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A Simultaneous, Validated RP-HPLC Method for Determination of Eight Cephalosporins in Pharmaceutical Formulations.

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ABSTRACT

A reversed phase high performance liquid chromatographic method with isocratic solvent system has been developed and validated for the simultaneous determination of eight of cephalosporins antibiotics (Cefepime Hydrochloride, Ceftazidime, Ceftiofur Sodium, Cefotaxime Sodium, Ceftriaxone Sodium, Cefoperazone Sodium, Cephradine and Cefazolin Sodium) in pharmaceutical formulations with run time of 35 min. The best separation was obtained by using a 250 mm × 4.6 mm i.d, 5.0 µm particle size C8 reversed phase Agilent column and 0.1 M ammonium acetate buffer (pH 5.6): Acetonitril, 95:5 (v/v) as mobile phase at a flow rate of 0.8 mL/min. UV detection was conducted at 250 nm and column temperature at 30° C. The method linearity achieved over the concentration range of 0.5-50 µg/ mL with correlation coefficient (r2= 0.9999) for each studied cephalosporins. The developed method achieved lower limits of detection (0.018 µg/mL: 0.03 µg/mL) and lower limits of quantification (0.056 µg/mL: 0.09 µg/mL).

The suggested method is highly sensitive, accurate, precise, and could be used for quality control assay and routine analysis for this group of cephalosporins in pharmaceutical formulations.

Keywords: Cephalosporins, Simultaneous, HPLC, Validation, Pharmaceutical formulations.

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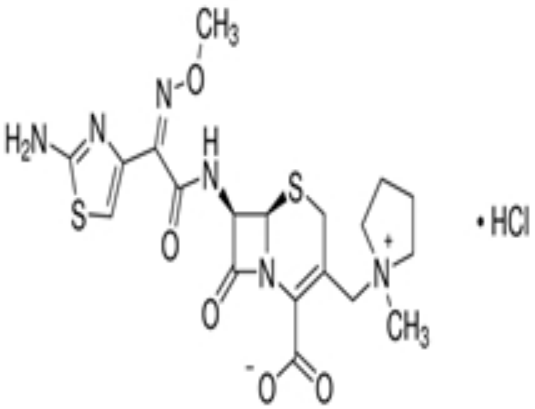
INTRODUCTON

Cephalosporins are β-lactam antibiotics that are structurally and pharmacologically related to penicillin [1, 2]. Cephalosporins are distinct from penicillins because the B ring is a 6-membered ring of dihydrothiazine. Variations between cephalosporins are reported either in the 7-position acyl side chain to change antibacterial function or in the 3-position to alter the pharmacokinetic profile [3]. By blocking transpeptidases, cephalosporins prevent synthesis of the bacterial cell wall [4].

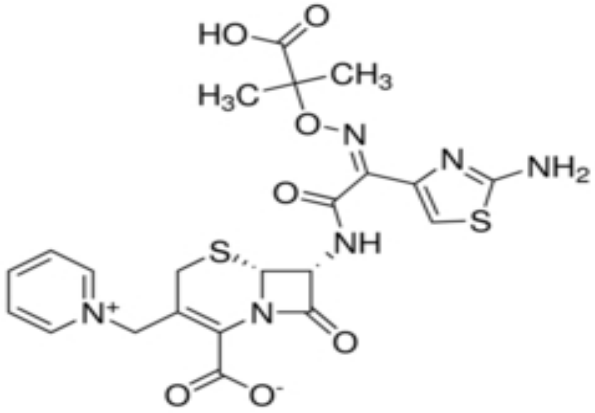
They are used for the control of Gram (+) and Gram (−) bacterial infections. They are among the safest and most potent broad-spectrum bactericidal antimicrobial agents, making them the most commonly prescribed antibiotic class [5, 6]. They are classified into five generations [7].

In the literature, a variety of methods for the determination of different cephalosporins in pharmaceutical preparations and biological matrices have been reported. These involve spectrophotometric [8-13], spectrofluorimetric [14-16], voltammetric [17], and chromatographic [18-26]. But only this proposed method analyzed the eight cephalosporins simultaneously.

Our study was performed to develop and validate a rapid, sensitive, cost effect and time-saving method for the simultaneous determination of eight of the most commonly prescribed cephalosporins in pharmaceutical formulations. The simultaneous quantification of these essential cephalosporins with an isocratic solvent system in the same run not only saves the solvent but also with a short run time makes it a better option for the determination of these drugs in analysis and quality control labs. According to ICH guidelines, this suggested method was validated with well-resolved peaks without interference [27].



Cefepime hydrochloride



Ceftazidime

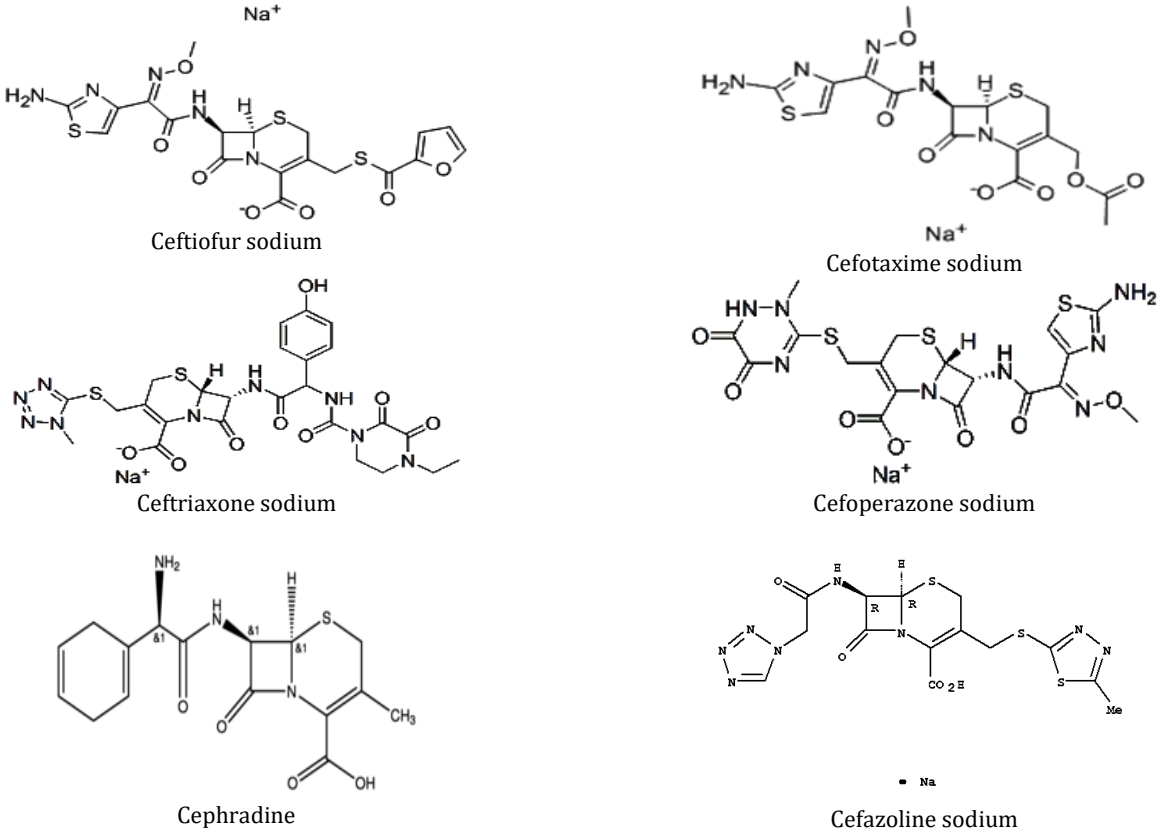


Figure 1. Chemical structure of the cephalosporins studied.

EXPERIMENTAL

Materials and Reagents

Acetonitril, ammonium acetate and glacial acetic acid were HPLC or high-grade purity (LOBA CHEMIE PVT.LTD.).

De-ionized water (Type 1-Ultra-pure water) was obtained from a Milli-Q-system (Millipore, Molsheim, France).

Standard reference materials of (Cefepime Hydrochloride, Ceftazidime, Ceftiofur Sodium, Cefotaxime Sodium, Ceftriaxone Sodium, Cefoperazone Sodium Cephradine and Cefazolin Sodium) were provided by Sigma-Aldrish and Supelco. Pharmaceutical formulations were purchased from local market.

Preparation of standard solutions

A stock solution of each of cephalosporins was prepared by dissolving 10 mg of each standard powder in 10 mL De-ionized water (1000 µg/mL). Stock standard solutions stored at 4 °C. Intermediate mixture solutions of all cephalosporins were prepared by mixing standard stock solution of each standard of selected cephalosporins in equivalent concentration units in 10 mL volumetric flask and diluted with De-ionized water to give final concentration (100 µg/mL) from which working mixture solutions were prepared (0.5, 1, 2, 5, 10, 20, 50 µg/mL).

Preparation of sample solution

Ten mg was accurately weighed from the powder of each drug and transferred to a 10 mL volumetric flask and De-ionized was added to volume to give a final concentration of (1000 µg/mL) (stock solution). One mL of each solution was transferred to 10 mL volumetric flask and diluted to volume with De-ionized water, giving a final concentration of 100 µg/mL (intermediate solution) and sonicated for a minimum 30 minute.

Preparation of buffer solution

By dissolving about 7.708 g of ammonium acetate in 1000 mL of De-ionized water, a concentration of 0.1 M of the buffer solution was obtained. 0.1 M acetic acid prepared by adding 5.7 mL 17.4 M glacial acetic acid to 1000 mL of De-ionized water used to adjust pH at 5.6 ± 0.2.

Instrumentation and Chromatographic Conditions

The HPLC Agilent 1200 system consists of quaternary pump, Auto sampler, UV-VIS detector, and 2D Chemstation software (HP, Les Ulis, France). The detector was set at 250 nm. Agilent Zobrax C8 column (5µm, 250 mm×4.6 mm) was used at 30ºC. The isocratic elution was applied by using mixture of 0.1 M ammonium acetate buffer and acetonitril in 95:5 (v/v) (pH 5.6) with a flow rate of 0.8 mL/min. Before using the mobile phase it should be filtered through a 0.45 µm membrane filter and degassed by sonication, the injection volume was 25 µL.

Method Validation

It is the technique which applied by laboratory studies in order to verify that the performance characteristics of the method fulfill the requirements for the proposed analytical application according to International conference on harmonization of technical requirements for registration of pharmaceuticals for human use (ICH, 2005) [27].

1-Selectivity and specificity:

Verification of selectivity and specificity is conducted by evaluating the standard addition on the mixture of drug samples. Criteria for acceptance: There is no interference between the pure standard peaks and peaks of any impurities.

2-Linearity and range:

Linearity is established by preparing 7 different concentrations of drug standard mixture. Linearity is evaluated by the squared correlation coefficient, which should be (r^2) 0.999.

3-Limit of Detection (LOD) and Limit of Quantification

(LOQ):
They were calculated based on standard deviation of intercept (S) and slope (b)
LOD = 3.3×S/b, LOQ = 10×S/b

Method Precision:
It is performed by using 5 replicates of standard mixture solutions. Criteria for acceptance: Relative standard deviation percentage (RSD) ≤ 2%.

Accuracy and recovery:
The standard additions at different concentrations are prepared by adding known quantities of mixture standard solution on mixture of drug samples. These samples were analyzed against standard solutions of same concentrations. From the test results, the accuracy is then calculated as a percentage recovery.

Robustness:
The robustness of an analytical method is the degree to which it is able to remain unaffected by minor but deliberate changes in the parameters of the system and to achieve its reliability during normal use. The Detection wavelength (nm), temperature (°C), and mobile phase pH parameters were chosen for this study. Criteria for acceptance: Relative standard deviation percentage is not more than 2% in every change item.

System Suitability Test:
To assess system suitability, relative standard deviations of retention time, tailing factor, number of theoretical plates and peak area were calculated as specified in United States pharmacopeia, 2019) [28].

RESULTS AND DISCUSSION

Optimization of experimental conditions

The potential effect of a number of experimental conditions on the resolution of chromatographic peaks of the studied group of drugs was tested. Three experimental parameters, mobile phase composition, wavelength and buffer pH have been chosen to properly investigate their effect on the analysis of mixture of the selected drugs. Different proportions of a mixture of 0.1 M ammonium acetate buffer and acetonitrile such as 85:15, 90:10, and 95:5 were examined for their separation using reversed phase High Performance Liquid Chromatographic technique. Proper separation with symmetrical peaks was achieved by using mobile phase containing 0.1 M ammonium acetate buffer and acetonitrile in 95:5 (v/v) at flow rate 0.8 mL/min. The UV detector was adjusted at different wavelengths, 230 nm, 240 nm, 250 nm, 260 nm, 270 nm, and 280 nm then the peak heights were estimated. At 250 nm, which was chosen as the optimal wavelength for the rest of the measurements, not only higher peak heights but also the best separation for the mixture of drugs was achieved. It was found that pH influenced the chromatographic separation but not to a wider extent so, pH 5.6 was used in further analysis.

Method Validation:

Selectivity and specificity

Demonstration of selectivity and specificity requires that the procedure is unaffected by the presence of impurities or excipients. There no interferences were observed on the chromatograms as no interfering peaks were obtained with the same retention time of drug substances.

Table 1: Summery of retention time data:

Compound	Retention time
Cefepime Hydrochloride	6.08
Ceftazidime	8.81
Ceftiofur Sodium	9.85
Cefotaxime Sodium	10.94
Ceftriaxone Sodium	17.51
Cefoperazone Sodium	23.58
Cephradine	25.67
Cefazolin Sodium	29.44

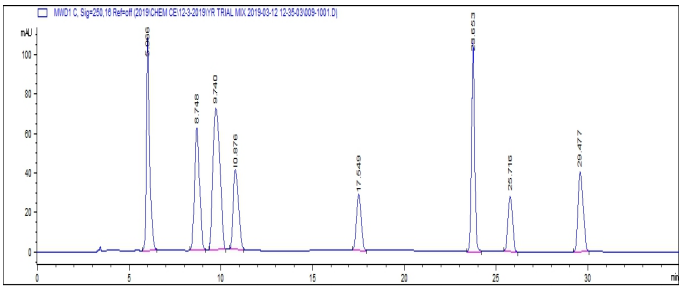


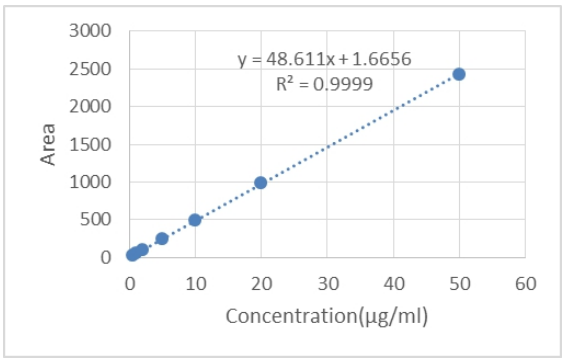
Figure 2. Chromatogram of a standard solution with the eight cephalosporins in a concentration of 20 µg/mL.

Linearity and Range

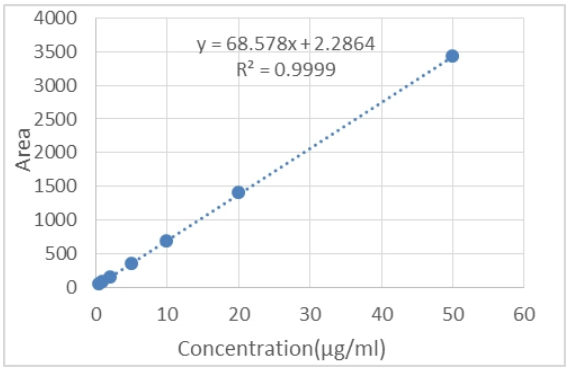
Linear correlation was obtained between peak area and concentration of each compound of the mixed cephalosporins standard solution in the range of 0.5 µg/mL -50µg/mL, respectively. The linearity of the calibration curves was validated by the value of correlation coefficients of the regression (r^2) which found to be 0.9999 for each compound of the selected group.

Table 2: Regression statistics:

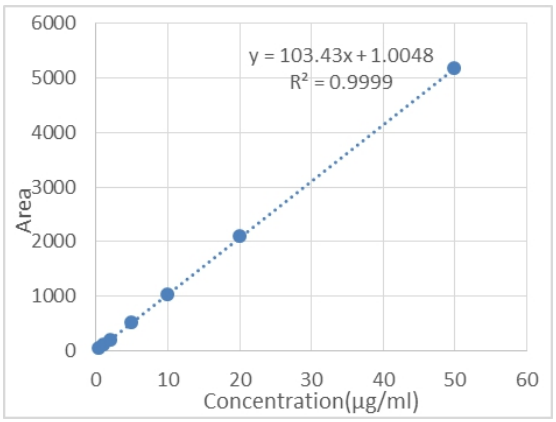
Drug	Regression equation
Cefepime Hydrochloride	y = 68.578x + 2.2864
Ceftazidime	y = 48.611x + 1.6656
Ceftiofur Sodium	y = 103.43x + 1.0048
Cefotaxime Sodium	y = 36.455x + 0.7775
Ceftriaxone Sodium	y = 31.416x + 0.1929
Cefoperazone Sodium	y = 60.32x + 1.2917
Cephradine	y = 31.027x - 0.4877
Cefazolin Sodium	y = 43.357x + 1.3028



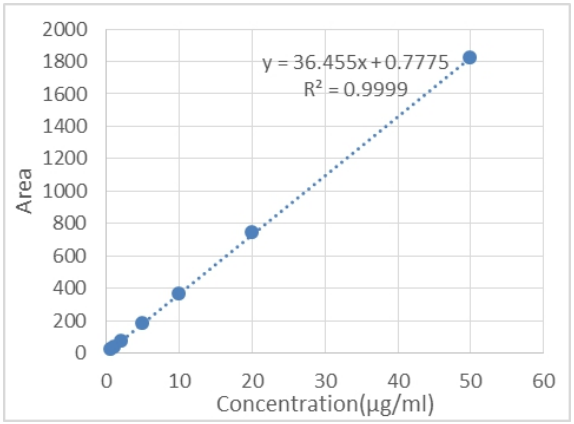
Calibration curve of Cefepime Hydrochloride.



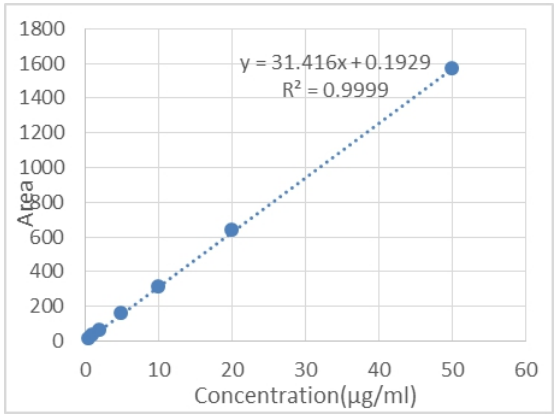
Calibration curve of Ceftazidime.



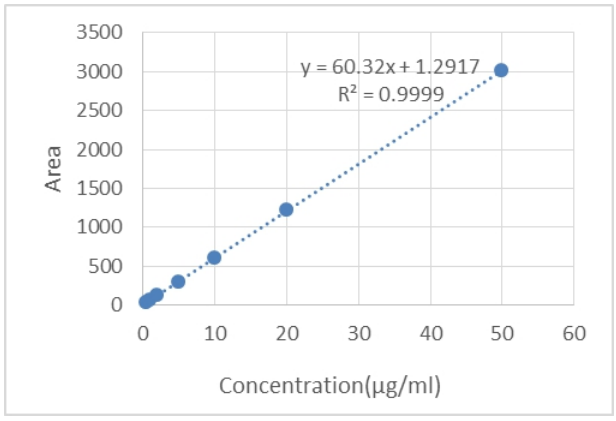
Calibration curve of Ceftiofur Sodium.



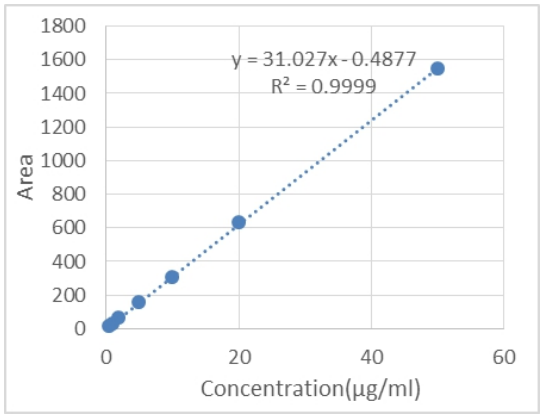
Calibration curve of Cefotaxime Sodium.



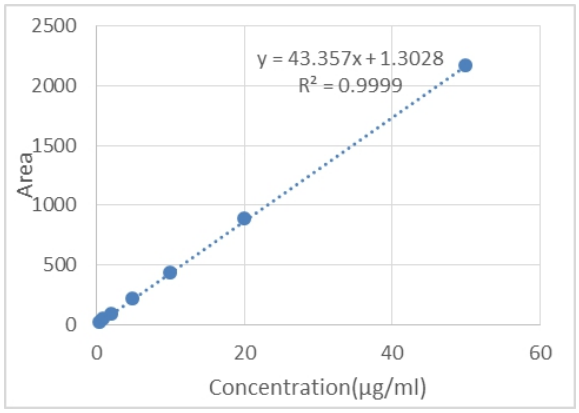
Calibration curve of Ceftriaxone Sodium



Calibration curve of Cefoperazone Sodium



Calibration curve of Cephradine



Calibration curve Cefazolin Sodium

Figure 3: Linearity data of cephalosporins.

Limit of detection and Limit of quantification

Based on standard deviation (S) of response and slope (b) of each compound of selected cephalosporins drugs under stated experimental conditions using linear range for lowest concentration levels (LOD= σ /S × 3.3 and LOQ= σ /S × 10).

Table 3: LOD and LOQ results:

Compound	LOD (µg/mL)	LOQ(µg/mL)
Cefepime Hydrochloride	0.03	0.09
Ceftazidime	0.027	0.081
Ceftiofur Sodium	0.018	0.056
Cefotaxime Sodium	0.026	0.08
Ceftriaxone Sodium	0.022	0.067
Cefoperazone Sodium	0.018	0.056
Cephradine	0.019	0.057
Cefazolin Sodium	0.026	0.08

Precision

The intra-day and inter-day precision of the method were determined by using 6 replicate injections of 100% of test concentration (10 µg/mL) for each drug and analyzed on the same day (repeatability) and different six days (reproducibility). Acceptance criteria that the relative standard deviation % not more than 2.0%.The RSD values for intra-day and inter-day precision study were < 2.0 % for studied group of drugs. Which confirm that the method was precise.

Table 4: Results of precision study

Compound	Intra-day precision			Inter-day precision		
	Mean(peak areas n=6)	SD	RSD%	Mean(peak areas n=6)	SD	RSD%
Cefepime Hydrochloride	677.69	0.72	0.11	677.38	1.07	0.16
Ceftazidime	481.76	0.72	0.15	481.56	0.1	0.21
Ceftiofur Sodium	1026.59	0.79	0.08	1026.86	0.99	0.1
Cefotaxime Sodium	361.04	0.66	0.18	360.59	0.97	0.27
Ceftriaxone Sodium	309.01	0.33	0.11	308.93	0.47	0.15
Cefoperazone Sodium	599.13	0.86	0.14	598.83	0.93	0.16
Cephradine	304.95	0.42	0.14	304.92	0.53	0.17
Cefazolin Sodium	430.84	0.21	0.05	430.74	0.29	0.07

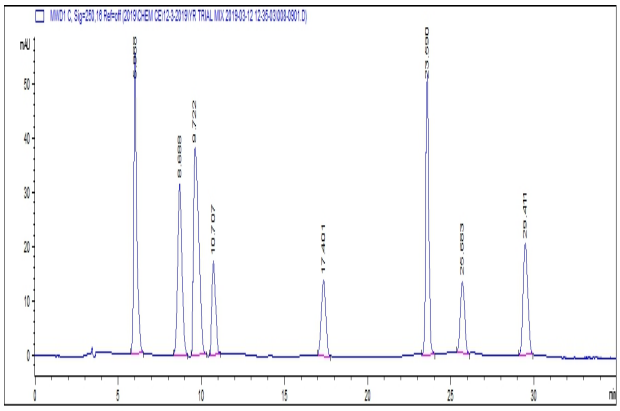


Figure 4: Chromatogram of precision result level 100% at concentration 10 µg/mL.

Accuracy and recovery:

To assess the accuracy, samples at three different concentrations levels 50%, 100%, and 200% corresponding to concentrations (5 µg/mL,10 µg/mL and 20 µg/mL) , respectively were prepared by standard addition and then analyzed against standard solution of the same concentration, in each level of concentration the injection is triplicate. The mean recoveries for selected cephalosporins were ranged from 98.25% to 101.21%.The result indicating that the method was accurate. The results were illustrated in table 5.

Table 5: Results of accuracy study

Compound	Level Conc. (µg/mL)	Mean (N=3)	SD	RSD	Average Recovery %
Cefepime Hydrochloride	5	4.98	0.02	0.35	99.69
	10	9.85	0.01	0.14	98.49
	20	20.18	0.1	0.5	100.92
Ceftazidime	5	4.96	0.03	0.51	99.23
	10	9.87	0.03	0.27	98.71
	20	20.22	0.05	0.25	101.12
Ceftiofur Sodium	5	4.96	0.01	0.23	99.2
	10	9.92	0.01	0.13	99.17
	20	20.24	0.04	0.18	101.21
Cefotaxime Sodium	5	4.96	0.03	0.53	99.15
	10	9.89	0.01	0.1	98.9
	20	20.22	0.06	0.32	101.1
Ceftriaxone Sodium	5	4.95	0.02	0.39	98.94
	10	9.83	0.02	0.18	98.25

	20	20.17	0.12	0.61	100.85
Cefoperazone Sodium	5	4.94	0.01	0.18	98.85
	10	9.88	0.04	0.39	98.81
	20	20.16	0.15	0.73	100.79
Cephradine	5	4.97	0.03	0.53	99.34
	10	9.84	0.02	0.22	98.42
	20	20.17	0.1	0.47	100.84
Cefazolin Sodium	5	4.94	0.02	0.46	98.75
	10	9.89	0.03	0.29	98.88
	20	20.18	0.09	0.45	100.9

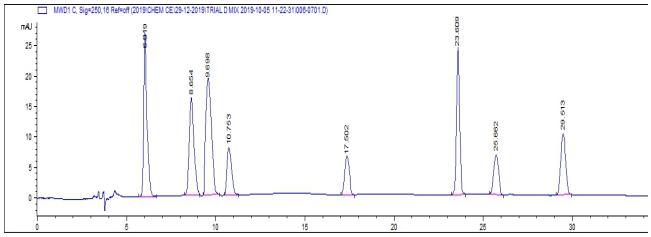


Figure 5: Accuracy results level 50 % at concentration 5 µg/mL.

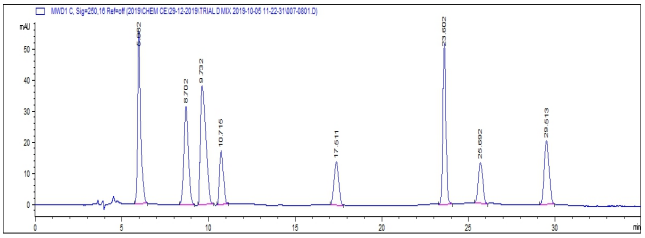


Figure 6: Accuracy results level 100% at concentration 10 µg/mL.

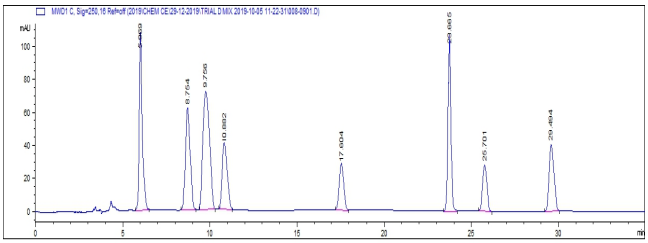


Figure 7: Accuracy results level 200% at concentration 20 µg/mL.

Robustness:

The robustness of the method was investigated by varying procedure parameters and observing to which extent the sensitivity of the responses is to slight changes in the setting conditions, the number of replicates (3) for the concentration level 100% was analyzed based on the evaluation of system suitability parameters on recovered amounts, compared to data obtained by using the original method. The following changes were done separately, including pH of mobile phase (5.4 – 5.8), detector wavelength (247 nm – 253 nm) and temperature (25°C – 30°C). The result showed that the values of the test preparation solutions were not affected at all variance conditions. The system suitability parameters are passed for all the conditions; therefore, the analytical method would be supposed as robust.

Table 6: Summary of robustness study

Compound	**parameter**	**Standard**	**pH of M.ph.**		**Wavelength**		**Temperature**	
	Variation	-	**+2**	**-2**	**+3**	**-3**	**+5**	**-5**
Cefepime Hydrochloride	**Mean(n=6)**	9.85	9.84	9.83	9.83	9.82	9.83	9.84
	RSD%	0.14	0.25	0.3	0.3	0.25	0.32	0.23
Ceftazidime	**Mean(n=6)**	9.87	9.85	9.85	9.84	9.86	9.86	9.86
	RSD%	0.27	0.45	0.4	0.51	0.34	0.32	0.48
Ceftiofur Sodium	**Mean(n=6)**	9.92	9.9	9.89	9.89	9.9	9.9	9.89
	RSD%	0.13	0.3	0.39	0.33	0.41	0.26	0.39
Cefotaxime Sodium	**Mean(n=6)**	9.89	9.85	9.86	9.87	9.84	9.84	9.86
	RSD%	0.11	0.45	0.39	0.31	0.49	0.26	0.3
Ceftriaxone Sodium	**Mean(n=6)**	9.82	9.83	9.84	9.84	9.84	9.83	9.83
	RSD%	0.18	0.26	0.27	0.25	0.3	0.24	0.28
Cefoperazone Sodium	**Mean(n=6)**	9.88	9.88	9.87	9.85	9.89	9.86	9.87
	RSD%	0.39	0.44	0.55	0.41	0.64	0.4	0.44
Cephradine	**Mean(n=6)**	9.84	9.85	9.86	9.86	9.86	9.85	9.85
	RSD%	0.23	0.26	0.31	0.29	0.33	0.28	0.35
Cefazolin Sodium	**Mean(n=6)**	9.89	9.87	9.86	9.89	9.88	9.89	9.88
	RSD%	0.26	0.28	0.27	0.27	0.33	0.31	0.28

System Suitability Test

An essential part of an analytical technique is the system-suitability test, which defines the compatibility and efficacy of the system used. System-suitability studies were conducted as specified in USP (United States Pharmacopeia, 2019) [28]. The characteristic measures were retention time, tailing factor, column efficiency and peak area. Criteria for acceptance: theoretical plate not less than 2000 and relative standard deviation

percentage of peak area not more than 2.0. The values obtained are listed in Table 7.

Table 7: Evaluation data of System Suitability study

Compound	parameter	Retention time	Tailing factor	Theoretical plates	Peak area
Cefepime Hydrochloride	**Mean(n=6)**	6.08	1.18	7775.83	677.69
	RSD%	0.19	0.47	0.05	0.11
Ceftazidime	**Mean(n=6)**	8.81	1.28	9676.5	481.76
	RSD%	0.56	0.63	0.08	0.15
Ceftiofur Sodium	**Mean(n=6)**	9.85	1.34	10544	1026.59
	RSD%	0.43	0.67	0.09	0.08
Cefotaxime Sodium	**Mean(n=6)**	10.94	1.12	7830	361.04
	RSD%	0.42	0.8	0.06	0.18
Ceftriaxone Sodium	**Mean(n=6)**	17.505	1.26	8220.33	309.01
	RSD%	0.29	0.71	0.1	0.11
Cefoperazone Sodium	**Mean(n=6)**	23.58	1.09	11353.5	599.13
	RSD%	0.21	0.82	0.08	0.14
Cephradine	**Mean(n=6)**	25.67	1.215	9319.83	304.95
	RSD%	0.11	0.86	0.11	0.14
Cefazolin Sodium	**Mean(n=6)**	29.44	1.24	11562	430.84
	RSD%	0.11	0.72	0.06	0.05

CONCLUSION

For drug analysis, requiring a high degree of specificity and selectivity HPLC techniques are often the first choice. In determining the suitability of the method for particular analysis, the running cost and speed of the HPLC method play an important role. The proposed RP-HPLC method is simple, specific, precise, accurate, and reproducible for simultaneous analysis of some cephalosporins antibiotics from different generations with a single run and low cost. The simultaneous determination of these significant cephalosporins with an isocratic solvent system in the same run not only saves the solvent but also with a short run time makes it a better option for the determination of these drugs in analysis and quality control labs. Therefore, this HPLC method reported by this study has a lot of merits over the previous methods reported; it is more efficient and simpler to apply compared to reference methods where each drug needs a separate solvent system.

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