying sound analytical knowledge and definitions and coping with the terminology and definitions presented by the multitude of global regulatory guidance(s) and expectations. (4) despite the several scientific meetings and conferences, until recently, regulators could not draw a line between method development and method validation experiments.

The present report demonstrates that despite of the above challenges, the developed and validated method for the determination of cefdinir in human plasma produced quite accurate, precise, and reproducible data. This was only accomplished when the bioanalysis is performed with proactive high quality strategy.

Method development experiments have included the evaluation of (1) selectivity, (2) matrix effect and recovery (3) choice of the calibration model and linearity (4) lower and upper limits of quantitation and dilution integrity (5) sensitivity and (6) stability. Method performance in this context, is characterized by the merits of method performance which eventually determines fitness for purpose and its successful application to the present BE study.

Method validation assesses the most critical parameters including accuracy, which is a combination of trueness and precision as well as reproducibility to ensure that the validated method remained valid until the last sample was analyzed.

After optimizing the cefdinir and cephalexin analytical signals, the experimental conditions were fixed and used for evaluating merits of method performance (Table 1). High selectivity was demonstrated by optimizing (1) separation selectivity (2) extraction selectivity (3) and detection selectivity. Separation selectivity was optimized by chromatographic conditions, extraction selectivity was demonstrated through an optimum sample preparation which involved a simple de-protonization procedure followed by dilution (20 times). The described procedure gave good analytical signals using a highly sensitive triple quadruple MS/MS. Furthermore, detection selectivity, sensitivity and signal to noise ratio were optimized by the choice of the MS/MS experimental condition coupled by the MRM analytical method.

5.3 Cefdinir Pharmacokinetic Characteristics

In adults, cefdinir is absorbed rapidly from the suspension formulation (125 mg/ 5mL). The mean maximum plasma concentration (C_{max}) of 800 (ng/ml) after a single dose was measured. A T_{max} of 3 hours was observed after administration. Cefdinir is eliminated fairly rapidly with a plasma elimination half-life ($t_{½}$) of 1.3-1.8 hours in adults. The mean concentration time profile is illustrated in figure 4. The pharmacokinetic parameters of C_{max}, T_{max}, $t_{½}$, $AUC_{0\text{-}Last}$, $AUC_{0\text{-}\infty}$ and the statistical parameters for the pharmacokinetic study are summarized in Table 11 and 12 respectively.

5.4 Bioequivalence Decision Making

Cefdinir is not metabolized to an appreciable extent and is eliminated via the kidneys. After single oral doses of 300 or 600 mg, renal clearance was about 2ml/min/kg with apparent oral clearance values of 11.6 and 15.5 mg/min/kg. The plasma elimination half life of cefdinir is 1.5-1.7 hours in adults and 1.2 - 1.5 hours in healthy infants and children.

Cefdinir has an estimated BA of 21% and 16% after administration of single 300 and 600 mg capsules and an estimated absolute bioavailability of 25% after administration of the suspension. The rate and extent of absorption of cefdinir, decrease although not clinically significant when the drug is taken with a high fat meal. Consequently, cefdinir may be taken without regard to food. The mean volume of distribution of cefdinir is 1.56-2.09 L/kg in adults and 0.67 L/kg in pediatric patients.

Cefdinir is 60-73% plasma protein bound and is widely distributed and achieves clinically relevant concentrations in bronchial mucosa, epithelial lining fluid, tonsillary tissue, sinus tissue, skin blister fluid, and

middle ear fluid. (1)

6. CONCLUSION

The developed and validated bioanalytical method was fit for the purpose and was successfully applied for the determination of plasma concentration of cefdinir harvested during a bioequivalence study on healthy male volunteers (N=26). Sampling lasted for 12 hours after dosing in the fasted state. The resulting concentration time profiles were utilized for evaluating the main PK parameters including C_{max}, T_{max}, $t_{½}$, $AUC_{0\text{-}Last}$, $AUC_{0\text{-}\infty}$. The results were in line with expectations. The statistical evaluation demonstrated that the two products are bioequivalent.

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قياس تراكيز السيفدينير في البلازما البشرية باستخدام تقنية الكروماتوغرافيا السائلة ذات الكفاءة العالية المرتبطة بكاشف المطياف الكتلي: التطبيق على دراسة التكافؤ والتوافر الحيوي

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ملخص

تم تطوير طريقة تحليلية حساسة وانتقائية لقياس تراكيز مادة السيفدينير الفاعلة في عينات بلازما بشرية بعد أن تم إجراء جميع التجارب التثبيتية المطلوبة. الطريقة التحليلية التي تم اعتمادها هي تقنية الكروماتوغرافيا السائلة المرتبطة بكاشف المطياف الكتلي الحساس. تم فصل مادة السيفدينير الفاعلة ومادة المعايرة الداخلية السيفاليكسين عن مكونات البلازما البشرية باستخدام عامود الفصل المعتمد على الطور المعكوس (Reverse Phase). تم استخدام عامود بيوروسفير من شركة ميرك الألمانية (الطول 30 ملم، القطر الداخلي 4.6 ملم، قطر الحبيبات 3 ميكروميتر). أم الطور المتحرك مكون من محلول مائي يحتوي على حامض الفورميك والأسيتونيترال بنسبة 85:15%. تم تثبيت سرعة الطور المتحرك لتصبح 0.5 ملم في الدقيقة. كما تم استخدام كاشف المطياف الكتلي الثلاثي بعد تأيين المراد الكشف عنها باستخدام تقنية الرذاذ الكهربائي (Electrospray Ionization) الموجب. تم اعتماد قراءات الإشارات التحليلية ومتابعتها بوساطة القيم الانتقالية 226.90 → 396.10 لمادة السيفدينير و158.10 → 348.24 لمادة السيفاليكسين. تم الحصول على معادلة الخط المستقيم للتراكيز الواقعة بين 10–1200 نانوغرام/ملم بنجاح. كما تم إجراء جميع التجارب المعتمدة للقيام بالتجارب التثبيتية اللازمة وتطبيق الطرق التحليلية على دراسة التكافؤ والتوافر الحيوي لمحلول معلق يحتوي على 125/5 ملم من الـ26 متطوعاً بعد امتناع المتطوعين عن الطعام لمدة 10 ساعات قبل إعطاء جرعة الدواء المطلوبة.

الكلمات الدالة: تراكيز السيفدينير، البلازما البشرية، تقنية الكروماتوغرافيا.

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