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Original article

Simple rapid eco-friendly chromatographic method for the determination of ten residual cephalosporin antibiotics in wastewater after their pharmaceutical production

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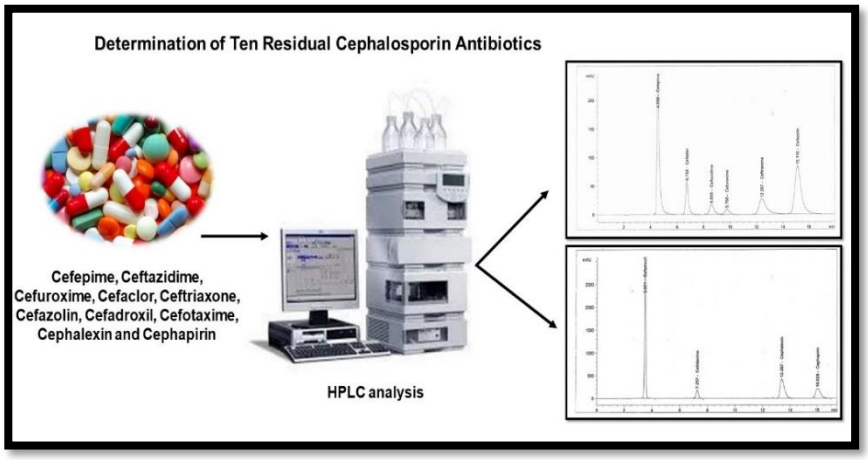
Abstract:
A simple, reliable, and rapid HPLC analysis is proposed for the quantification of ten cephalosporins; Cefepime, Ceftazidime, Cefuroxime, Cefaclor, Ceftriaxone, Cefazolin, Cefadroxil, Cefotaxime, Cephalexin and Cephapirin, simultaneously, in wastewater from factories as a tool at the local level for regular environmental monitoring program. The water samples are directly used with no complicated sample pretreatment steps. The separation was accomplished on the C_{18} reversed-stationary phase and the mobile phases used were composed of methanol:0.1% pentane sulfonic acid (22:78) at pH 2.5 with 1.2 mL/min flow rate (system 1) and methanol: phosphate buffer (27:73, pH 7) with 1.0 mL/min flow rate (system 2). 265 and 235 nm are the optimum wavelengths used for UV detection for system 1 and system 2, respectively. Under the optimized conditions, very low LODs and LOQs were obtained for all the studied compounds. The quantification of various cephalosporins in real water was accomplished. Both the effluent and influent of the local wastewater showed negative results, indicating that the obtained wastewater is free from cephalosporin antibiotics.



Keywords: Cephalosporin antibiotics; HPLC; wastewater

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1. Introduction

Cephalosporins, are one of the ß-lactam antibiotics that are widely used in many human and veterinary practices. Their chemical structure includes a ß-lactam ring attached to a dihydrothiazine six-membered ring allowing them to have strong antimicrobial activity against gram-negative bacteria. Such chemicals are repeatedly liberated into the environment either after their manufacture or after animal or human excreation[(1)]. It has been reported that antibiotics were determined in various water types as hospital wastewater, sewage water, and river water[(2-7)]. Environmental pollution caused by antibiotic residues would undoubtedly lead to resistance in bacterial strains which would present a serious threat to public health [(8)]. The fact that ß-lactam antibiotics may cause allergenic reactions in some individuals adds to the dangerous effects of high levels of antibiotic residues in food and drinking water [(9)]. Also, the induction of bacterial-resistant strains because of such antibiotic residues is another dangerous factor. Therefore, the quantification of antibiotics in the environment and wastewater is of extreme importance to track and manage such pollution [(10)]. Such residues exist in trace levels in complex environmental and wastewater samples; thus, their determination necessitates the development of selective and sensitive methods of analyses. Unfortunately, environmental analysis of cephalosporins in water is not well reported in the literature. It is worth noting that developing specific methods for cephalosporin determination is challenging because of the β-lactam instability and the small variations of the structure between different members. Chromatographic techniques were mostly used for the analysis[(11-14)]. Niu et al.[12] succeeded in using carbon nanotubes for the extraction of several sulfonamides, phenolic compounds, and cephalosporin antibiotics, but this procedure was successful only for the analysis of sulfonamides in real water. Also, a CE analysis was presented by Puig et al.[(13)] for the quantification of Cefoperazone and Ceftiofur, in water samples. While Cha et al. [(3)] and Rao et al.[14] reported two LC-MS analyses for the determination of Cephapirin and Cefadroxil, Cefuroxime, Cefprozil, Cefdinir, and Ceftiofur cephalosporins, respectively, in surface water samples. Such methods used HPLC-MS/MS for the analysis and offered high selectivity and sensitivity. However, the high cost of LC-MS/MS instrumentation may affect its availability and applicability in many laboratories. It is obvious that there is a need to discuss the occurrence, behaviour, and fate of cephalosporins in water. In addition to developing simple rapid methods for concentrations assessment of such antibiotics in various types of waters.

Thus, the main goal here is to develop a simple, and rapid method for the simultaneous sensitive and selective quantification of mixtures of the most commonly used cephalosporin antibiotics in wastewater from factories producing cephalosporins. This is of great importance as it can be applied to environmental monitoring at the local level which is a part of the factory quality control. In this study, two mixtures of some selected cephalosporin antibiotics in water samples are simultaneously separated using a rapid simple HPLC method with DAD. One mixture is composed of six cephalosporin antibiotics (Cefepime, Ceftazidime, Cefuroxime, Cefaclor, Ceftriaxone, and Cefazolin) and the other is composed of four cephalosporin antibiotics (Cefadroxil, cefotaxime, Cephalexin, and Cephapirin). The presented method is applied for the quantification of the selected ten cephalosporins in water.

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2. Experimental

2.1. Materials and Methods

Cefepime (CP), Ceftazidime (CT), Cefuroxime (CR), Cefaclor (CC), Ceftriaxone (CX), Cefazolin (CZ), Cefadroxil (CD), cefotaxime (CM), Cephalexin (CL) and Cephapirin (CPR) were kindly supplied from Pharco B International Pharmaceutical Company, Borg El Arab, Alexandria, Egypt. All the information on the studied cephalosporins is summarized in **Table 1**. Methanol (HPLC grade), triethylamine, and phosphoric acid are obtained from Merck (Darmstadt, Germany). Analytical grade pentane sulfonic acid sodium, anhydrous dibasic sodium phosphate, and monobasic potassium phosphate (Sigma Aldrich, St. Louis, MO, USA) were used in the mobile phases.

1 mg mL⁻1 of CT, CR, CX, CPR, and CM were prepared in distilled water. The other five reference standards are freshly prepared before measurements. Two working standard solutions (WSS) were prepared. The first working standard solution (WSS-1) is a mixture of CP, CT, CR, CC, CX, and CZ and the second WSS (WSS-2) is a mixture of CD, CM, CL, and CPR.

Table 1: Basic information on the selected cephalosporin antibiotics in this study

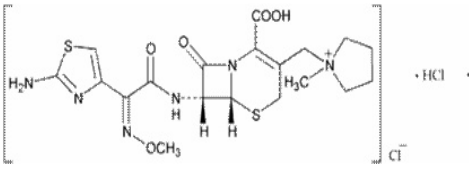
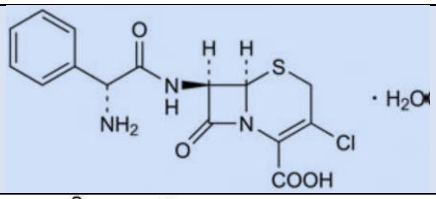
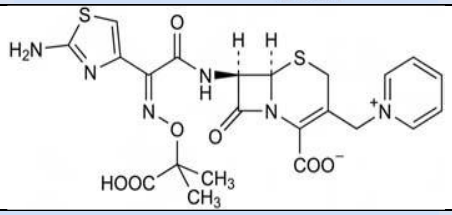
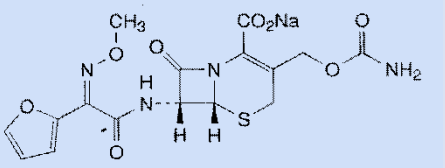
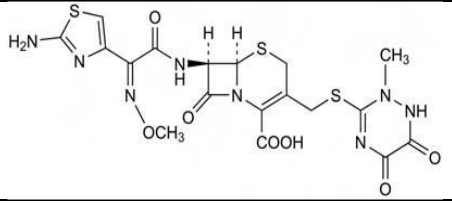
Antibiotics	CAS Number	Molecular formula and weight	pKa	Log Kow	Chemical structure
Cefepime hydrochloride (CP)	88040-23-7	$C_{19}H_{24}N_6O_5S_2$ 480.56	2.46, 1.7	-1.80	
Cefaclor monohydrate (CC)	70356-03-5	C15H14ClN3O4S, H2O 385.8	1.5, 7.2	0.35	
Ceftazidime pentahydrate (CT)	78439-06-2	C22H22N6O7S2, 5H2O 636.7	1.9, 2.7, 4.1.	-1.36	
Cefuroxime (CR)	55268-75-2	C16H16N4O8S 424.4	2.5, 5.1	-0.16	
Ceftriaxone (CX)	73384-59-5	C18H18N8O7S3 554.6	3.0	-1.9	

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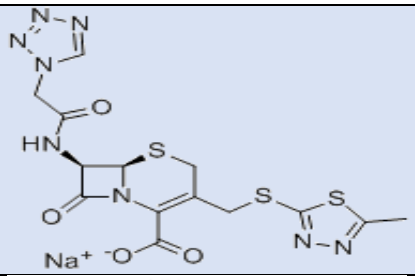
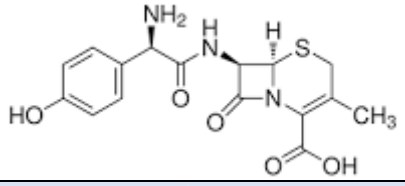
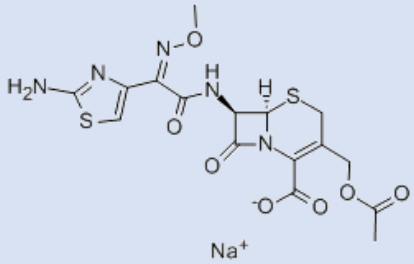
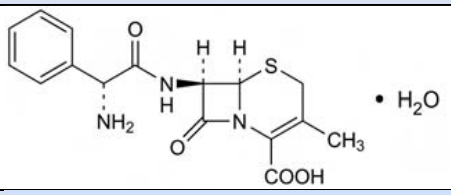
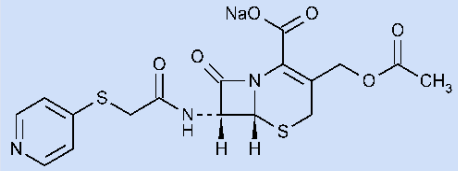
Table 1: Continue

Cefazolin (CZ)	25953-19-9	C14H14N8O4S3 454.5	3.6	-0.58	
Cefadroxil (CD)	66592-87-8	C16H17N3O5S 363.4	2.64, 7.30, 9.69	-0.08	
Cefotaxime sodium (CM)	64485-93-4	C16H16N5NaO7S2 477.45	2.1, 3.4, 10.9	0.64	
Cephalexin monohydrate (CL)	23325-78-2	C16H17N3O4S, H2O 365.4	2.5, 5.2, 7.3	0.60	
Cephapirin sodium (CPR)	24356-60-3	C17H16N3O6S2 · Na 445.45	2.15, 7.3	-0.80	

2.2. Instrumentation and software

Agilent 1200 series (Agilent Technologies, CA, USA) was used. Data acquisition and analysis were accomplished using Agilent chem station software. Crison pH 2000, a Model Universal 320R centrifuge, and a vortex-2 Genie were used.

2.3. HPLC conditions

LUNA C-18 (5 µm, 100 Å, 250 x 4.6 mm) column was used. For the separation of the WSS-1 mixture, methanol (A) and 0.1% pentane sulfonic acid in water (B) in a ratio of A/B = 22/78 adjusted to pH 2.5 by 1% triethylamine and phosphoric acid was used at a 1.2 mL/min flow rate. While for the separation of the WSS-2 mixture, methanol (A) and phosphate buffer pH 7 (B) in a ratio of A/B = 27/73 was used at a 1.0 mL/min flow rate. 20 µL injection volume and 265 and 235 nm wavelength for UV detection were used for WSS-1 and WSS-2, respectively.

2.4. Sample preparation

Water samples were collected from the influent and effluent water of Pharco B International Pharmaceutical Company in Borg El Arab, Alexandria, Egypt. Samples of water were directly collected in PVC containers, filtered through 0.2 mm nylon membranes, and stored in the dark (4 ºC). A

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volume of 200 mL of the untreated water samples was used per analysis. We determined the selected cephalosporins in three separate influents samples and in their corresponding effluents.

3. Results and discussion

3.1. Optimization of the chromatographic separation

For the optimization of the mobile phase, in the organic fraction, methanol, acetonitrile, and mixtures of both were assessed with different ratios of formic acid, acetic acid, pentane sulfonic acid, triethylamine, and phosphoric acid in the aqueous fraction. For the best separation, methanol was used as the organic phase, and 0.1% pentane sulfonic acid was used as an aqueous portion for the separation of WSS-1, the addition of an aliphatic sulfonate as a counter ion was necessary to enhance the separation of cephalosporins and especially to achieve the resolution between Ceftazidime, Cefuroxime, and Cefaclor, while aqueous phosphate buffer was used as aqueous fraction for the separation of WSS-2. The ratio of organic/aqueous fractions of the mobile phase was 22/78 and 27/73 for WSS-1 and WSS-2, respectively.

Several experimental trials using various pH values of mobile phase, flow rates, and injection volumes were examined. Cephalosporins are quite polar compounds, with low pKa values (**Table 1**) due to the presence of carboxylic groups. Under neutral conditions they are in anionic form and highly soluble in an aqueous solution, resulting in poor chromatographic separation. The results showed that maximum resolution between peaks was achieved at pH 2.5 adjusted by 1% triethylamine and phosphoric acid for the separation of WSS-1. pH 2.5 was the optimum pH to obtain well-resolved peaks for the separation of the WSS-1 mixture. Upon comparison with the pKa values of the selected cephalosporins, pH 2.5 will enhance their deprotonation forming the conjugate bases that interact with pentane sulphonate producing well-resolved symmetrical peaks. On the other hand, pH 7 for the separation of WSS-2 and the optimum flow rate was 1.2 and 1.0 mL min^{-1} for WSS-1 and WSS-2, respectively. 5 to 20 µL injection volumes were examined. 15 µL showed high sensitivity with good resolution. Figure 1 shows a water sample chromatogram spiked with 100 mg/L of the selected cephalosporins. Results show well-resolved peaks with analysis time not more than 18 min.

3.2. System suitability parameters

The system suitability parameters are an essential part of accurate chromatographic methods. They are valuable for the assessment of the efficiency of separation, peak symmetry, and resolution of peaks. As shown in **Table 2**, the peaks of the investigated cephalosporins are well separated with high efficiency of separation with short analysis time.

3.3. Validation

3.3.1. Calibration plots

Duplicates of each concentration were measured three times. The coefficients of determination (r^2) ranging from 0.997 to 0.999 were obtained for all the analytes (CP, CC, CT, CR, CX, CZ, CD, CM, CL, and CPR). **Table 2** demonstrates all the analytical parameters. LODs and LOQs were estimated using Signal/Noise of 3 and 10, respectively. As shown in **Table 3**, very low LODs and LOQs were obtained for all the studied compounds.

3.3.2. Precision

Intraday-precision was evaluated on the same day by repeating the separation procedure of water samples containing cephalosporins of concentrations of 50, 100, and 150 µg/L with triplicate measurements for each sample. Interday-precision was similarly evaluated but for five consecutive days. Results show good precision with %RSD were in the range

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of 0.220–1.46% to be less than 2.0%, (**Table 4**).

Table 2. System suitability parameters for the analysis of the studied cephalosporins using the proposed methods

Parameters	WSS-1					
	Cefepime	Cefaclor	Ceftazidime	Cefuroxime	Ceftriaxone	Cefazolin
tm ± SD, min	4.56± 0.31	6.73± 0.26	8.60± 0.33	9.77± 0.47	12.40± 0.73	15.12± 0.52
α	-	1.48	1.28	1.14	1.27	1.22
Rs	-	6.67	3.94	2.05	3.62	3.11
N	3688	5880	3345	5162	2993	5201
A_f	0.9	1.02	0.93	0.94	1.13	1.04

Parameters	WSS-2			
	Cefadroxil	cefotaxime	Cephalexin	Cephapirin
tm ± SD, min	3.5 ± 0.42	7.26 ± 0.75	13.39 ± 0.35	16.03 ± 0.73
α	-	2.07	1.84	1.20
Rs	-	15.95	14.13	4.46
N	7989	8579	9389	10324
A_f	0.85	0.88	0.8	0.974

Table 3: Analytical performance of the proposed method

System 1				
Antibiotic	**LOD (µg/mL)**	**LOQ (µg/mL)**	**Linearity range (µg/mL)**	**r^2**
Cefepime hydrochloride (CP)	0.0226617	0.07553901	0.350 - 1.05	0.999798
Cefaclor monohydrate (CC)	0.01218188	0.04060627	0.15 - 0.45	0.999122
Ceftazidime pentahydrate (CT)	0.005524478	0.01841493	0.0575 - 0.1725	0.998770
Cefuroxime (CR)	0.00365861	0.01219537	0.025 - 0.075	0.997152
Ceftriaxone (CX)	0.006380345	0.02126783	0.1 - 0.3	0.999458
Cefazolin (CZ)	0.0174549	0.03818299	0.5 - 1.5	0.999838
System 2				
Antibiotic	**LOD (µg/mL)**	**LOQ (µg/mL)**	**Linearity range (µg/mL)**	**r^2**
Cefadroxil monohydrate (CD)	0.092397	0.3079884	0.025 - 0.075	0.99701
Cefotaxime sodium (CM)	0.015832	0.052772	0.1 - 0.3	0.99946
Cephalexin (CL)	0.00056166	0.0018722	0.25 - 0.75	0.99999
Cephapirin (CPR)	0.0185741	0.061913	0.25 - 0.75	0.99920

Table 4: Precision study (RSD%) of the proposed method for different concentration levels

Antibiotic	Intra-day			Inter-day		
	50 µg/L	**100 µg/L**	**150 µg/L**	**50 µg/L**	**100 µg/L**	**150 µg/L**
Cefepime hydrochloride (CP)	0.377	0.465	0.850	0.932	1.05	0.798
Cefaclor monohydrate (CC)	0.426	0.422	1.25	0.899	0.740	1.22
Ceftazidime pentahydrate (CT)	0.365	0.899	0.574	0.850	1.25	0.877
Cefuroxime (CR)	0.372	0.564	0.692	0.791	0.705	0.971
Ceftriaxone (CX)	1.46	0.896	0.732	0.548	0.812	0.948
Cefazolin (CZ)	0.509	0.803	1.19	0.838	1.35	1.43
Cefadroxil monohydrate (CD)	0.750	0.461	0.951	0.701	0.745	1.06
Cefotaxime sodium (CM)	0.220	0.431	0.375	0.946	0.993	0.654
Cephalexin (CL)	0.454	0.318	0.784	1.27	0.675	0.999
Cephapirin (CPR)	0.330	0.684	1.12	0.920	0.805	1.29

RSD, Relative standard deviation, n = 9 for intra-day study; n= 15 for inter-day study

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3.3.3. Recovery of cephalosporins from spiked water samples

Recovery was performed in water samples containing 50 µg/L of each antibiotic. The water samples were spiked by 25 - 125 µg/L of each of the selected cephalosporins before analysis in triplets using the proposed method. Results are reported in **Table 5.** The % recoveries of cephalosporins in spiked water samples are between 92.4 –99.9 %. **Fig. 2** demonstrates the Chromatograms of water samples spiked with each of the studied cephalosporins.

The developed method showed good precision and trueness for the quantification of residual cephalosporins in water, thus, proving the accuracy of the developed method in such a matrix.

3.3.4. Specificity

Method specificity was demonstrated by the successful separation of the ten studied cephalosporins from their potential impurities and related substances **(Fig. 1).** Moreover, using DAD confirms the specificity of the method as it confirms peak purity with no signs of coelution of inactive interferents.

3.4. Environmental analysis

Quantification of cephalosporins was accomplished in wastewater located in the area of Pharco B International Pharmaceutical Company in Borg El Arab, Alexandria, Egypt. We determined the selected cephalosporins in three separate influents samples and their corresponding effluents. Collected samples were analyzed using the proposed method. In the collected wastewater samples, none of the selected cephalosporins were found. Thus, the procedures applied by the factory for the purification of wastewater are successful regarding the selected cephalosporins.

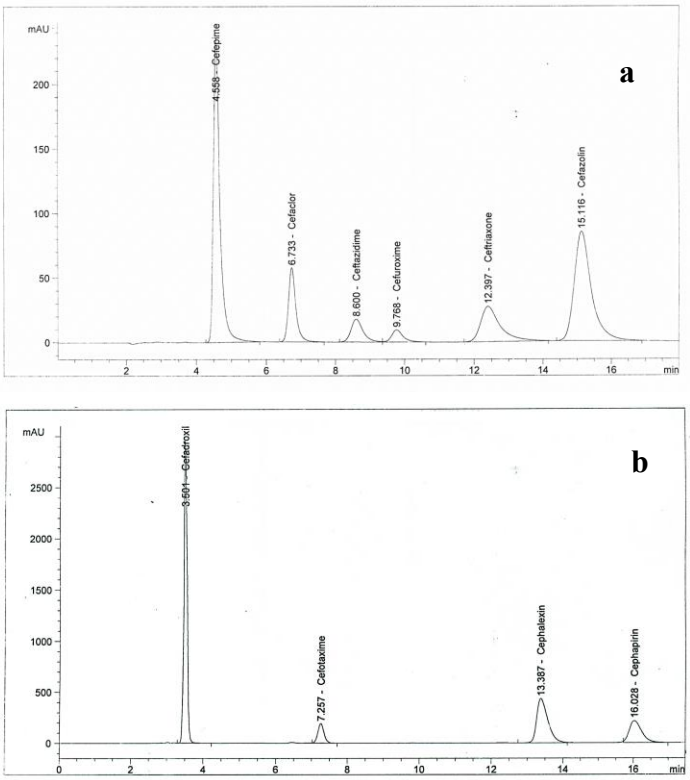

Fig. 1: Chromatogram of water sample spiked with 100 mg/L of each studied cephalosporins using C18 stationary phase and a mobile phase of methanol:0.1% pentane sulfonic acid (22:78) at pH 2.5 with flow rate of 1.2 mL/min (a) and a mobile phase of methanol: phosphate buffer (27:73) at pH 7 with a flow rate of 1.0 mL/min (b)

4. Conclusions

A simple rapid HPLC method with DAD detection was proposed and validated for the analysis of ten commonly used cephalosporins (Cefepime, Ceftazidime, Cefuroxime, Cefaclor, Ceftriaxone, Cefazolin, Cefadroxil, cefotaxime, Cephalexin, and Cephapirin). The proposed method showed good resolution with low quantification limits, introducing an easy and efficient alternative for cephalosporins determination. Results show that the method is sensitive and can be applied successfully for the routine determination of cephalosporins in various laboratories. Upon

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applying this procedure to wastewater samples, no cephalosporins were detected in various wastewater samples in the area of Pharco B International Pharmaceutical Company in Borg El Arab, Alexandria, Egypt. This can be attributed to the efficient purification protocols applied by the factory.

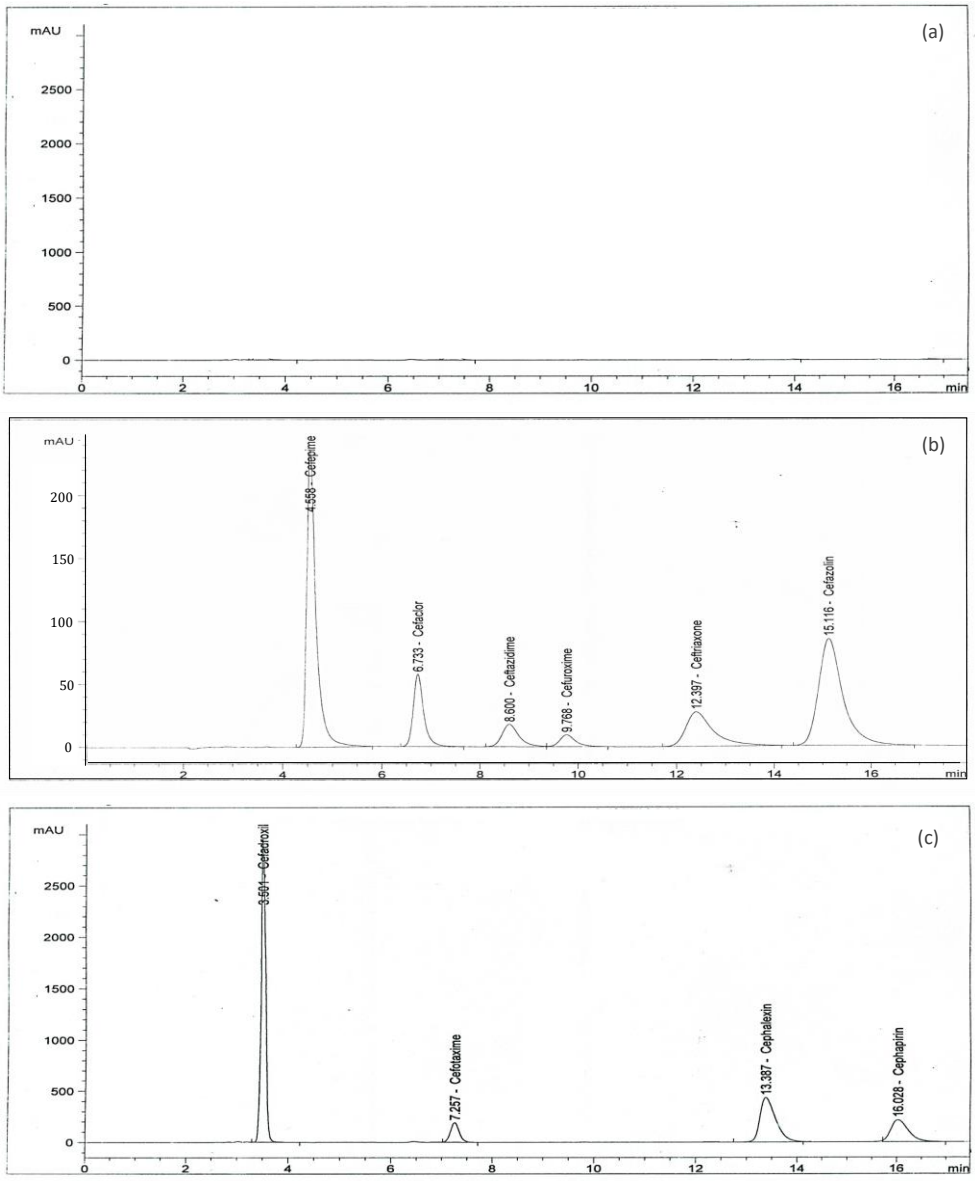

Fig. 2: (a) Chromatogram of a blank water sample. (b) Chromatogram of water sample spiked with each of the studied cephalosporins in WSS-1 and (c) Chromatogram of water sample spiked with each of the studied cephalosporins in WSS-2

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Table 5: Recoveries of cephalosporin antibiotics from water samples spiked at different concentration levels

Antibiotic	Added amount (µg/L)	Initial amount (µg/L)	Found amount (µg/L)	Recovery (%)	Mean recovery ± RSD%
Cefepime hydrochloride (CP)	100	50	143.5	95.7	98.1 ± 2.2
			148.0	98.7	
			149.8	99.9	
Cefaclor monohydrate (CC)	75	50	115.5	92.4	94.1 ± 1.8
			120.0	96.0	
			117.5	94.0	
Ceftazidime pentahydrate (CT)	50	50	94.8	94.8	95.3 ± 1.8
			93.7	93.7	
			97.3	97.3	
Cefuroxime (CR)	25	50	74.8	99.8	98.5 ± 1.2
			73.0	97.4	
			73.8	98.4	
Ceftriaxone (CX)	125	50	170.3	97.3	97.5 ± 0.78
			169.6	96.9	
			172.2	98.4	
Cefazolin (CZ)	150	50	197.8	98.9	96.8 ± 1.8
			190.8	95.4	
			192.4	96.2	
Cefadroxil monohydrate (CD)	25	50	71.8	95.7	96.0 ± 1.8
			73.4	97.9	
			70.7	94.3	
Cefotaxime sodium (CM)	100	50	149.6	99.7	98.6 ± 1.1
			146.1	97.4	
			147.9	98.6	
Cephalexin (CL)	25	50	72.3	96.4	96.5 ± 0.71
			71.9	95.9	
			73.0	97.3	
Cephapirin (CPR)	25	50	71.2	94.9	96.0 ± 1.2
			71.9	95.8	
			72.9	97.2	

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Highlights

- HPLC/DAD method is developed for the simultaneous determination of ten cephalosporin antibiotics in wastewater.
- The determined antibiotics are Cefepime, Ceftazidime, Cefuroxime, Cefaclor, Ceftriaxone, Cefazolin, Cefadroxil, Cefotaxime, Cephalexin and Cephapirin.
- The developed methods are simple, eco-friendly, and robust analytical method-based
- The proposed method serves as a tool for regular environmental monitoring programs at local level.

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