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Research Article

Simultaneous Determination of 11 Commonly used Cephalosporin Antibiotics Residue by High Performance Liquid Chromatography - Diode Array Detectors in Pharmaceutical Waste Water - A Tool for Controlling One of the Source of Antibiotic Resistance

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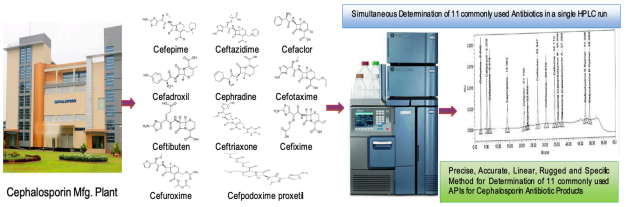
ABSTRACT

11 commonly used Cephalosporin drugs from 1st, 2nd & 3rd Generation in wastewater from Cephalosporin Antibiotics Manufacturing plant is developed, validated and proposed for routine analysis of wastewater collected from waste water pre-treatment plant (wwptp). The determined residues included the routinely manufactured cephalosporin drugs for the treatment as β-Lactams Antibiotic drugs like Cefepime, Cefadroxil, Ceftazidime, Cephradine, Cefaclor, Cefotaxime, Ceftibuten, Ceftriaxone, Cefixime, Cefuroxime Axetil and Cefpodoxime proxetil. A gradient program was developed with Xterra RP-18 (250 cm x 4.6 mm, 5 μm) column as stationary phase. All cephalosporin molecules were selective and separated with a 0.2 M Tetra-butyl Ammonium Hydroxide (TBAH) buffer and Acetonitrile in the ratio of 85:15 V/V (Solution-A) and 25:75 V/V (Solution-B), pH 6.8 was adjusted with O-phosphoric acid into the buffer solution as the mobile phase at flow of 1.2 mL min^{-1} with a UV detection at 254 nm using DAD. All peaks eluted within 60 minutes gradient run. The system suitability parameters such as theoretical plate count, tailing and resolution between the closest peaks were within the limit. The method was validated following all criteria regarding ICH (Q2) guidelines. Calibrations were linear over the concentration range of 0.5–150 μg mL^{-1} as indicated by correlation coefficient (r) of 0.999. The developed method can be the tool for determining the Cephalosporins residue as routine quantitative analysis of waste water discharged from the Antibiotic manufacturing plant.

Graphical Abstract



Keywords: Cephalosporins; Waste water; Method validation; Antibiotic Resistance; Antibiotic manufacturing plant

INTRODUCTION

Antibiotics are prescribed to treat or prevent Bacterial Infections. It also helps to prevent spreading of infections. Antibiotics are available with different types, but most of them can be classified into 6 groups, such as: Penicillins, Cephalosporin; Aminoglycosides; Tetracyclines;

Macrolides; Fluoroquinolone etc. There are series of drugs available with each groups and every antibiotic has its own criteria, thus, Chemical structure could be a great option to classify the Antibiotics[1,2]. Cephalosporins antibiotic have unique structure (Figure 1) with a common β-lactam ring that act to hinder Cell wall synthesis of the bacteria[3].

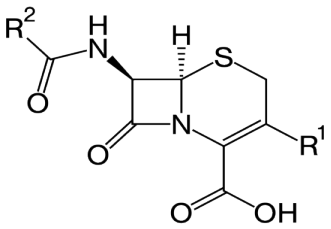


Fig. 1: Core Struture of Cephalosporings with β-Lactam ring ring

Cephalosporin Antibiotics are essentially most effective and most prescribed broad-spectrum antibiotics which are active against both Gram (+) and Gram (−) bacteria[4]. Although these antibiotics are helpful to protect human and animal heath from different bacterial infections but at the same time, presence of these antibiotics as residue level into the aquatic environment might initiate an unexpected issue like bacterial resistance to Antibiotics. Several scientists found resistant bacteria into the environment due to unconscious uses of Bacteria[5]

Cephalosporins Antibiotic resistant bacteria have already been detected into the aquatic environment or into different sewage treatment plants as well. These antibiotics are recognized as one of the main source of Antibiotic resistance from all pharmaceutical active components present into the environment[6–8]. The water quality in perspective of Cephalosporin Antibiotic residue is very censorious in developing countries where self-medication and untreated sewage into the environment is very common[9–11]. There are several other reasons also involved for the presence of mass of antibiotic contaminants into the environment, like, plant agriculture with antibiotics and also waste disposal from pharmaceutical manufacturing plants. Earlier pharmaceutical manufacturing plant was not in attention but recently it is identified as one of the main source for discharging effluent with Antibiotics in some Asian countries of up to several L mg L^{-1} (ppm) level for single compound[12,13]. Presently, Cephalosporins have five generations and most of them are used to treat as antibiotics to the patients. But cephalosporin 3rd Generations was mostly prescribed drugs (Table 1) than other Generations (1st or 2nd) in Bangladesh[14].

According to use of Antibiotics, the Pharmaceutical manufacturing company also manufacture the products to supply into the market to meet the demand of patients, thus, it is obvious that Cephalosporin Antibiotics are mostly manufactured antibiotics from Pharmaceutical Companies.

Table 1: Frequencyof suggested Antibiotics in groups in Bangladesh

Groups of Antibiotic	TSP	TAP (%)	FA-TAP (%)	FA-TSP (%)	Max
Cephalosporins			205 (34.28)	205 (18.64)	3rd Generation
Macrolides			78 (13.04)	78(7.09)	Azithromycin
Quinolones			99 (16.55)	99(9.00)	Ciprofloxacin
Penicillines	1100	598 (54.36)	45 (7.52)	45(4.09)	Flucloxacillin
Tetracyclines			28 (4.68)	28(2.54)	
Metronidazoles			95 (15.88)	95(8.64)	
Antifungals			24 (4.01)	24(2.18)	
Others			24 (4.01)	24(2.18)	

So, Cephalosporin antibiotics contaminants are also in high risk to be available in Pharmaceutical waste water during discharge to outside surface water. Pharmaceutical manufacturing site especially Cephalosporin manufacturing site is one of the main source of Antibiotic Resistance. As we already discussed that among all antibiotics, β-Lactams and Florfenicol are mostly stable into the soil mostly than other Antibiotics, thus, these groups have affinity to show activity of Antibiotic resistance[15]. There are several ways by which a Pharmaceutical company can be involved to contaminate the Environment with Antibiotic Pharmaceuticals. Waste water from Cephalosporin manufacturing plant is one of the source of multiple antibiotics from Cephalosporin groups (Cephems). Increasing concentration of these multiple antibiotics can lead to multi-resistant bacteria in the environment[16]. Bacteria strains can be resistant by frequently contact with minor concentrations of antibiotics[17]. Due to long term presence of multiple Antibiotics into the waste water system and also pressures selection at sub-inhibitory concentrations of various antibiotics, ARG and ARB can be developed[16].

Pre-treatment of Cephalosporin waste water from the WWPTP before transferring might be an option to degrade the Cephalosporin drugs. Identification and quantification of Cephalosporin residues in waste water is required for the quality control department of a pharmaceutical company to detect the Cephalosporin residues in waste water. Measurement of Cephalosporin residues in the aquatic environment is very much important to ensure the quality of the environment, especially in the surrounding of the pharmaceutical production plants. So, an analytical method is in high demand for analyzing all the Cephalosporin

drugs molecule in waste water. Literature survey reveals that the available analytical methods in the literature are either developed for formulation development/clinical/biological samples[2,18–25]. Analytical HPLC method for Environmental water samples is available with a single Cephalosporin group[5,26] or a multiple Cephalosporin group like[3,27] which have determined mostly 06 (six) Cephalosporin drugs. By evaluating all relevant literature it is found that very recently in 2021, maximum 08 (Eight) Cephalosporin molecules has been detected and quantified in Pharmaceutical formulation through a HPLC method[1,22].

But in this study, we aimed to develop an analytical method for determination of most frequently prescribed and mostly manufactured eleven Cephalosporin drugs, which will be applicable for the analysis of waste water transferred from Cephalosporin manufacturing unit to the outside surface water. It will be used as an important tool for regular environmental monitoring program of factory quality control to ensure one health also. Our developed method will be a cost-effective and time-saving method as eleven different Cephalosporin molecules (Figure 2) will be identified and quantified in a single HPLC run. The method was developed and validated with well separated peaks as per ICH Q2(R1) guidelines.

EXPERIMENTAL PROCEDURES

Instruments

Waters 2695 with 2998 PDA detector HPLC systems (Empower 3 software), Sartorius Electronic analytical balance, Ultra sonic bath, Mettler toledo pH meter and Waters XTerra RP18 (4.6 x 250 mm, 5 μm) Column were used.

Chemicals and reagents

Cefepime (potency: 83.8%), Cefadroxil (potency: 94.0%), Ceftazidime (potency: 84.3%), Cephradine (potency: 92.5%), Cefaclor (potency: 91.9%), Cefotaxime (potency: 92.5%), Ceftibuten (potency: 83.8%), Ceftriaxone (potency: 84.1%), Cefixime (potency: 85.6%), Cefuroxime Axetil (potency: 81.3%) and Cefpodoxime (potency: 67.4%) working standard obtained from The ACME Laboratories, Dhamrai, Dhaka, Bangladesh. And pharmaceutical waste water was achieved from the ETP pre-treatment plant of Cephalosporin unit, The ACME Laboratories Ltd., Bangladesh. HPLC grade methanol, acetonitrile, tetra butyl ammonium hydroxide 40% and phosphoric acid were procured from Merck, Germany. HPLC grade deionized water was used.

Buffer Solution

Tetra butyl ammonium hydroxide 40% solution (3.3 mL) was dissolved in 1000 mL of HPLC grade and adjusted the pH to 6.8 with Ortho-phosphoric acid.

Solution-A preparation

A mixture of buffer and acetonitrile (85:15) was prepared. It was filtered through 0.2μm nylon membrane filter and degassed. It was used as diluents for the preparation of different samples.

Solution-B preparation

A mixture of buffer and acetonitrile (25:75) was prepared. It was filtered through 0.2μm nylon membrane filter and degassed.

METHOD

Wavelength detection

Weighed Cefepime HCl, Cefadroxil monohydrate, Ceftazidime pentahydrate, Cephradine, Cefaclor monohydrate, Cefotaxime sodium, Ceftibuten dihydrate, Ceftriaxone sodium, Cefixime trihydrate, Cefuroxime axetil and Cefpodoxime proxetil equivalent to 25.0 mg each working standard and prepared a solution containing 5 μg/mL concentration of each component. Filtered the solution through PTFE syringe filter, 0.45 μm and collected the solution in a clean and dry vial and scanned between 200 and 400 nm with 2998 PDA detector of Waters 2695 HPLC system. The maximum absorbance of each molecule was around 254 nm, thus the wavelength detection had set at 254 nm.

Chromatographic conditions

Chromatographic conditions were finalized as 35 °C Column Temperature, 10°C Auto sampler (Cooling Temperature) with a 254 nm UV detection at a flow rate of 1.2 mL per min at gradient elution (Table 2) and run time was 60 minutes. Before injection, the column need to equalized with Mobile phase for 60 minutes. The injection volume was set to 20 μL. The solvent interference was checked with diluent (Figure 3).

Table 2: Gradient program of the M obile phase

Time (min)	Mobile phase A (percent V/V)	Mobile phase B (percent V/V)
0	100	0
15	100	0
25	85	15
50	60	40
55	100	0
60	100	0

System suitability solution preparation

Weighed accurately and transferred about 29.832 mg of Cefepime HCl equivalent to 25.0 mg Cefepime, 26.596 mg

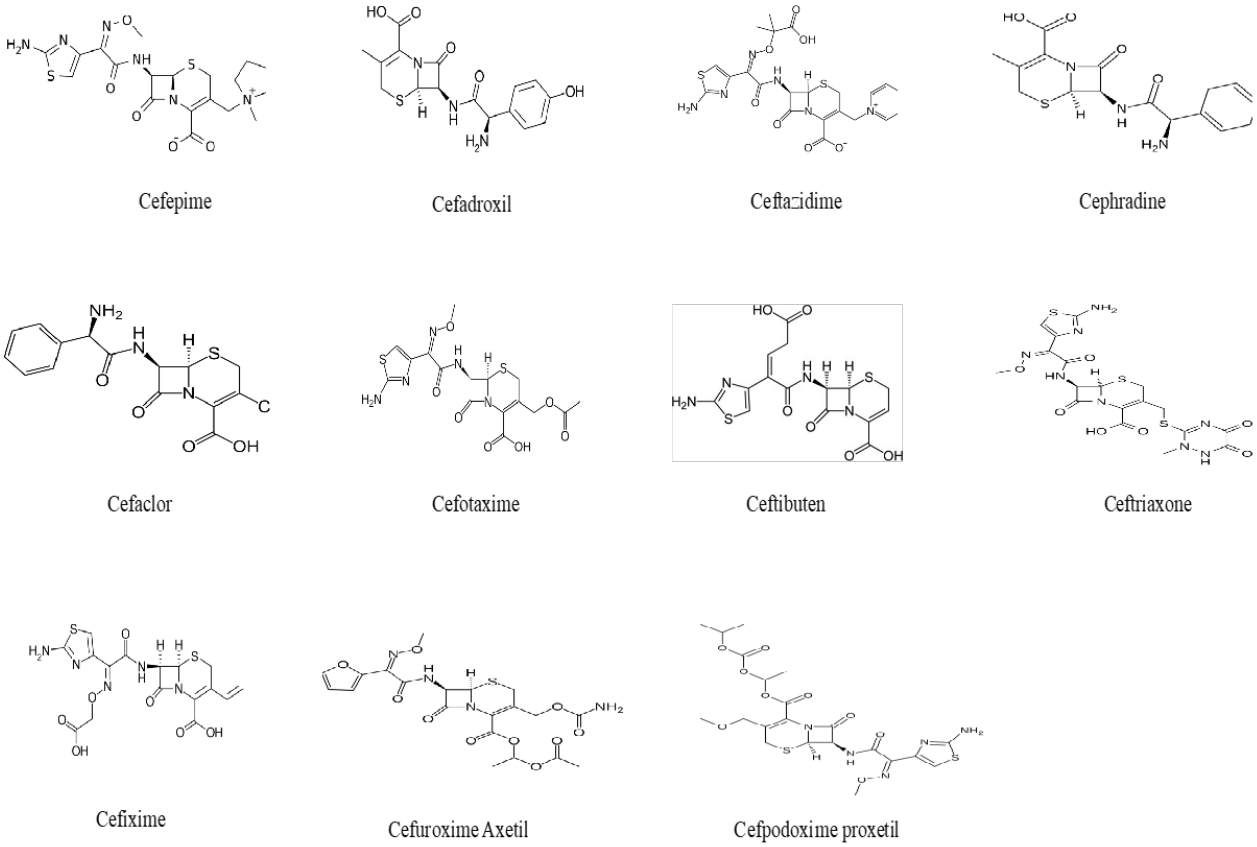


Fig. 2: Chemical structure of Cephalosporins studied

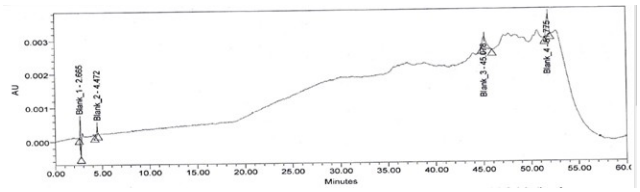


Fig. 3: Chromatogram (Diluent/Bank)

Cefadroxil monohydrate equivalent to 25.0 mg Cefadroxil, 29.657 mg Ceftazidime pentahydarte equivalent to 25.0 mg Ceftazidime, 27.027 mg Cephradine with Cephalexin equivalent to 25.0 mg Cephradine, 27.203 mg Cefaclor monohydrate equivalent to 25.0 mg Cefaclor, 27.027 mg Cefotaxime sodium equivalent to 25.0 mg Cefotaxime, 29.976 mg Ceftibuten dihydrate equivalent to 25.0 mg Ceftibuten, 29.727 mg Ceftriaxone sodium equivalent to 25.0 mg sodium, 29.206 mg Cefixime trihydrate equivalent to 25.0 mg Cefixime, 30.750 mg Cefuroxime axetil equivalent to 25.0 mg Cefuroxime and 37.092 mg Cefpodoxime proxetil equivalent to 25.0 mg Cefpodoxime working standard into a 100 mL clean and dried volumetric flask and added 5 mL methanol to dissolve then added 20 mL diluent and sonicated about 5 minutes to dissolve, made volume up to 100 mL with diluent and mix well. A 5 μg/mL system suitability solution was prepared through further dilution of this solution with diluent. Filtered the solution through PTFE syringe filter, 0.45 μm and collect the solution in a clean and dry vial.

Evaluation of system suitability

All System Suitability criteria met as per the requirements by injecting 20μL system suitability solution into the HPLC. The theoretical plat count for column efficiency was found more than 1000 USP, USP Tailing factor was more than 2.0 and resolution for the same peak is not less than 1.5 in the chromatogram as shown in Table 3 & Figure 4.

Sample preparation

Collect about 500 mL waste water from deactivation tank in a glass bottle. Filter the sample into a 500 mL conical flask through Whatman no. 1 filter paper. Again filter the filtrate through PTFE syringe filter, 0.45 μm and collect the filtrate in a clean and dry vial before injection.

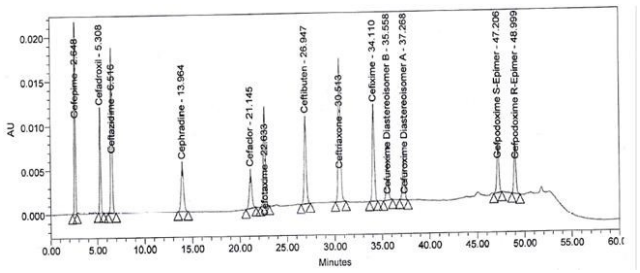


Fig. 4: Chromatogram (Standard preparation)

Table 3: System suitability Data

Peak Name		Retention Time	USP plate count	USP Tailing	Resolution
Cefepime		2.65	1181	0.77	N/A
Cefadroxil		5.31	7740	1.07	10.69
Ceftazidime		6.52	7979	1.02	4.47
Cephradine		13.96	12228	1.07	18.3
Cefaclor		21.15	26423	1	13.75
Cefotaxime		22.63	46469	0.99	3.12
Ceftibuten		26.95	66835	0.99	10.15
Ceftriaxone		30.51	81605	1.07	8.3
Cefixime		34.11	109393	0.99	8.42
Cefuroxime Axetil	Isomer B	35.56	114143	1.02	3.41
	Isomer A	37.27	129337	0.99	4.03
Cefpodoxime Proxetil	S-Epimer	47.21	204910	0.93	23.53
	R-Epimer	49	222262	1.05	4.24

Table 4: Data of Specificity

Name of the Peak		Retention Time	Purity Angle	Purity Threshold
Cefepime		2.6	0.51	1.09
Cefadroxil		5.33	0.26	1.12
Ceftazidime		6.51	0.11	1.08
Cephradine		14.03	0.33	1.36
Cefaclor		21.17	0.48	1.4
Cefotaxime		22.59	0.16	1.17
Ceftibuten		26.9	0.17	1.15
Ceftriaxone		30.46	0.11	1.08
Cefixime		34.06	0.16	1.12
Cefuroxime Axetil	Isomer B	35.63	2.18	1.25
	Isomer A	37.34	0.74	1.22
Cefpodoxime Proxetil	S-Epimer	47.25	1.52	1.25
	R-Epimer	49.04	0.7	1.26

VALIDATION PARAMETER[28]

Specificity

Purified water was used as Placebo solution. Placebo, All individual APIs, Standard solution, sample solution and spiked sample solution were injected and the responses of the peaks were noted to observe any interference of the other API at the retention time of Cefepime HCl, Cefadroxil monohydrate, Ceftazidime pentahydrate, Cephradine with Cephalexin, Cefaclor monohydrate, Cefotaxime sodium, Ceftibuten dehydrate, Ceftriaxone sodium, Cefixime trihydrate,Cefuroxime axetil and Cefpodoxime proxetil individually (Figure 5). The data demonstrated in specificity below (Table 4).

Method Precision & Intermediate precision

Method precision and Intermediate precision were carried out of the sample injection into two different HPLC system (Waters and Shimadzu). Spiked sample with known concentration has been injected and measure the peak area. The peak area of individual Cephalosporin component of six spiked sample solutions was calculated and reported along with the standard deviation and RSD of peak area of six sample solutions for Cefepime, Cefadroxil, Ceftazidime, Cephradine, Cefaclor, Cefotaxime, Ceftibuten,Ceftriaxone, Cefixime, Cefuroxime axetil, Cefpodoxime proxetil. This shows that the precision of the method is satisfactory as RSD is not more than 5% as shown in Table 5.

Linearity

The linearity of all 11 individual Cephalosporin compounds has been performed individually at concentration level 0.5 – 7.5 μg mL^{-1}. The statistical data for linearity has been calculated as per Linear regression line and found the linearity of Individual Cephalosporin compounds with a correlation coefficient (r) as equal or more than 0.999.

Result of LOD and LOQ

LOD means the lowest detectable amount of a sample solution which cannot be quantified but be detected with the analytical procedure. Standard deviation (SD) and slope values (S) were taken to calculate the LOD & LOQ calculated by using the calibration curve by with the formula LOD = 3.3 (SD/S) and LOQ = 10 (SD/S). The Final LOD and LOQ values for all 11 Cephalosporin compounds were shown in Table 6.

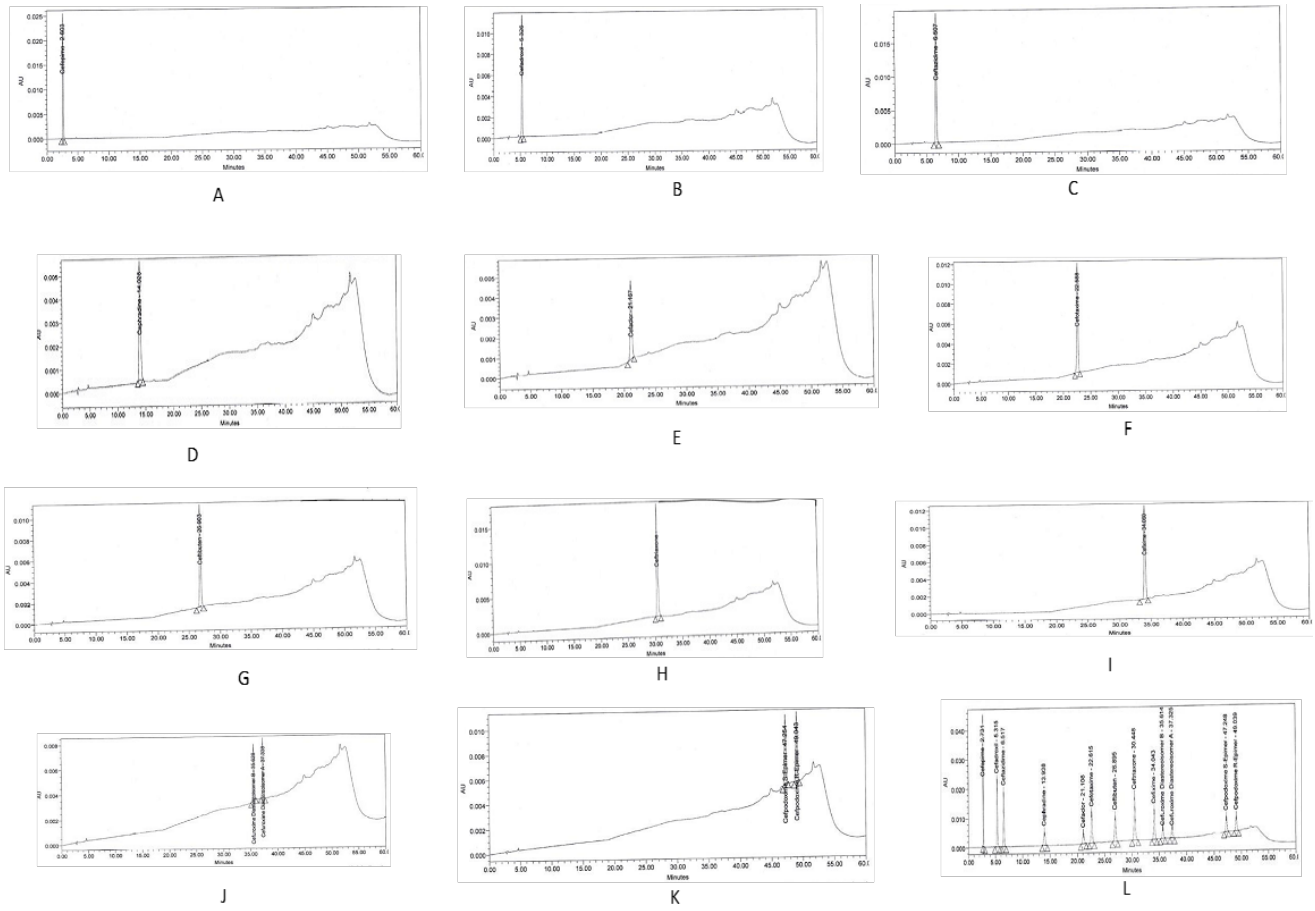


Fig. 5: Specificity Chromatograms; a) Cefepime, b) Cefadroxil, c)Ceftazidime, d) Cephradine, e) Cefaclor, f) Ceforaxime, g) Ceftibuten, h) Ceftriaxone, i) Cefixime, j) Cefuroxime Diastereoisomer, k) Cefprodoxime S-Epimer & R-Epimer, l) Sample spiked with 11 Cephalosporin Drugs

Stability studies for standard and sample solution

Stability of standard and sample solutions has been determined by keeping the samples in Ambient condition and in 2-8°C condition. The standard and sample solutions were stable for at least 24 h in refrigerator (2-8 °C).

Accuracy

A known quantity of pure Cephalosporin drugs different Accuracy level has been added with placebo and analyzed accordingly to determine the recovery and the percentage recovery. The solution was prepared by taking weigh equivalent to 25 mg each of Cefepime HCl, Cefadroxil monohydrate, Ceftazidime pentahydrate, Cephradine, Cefaclor monohydrate, Cefotaxime sodium, Ceftibuten dihydrate, Ceftriaxone sodium, Cefixime trihydrate, Cefuroxime axetiland Cefpodoxime proxetil accurately into a 100 mL volumetric flask. Final Accuracy solutions were prepared at LOQ level to 50%, 100% and 150% level of nominal test concentration. The resulting spiked sample solutions were assayed in triplicate and the results were compared and expressed as percentage. The mean percentage recovery of all Cephalosporin compounds were found to be in the range between 97.1 and 103.2 which are within the acceptance limits as shown in Table 7.

RESULTS AND DISCUSSION

All 11 Cephalosporin molecules were identified and quantified into a minimum level by the developed method with a Gradient mobile phase consisting of a TBAH buffer, pH 6.8 and Acetonitrile at the ratio 85:15 & 25:75 V/V as Solution A & B for the Gradient program with a 250 cm x 4.6 mm, 5 μ column as stationary phase at 35°C COT and 1.2 FR. The developed method demonstrated a unique chromatogram with separated individual specific and selective peaks for all 11 Cephalosporin compounds such as Cefepime, Cefadroxil, Ceftazidime, Cephradine, Cefaclor, Cefotaxime, Ceftibuten, Ceftriaxone, Cefixime, Cefuroxime and Cefpodoxime and placebo interference has also check by injecting Purified water as placebo, where the final sample is waste water.

Table 5: Method

Component Name	Parameters	Average area	% RSD	Acceptance Limit
Cefepime	Method Precision	168937	0.21	% RSD of all (Method precision & Intermediate precision) Cephalosporin peak area should be NMT 10.0%
Cefadroxil		102686	0.23	
Ceftazidime		196492	0.16	
Cephradine		90530	0.78	
Cefaclor		61275	0.97	
Cefotaxime		161593	0.2	
Ceftibuten		137248	0.34	
Ceftriaxone		238787	0.36	
Cefixime		160576	0.37	
Cefuroxime Axetil		130691	0.22	
Cefpodoxime Proxetil		157806	0.4	
Cefepime	Intermediate Precision	172965	0.12	
Cefadroxil		112960	0.1	
Ceftazidime		211218	0.32	
Cephradine		100207	1.52	
Cefaclor		55104	1.86	
Cefotaxime		175278	0.11	
Ceftibuten		134913	0.44	
Ceftriaxone		259606	0.41	
Cefixime		159139	0.19	
Cefuroxime Axetil		156462	0.55	
Cefpodoxime Proxetil		159938	0.75	

Table 6: LOD and LOQ determination

Name		LOD ppm	LOQ ppm	LOQ precision @ 0.1 ppm	
				Average Area	S/N of 1st Injection
Cefepime		0.03724	0.11285	3808	123
Cefadroxil		0.07248	0.21964	2224	53
Ceftazidime		0.03767	0.11417	4448	87
Cephradine		0.12507	0.37903	1601	20
Cefaclor		0.1266	0.38365	1445	15
Cefotaxime		0.05265	0.15956	3809	45
Ceftibuten		0.03084	0.09345	2848	36
Ceftriaxone		0.02692	0.08159	5526	72
Cefixime		0.03971	0.12033	3210	45
Cefuroxime Axetil	Isomer B	0.05376	0.16293	797	16
	Isomer A			1468	19
Cefpodoxime Proxetil	S-Epimer	0.16204	0.49104	5617	58
	R-Epimer			3509	45
Final LOQ			**0.1 ppm**		
Final LOD			**0.05 ppm**		

The stability of analytical solution was determined and got the stability up to 24 hours for the solutions in refrigerator (2-8°C) condition. The system was suitable during every analysis and the method was found precise during method precision and intermediate precision with different analyst and different instrument. The Method is linear for individual Cephalosporin compound by giving a correlation coefficient (r) value ≥0.999 at the concentrations level 0.5 – 7.5 μg mL^{-1} for each Cephalosporin compound. A precise LOQ level was found with the concentration value as 0.10 ppm (100 ppb) and Lo=OD level was 0.05 ppm (50 ppb) for each Cephalosporin compound. The % Recovery for individual Cephalosporin compound was determined for LOQ, 50%, 100% & 150% level and found the % recovery between 95.0% to 105.0% that were within limit.

Table 7: Accuracy

Name of the Cephalosporin components	LOQ Level	50% Level	100% Level	150% Level	Acceptance Limit
	Average	Average	Average	Average	
Cefepime	101.5	97.9	99.6	97.1	Recovery at each Level should be between 95.0% to 105%
Cefadroxil	101.2	98.2	100.9	99.5	
Ceftazidime	102.3	99.1	99.4	99.1	
Cephradine	100.2	100.8	103.2	101.7	
Cefaclor	100.2	101	102.3	99.2	
Cefotaxime	102	101.6	99.8	102.2	
Ceftibuten	102.7	101.8	101.1	100.5	
Ceftriaxone	99.6	101.7	102.8	101.9	
Cefixime	101	99.4	100.2	102.7	
Cefuroxime Axetil	99.9	101.1	101	101.7	
Cefpodoxime Proxetil	101.1	101	102.7	102.7	

CONCLUSION

Waste water from any pharmaceutical plant is transferred to Central Effluent treatment plant to surrounding outside water like Ground water, surface water etc. Waste water from

antibiotic plant like Cephalosporin unit must be pre-treated with chemical before transferring to Central ETP or surface water to conform the absence of Cephalosporin compounds in the waste water. There should have a specific method which can determine the Cephalosporin compounds. In this method, 11 most commonly manufactured Cephalosporin compounds in Bangladesh has been considered for the development of a HPLC method to quantify all these Cephalosporins into the residue level in the waste water. The developed and validated method for the analysis of waste water was is very simple method to be executed. HPLC method is modernized in respect of cost, and time by changing the Mobile phase, stationary phase and chromatographic conditions. The validation results indicated that the developed HPLC method for determination of 11 Cephalosporin antibiotics from different generations such as Cefepime, Cefadroxil, Ceftazidime, Cephradine, Cefaclor, Cefotaxime, Ceftibuten, Ceftriaxone, Cefixime, Cefuroxime Axetil and Cefpodoxime proxetil in a single run is precise, accurate, linear over the test concentration range, rugged and specific.

The gradient solvent system to determine all these Cephalosporins in a single run will not only save the cost and time but also it will be a unique method to control all type of Cephalosporin wastes discharged from the manufacturing unit to the environment and it will be a better option for the analytical & quality control labs to control Cephalosporin residues. Therefore, this HPLC method by this study will be helpful and distinctive from the previous methods published into different journals. This method will be more efficient and simple to apply where, each drug from different Cephalosporin compounds need a specific method and a separate solvent system.

ABBREVIATIONS

HPLC : High Performance liquid chromatography; DAD : Photodiode-array Detection; TSP : Total survey prescriptions; TAP : Tital antibiotic suggested prescriptions; FA-TAP : Frequency of Antibiotics out of TAP; FA-TSP : Frequency of antibiotics out of the TSP; Max : Maximally prescribed antibiotics in their respective group; ICH : International Council for Harmonisation; ARG : Antibiotic-resistant Gene; ARB: Antibiotic-resistant bacteria; RSD : Relative standard Deviation; WWPTP : Waste water pre-treatment plant; LOD : Limit of detection; LOQ : Limit of quantification; COT : Column oven temperature; FR : Flow rate; TBAH : Tetra-butyl Ammonium Hydroxide

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Competing interests

The authors declare no conflict of interes

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