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Simultaneous determination of Ceftriaxone and Cefpodoximeproxetil in Commercial Formulations and Spiked Human Plasma using Reversed-Phase High Performance Liquid Chromatography

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ABSTRACT

A simple and sensitive reversed phase high performance liquid chromatographic (RP-HPLC) method has been developed for the simultaneous quantification of ceftriaxone and cefpodoximeproxetil in artificial mixtures of commercial formulations and spiked human plasma. The separation was carried on C_{18} (4.6 mm x 250 mm) column with a mixture of acetonitrile and 0.03 M triethylamine (3:1 v/v) adjusted to pH 7.0, with acetic acid, as mobile phase at flow rate of 0.9 $mLmin^{-1}$ with UV detector at 240 nm. The chromatographic peaks were recorded at detection wavelength of 240 nm. The retention times of ceftriaxone and cefpodoximeproxetil were 2.111±0.014 min and 4.053±0.013 min respectively. The concentration versus detector response (Peak height) curve is linear in the range of 0.5- 250 µg mL^{-1} for ceftriaxone (R^2=0.9996), and 0.5-500 µg mL^{-1} for cefpodoximeproxetil (R^2=0.9995). The limit of detection (3.3σ/S) was found to be 6.62 ng mL^{-1} and 14.2 $ngmL^{-1}$, respectively, for ceftriaxone and cefpodoximeproxetil. Similarly limit of quantification (10σ/S) was 20 ng mL^{-1} for ceftriaxone and 42.9 $ngmL^{-1}$ for cefpodoximeproxetil. The developed method was applied to simultaneous determination of these antimicrobials in the artificial mixtures of commercial formulations and spiked human plasma.

Keywords: Ceftriaxone; cefpodoximeproxetil; simultaneous determination; artificial mixture; commercial formulations; spiked human plasma.

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INTRODUCTION

All cephalosporins are semisynthetic antibacterial agents derived from the 7-aminocephalosporanic acid and belong to the β-lactam family of antibiotics. They exhibit enhanced bactericidal activity and resistance to β-lactamase deactivation as compared to penicillins and therefore preferentially prescribed in the clinical practices to cure severe infections. The cephalosporins act as bactericidal by inhibiting the bacterial cell wall synthesis and disrupting its multiplication. The substituent at position 7 is responsible for the spectrum of activity and stability towards β-lactamase deactivation while substituent at position 3 decides its pharmacokinetic disposition[1].

Ceftriaxoneis third generation parenteral cephalosporin possessing broad spectrum of activity against both gram positive and gram negative pathogens. The MIC_{90} (minimum concentration of drug to inhibit the growth of 90 % strains) of ceftriaxone for most strains of the Enterobacteriaceae is 1 μg mL^{-1} while 3.1 to 4 μg mL^{-1} is sufficient to kill 90 % strains of staphylococcus aureus[2].

Cefpodoximeproxetilis third generation oral cephalosporin antibacterial agent having high rate of absorption (almost 50 %) as compared to other oral cephalosporins. It is effective against a number of gram positive and gram negative bacteria like Haemophilusinfluenzae, Moraxella catarrhalis, Streptococcus pyogenes,Streptococcus Pneumoniae,and most of theEnterobacteriaceae[3].

A number of methods have been given in the literature for determination of ceftriaxone and cefpodoximeproxetil in pharmaceutical formulation and biological samples. These include spectrophotometric[4–7], spectrofluorimetric[8–10], voltammetric[11], and chromatographic[12–22]. But the simultaneous determination of ceftriaxone and cefpodoximeproxetil has not been reported in the literature so far and a single analytical procedure for its simultaneous determination could be cost effective and time saving in routine laboratory analysis.

EXPERIMENTAL

Materials and Reagents

All reagents were of HPLC grade purity or high grade purity. Triethyl amine (TEA), glacial acetic acid, acetonitrile and methanol (BioM laboratories Cerritos, USA) were used in this work. Standard reference ceftriaxone and cefpodoximeproxetil were provided by Cirin Pharmaceutical (Pvt) Ltd., Hattar, Pakistan. Commercial formulations were purchased from local market.

Preparation of reagent solutions

Glacial acetic acid (0.05M) was prepared by diluting 0.3 mL of 17.5 M glacial acetic acid to 100 mL with distilled water. TEA solution(0.2 M) was prepared by diluting 2.8 mL of pure liquid TEA (7.2 M) to 100 mL with distilled water. TEA (0.03 M)buffer solution of pH 7 was prepared by diluting 37.5 mL of 0.2 M stock solution of TEA and adjusting the pH to 7 with 0.05 M acetic acid solution. The HPLC grade acetonitrile and TEA buffer were mixed in 3:1 (v/v). The mobile phase was filtered through 0.45 µm nylon membrane filter paper (Millipore).

Preparation of standard solution

Standard ceftriaxone solution was prepared by dissolving 0.005 g of pure drug in water and diluted to 25 mL with distilled water to get stock solution (200 µg mL^{-1}). Similarly, 200 µg mL^{-1} stock solution of cefpodoximeproxetil was prepared by dissolving 0.005 g of the standard in 10 mL methanol and diluting to 25 mL with distilled water. The working standards were prepared by dilution of appropriate volume of stock solutions with mobile phase. The artificial drug mixture, for optimization studies was prepared by mixing standard stock solution of both the drugs in 1:1 (concentration units).

Instrumentation and chromatographic conditions

The chromatographic system consisted of an Acme 9000 Series HPLC equipped with SP 930 isocratic pump and UV 730 D detector (Young Lin, Korea). All the separations were carried out on C_{18} column (5µm, 4.6 mm Ø x 250 mm, Teknokroma S. Coop. C. Ltd. Barcelona, Spain). A guard column containing with identical packing material to that of the analytical column was used. Degassing of the mobile phase was carried out with a ks300 KUM SUNG ultrasonic (Korea) sonicator. The biological samples were centrifuged by using CL international clinical centrifuge (USA). A binary mixture of acetonitrile and triethyl amine (TEA) buffer (pH 7) in ratio of 3:1 v/v was used as mobile phase. The mobile phase was filtered through 0.45 µm membrane filter (Millipore), degassed by sonication for 30 min and delivered at rate of 0.9 $mL.min^{-1}$. All the sample solutions were also filtered through nylon membrane filter (Millipore) before injection. A 20 µl sample was injected to the column by using a 100 µL syringe (Hamilton Co., Reno. Nevada USA) and the column effluents were monitored with UV detector at 254 nm. All measurements were performed at ambient temperature i.e. 25 ºC with run time of 10 min.

Construction of calibration curve

Artificial mixtures of the standard drugs in the range of 0.5–250 µg mL^{-1} for ceftriaxone and 0.5–500 µg mL^{-1} for cefpodoximeproxetil were prepared by mixing appropriate volumes of the standard stock solutions and diluted with mobile phase to the required volume. All standard

solutions were filtered through 0.45 µm nylon membrane filter and injected into the column under the chromatographic conditions already mentioned. The peak height was plotted against the concentration to construct the calibration curve.

Validation of the method

According to the International Conference on Harmonization (ICH) of Technical Requirements for the Registration of Pharmaceuticals for Human Use, guidelines for the validation of analytical procedures, the newly developed method was validated in terms of precision, accuracy, specificity, linearity, limit of detection (LOD) and limit of quantification (LOQ).

Specificity is studied by comparing the chromatograms of standard and commercial formulations.The linear range of the investigated method was studied in the range of 0.5–500 µg mL^{-1} for ceftriaxone and 0.5 to 1000 µg $mL^{-1}$for cefpodoxime proxetil. The limit of detection (LOD) and limit of quantification (LOQ) were evaluated on the basis of standard deviation (s) of response and slope (b). Six replicates at the lowest concentration level of the linear range of both the studied drugs were analyzed by the investigated procedure. The standard deviation and the relative standard deviation of the response factor were calculated.

Precision and accuracy of the proposed method was studied for quantification of ceftriaxone and cefpodoxime proxetil in artificial mixtures of commercial formulations and spiked human plasma. Commercial formulations and plasma samples containing known concentration of both the studied drugs were spiked with known quantities of the standards at three different concentration levels within the linear range of the proposed method and analyzed for the percent recoveries of the target analyte by the chromatographic procedure.

The proposed method was also evaluated for inter- and intraday precision. Binary mixtures of the studied drugs in three different concentrations of the pure drug standards were analyzed by the proposed procedure on different days and at different times of the same day.

Application to artificial mixtures of commercial formulations

Contents of the five vials labeled to contain 250 mg of the ceftriaxone sodium were weighed and mass of active ingredient per vial was calculated. Similarly the average weight of active ingredient per capsule of cefpodoximeproxetil was also calculated. Mass of powdered commercial formulations claimed to contain 0.005 g of ceftriaxone was dissolved in water and diluted to 25 mL with distilled water to get stock solution (200 µg mL^{-1}). Similarly, 200 µg mL^{-1} stock solution of cefpodoximeproxetilcommercial formulation was prepared by dissolving mass of powdered commercial formulations claimed to contain 0.005 g of cefpodoximeproxetil in 10 mL of methanol and diluted to 25 mL with distilled water. Artificial mixture of the commercial

formulations (1:1) in the concentration range of 2.5 µg mL^{-1}, 5.0µg mL^{-1} and 7.5 µg mL^{-1} were prepared by mixing appropriate volumes of stock solutions of the commercial formulations and diluting to appropriate volume with the mobile phase. All sample solutions were filtered through 0.45 µm membrane filter before injection to the chromatographic system.

Application to spiked plasma samples

Blood samples, provided by Khyber Teaching Hospital, Peshawar, were centrifuged at 3500 rpm for 10 min after coagulation and stored at -20 ºC. 500 µg standard of each of the drug i.e. ceftriaxone and cefpodoximeproxetil, was added to 5 mL plasma and deproteinized by mixing with 15 mL of acetonitrile. The mixture was centrifuged at the rate of 3500 rpm for 5 minutes. The supernatant was removed and diluted to 50 mL with distilled water. The resulting plasma solution has a concentration of 10 µg mL^{-1} with respect to both drugs. Plasma samples in concentration range of 2.5 µg mL^{-1}, 5.0 µg mL^{-1} and 7.5 µg mL^{-1} were prepared by dilution with mobile phase. All spiked plasma samples were filtered through 0.45 µm membrane filters and injected to the chromatographic system under the optimum chromatographic condition already mentioned.

RESULTS AND DISCUSSION

Optimization of experimental conditions

Both the studied drugs are third generation cephalosporin antibacterial agents and have closely related structures, therefore, a number of solvents combinations were screened for their separation and different chromatographic conditions were investigated for their effect on the separation and resolution of the chromatographic peaks of the studied drugs using reversed phase HPLC technique.

The effect of TEA concentration on retention time, peak height and peak shape was studied in the range of 0.01 to 0.05 M. By increasing concentration of TEA beyond 0.05 M the peak height was decreased along with the distortion of the peak shape but retention time was unaffected. It was observed that by increasing the concentration of TEA, pump pressure was also increased. Based on the peak height, peak shape and operating pressure of the pump, 0.03 M TEA was taken as optimum and used for further analysis (Fig 1).

The ceftriaxone is relatively more polar than cefpodoximeproxetil and, therefore, eluted first under the reversed phase conditions. Three different mobile phase compositions of acetonitrile: TEA buffer i.e. 2:1, 3:1, 4:1, (v/v), were investigated. It was found that by increasing the proportion of acetonitrile in the mobile phase, the separation between the peak increases and vice

versa (Fig. 2). The mobile phase having acetonitrile and TEA buffer in the ratio of 3:1 (v/v) produced better results in terms of the peak shape, peak height and peak separation.

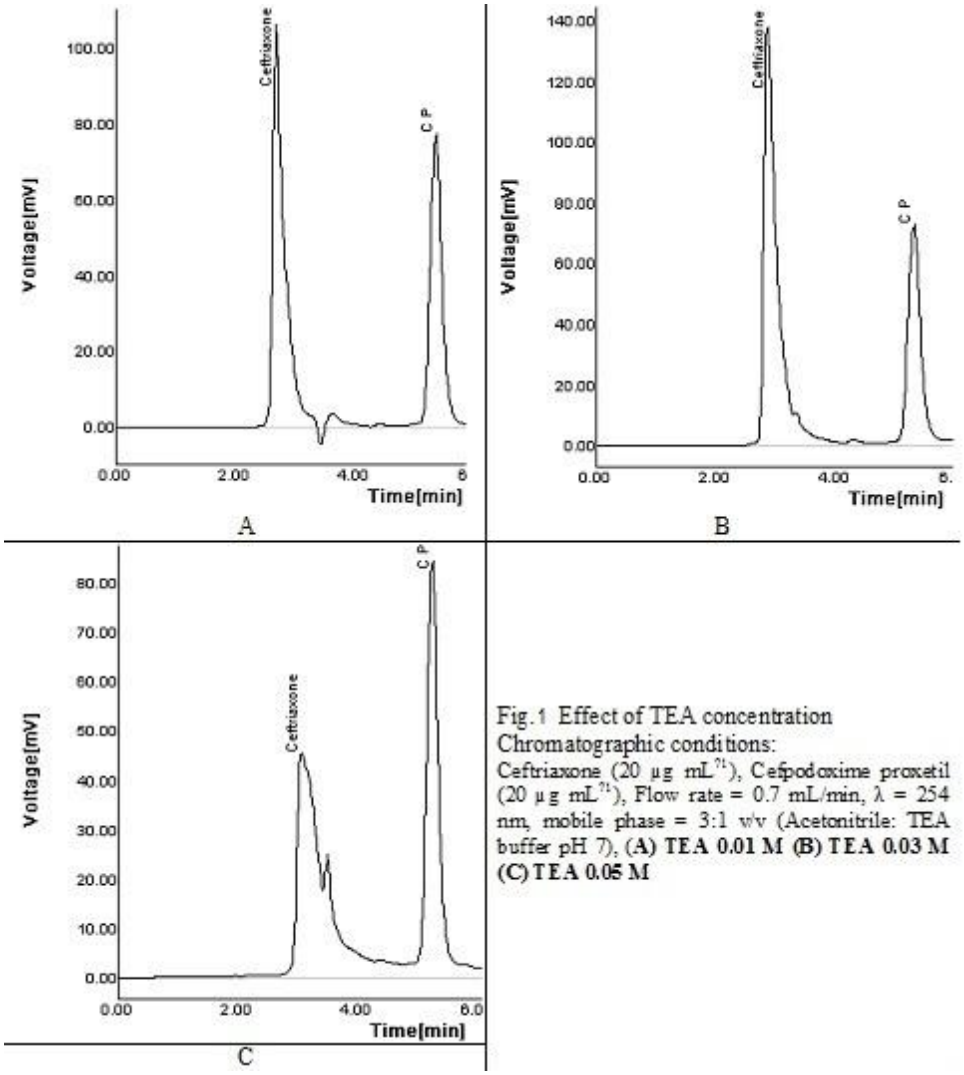

Fig.1 Effect of TEA concentration
Chromatographic conditions:
Ceftriaxone (20 µg mL^{-1}), Cefpodoxime proxetil (20 µg mL^{-1}), Flow rate = 0.7 mL/min, λ = 254 nm, mobile phase = 3:1 v/v (Acetonitrile: TEA buffer pH 7), **(A) TEA 0.01 M (B) TEA 0.03 M (C) TEA 0.05 M**

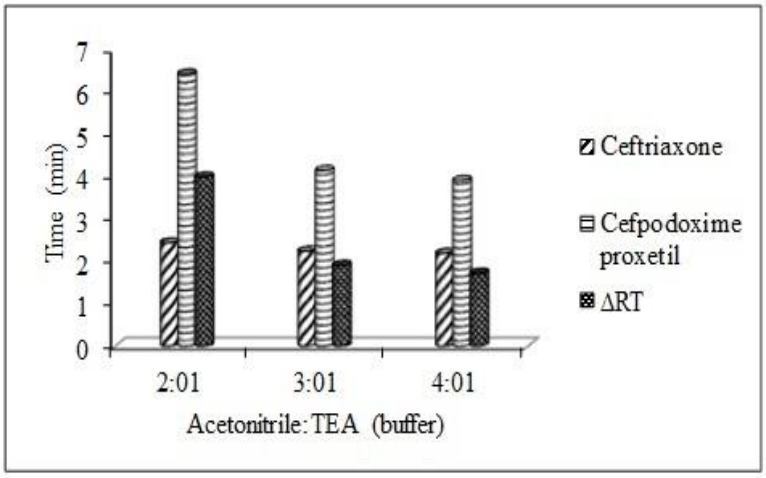

Fig.2. Effect of mobile phase composition

Chromatographic conditions: Ceftriaxone (20 µg mL^{-1}), Cefpodoximeproxetil (20 µg mL^{-1}), Flow rate = 0.7 mL/min, λ = 254 nm, mobile phase composition varied in the range of **2:1, 3:1 and 4:1** v/v (Acetonitrile: TEA buffer pH 7), TEA 0.03 M

The effect of pH of TEA buffer on separation of the two drugs at three different pH levels (pH5, 7, and 9) was investigated. There was a negligible change in retention time with change in pH.However, the pH above 7 resulted in the distortion of ceftriaxone peak (Figure 3). The TEA buffer with pH 7 was selected suitable because it not only increases the analytical column life but also the stability of the target drugs.

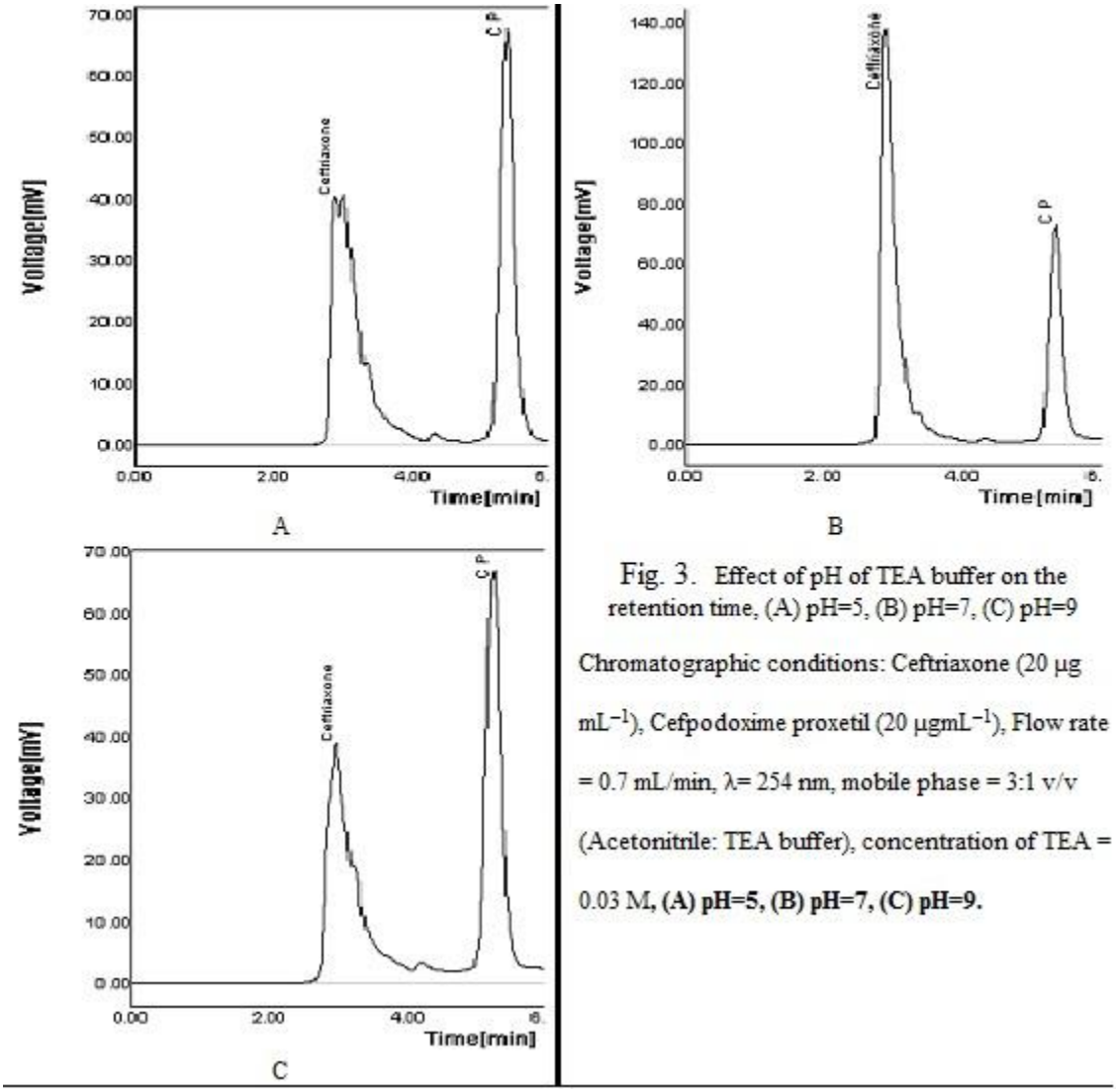

Fig. 3. Effect of pH of TEA buffer on the retention time, (A) pH=5, (B) pH=7, (C) pH=9

Chromatographic conditions: Ceftriaxone (20 µg mL^{-1}), Cefpodoxime proxetil (20 µgmL^{-1}), Flow rate = 0.7 mL/min, λ= 254 nm, mobile phase = 3:1 v/v (Acetonitrile: TEA buffer), concentration of TEA = 0.03 M, **(A) pH=5, (B) pH=7, (C) pH=9.**

The solvent flow rate was varied in the range of 0.5 $mL.min^{-1}$ to 1.3 $mL.min^{-1}$. The increase in the flow rate decreases the retention time of both the drugs to different extent. This caused a decrease in the separation of the analyte peaks accompanied by a proportional increase in the operating pressure of the isocratic pump with increase in flow rate (Figure 4). By an intelligent compromise between retention times of both drugs, separation of peaks (difference in the

retention times, ΔRT), peak shapes and operating pressure of the isocratic pump, 0.9 mL.min^{-1} flow rate was used in rest of the investigations.

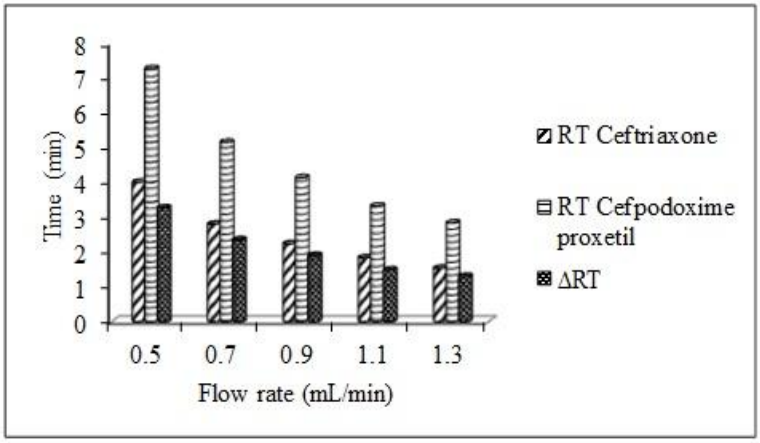

Fig.4. Effect of flow rate of the mobile phase

Chromatographic condition: Ceftriaxone (20 µg mL^{-1}), Cefpodoximeproxetil (20 µg mL^{-1}), λ=254 nm, mobile phase = 3:1 v/v (Acetonitrile: TEA buffer), concentration of TEA = 0.03 M, pH = 7, **Flow rate varied from 0.5 to 1.3 mL/min.**

Validation of method

Specificity

Specificity of the method was checked by comparing the chromatograms of standards of the two studied drugs in isolated as well as in mixture form with those of the commercial formulations and plasma samples (Figure 5). It is clear from these chromatograms that the retention time and shape of the peaks remain unchanged in isolated and mixture form, therefore, the method is specific for the studied drugs and the components of the sample matrix are not interfering in the assay.

Linearity and linear range

Linearity of the method was determined by plotting the peak height of drug against the concentration. It was found that the peak height increased linearly with increase in concentration from 0.5 to 250 µg mL^{-1} for ceftriaxone (Figure 6a)with regression coefficient (r^2) of 0.9996and 0.5 to 500 µg mL^{-1} for cefpodoximeproxetil (Figure 6b) with r^2 of 0.9995, above which a positive deviation was observed in both cases.

Sensitivity

The LOD (3.3s/b) and LOQ (10s/b) were calculated according to the ICH guidelines for the validation of analytical procedures.The LOD and LOQ were found to be 6.62 ng mL^{-1}, 20 ng mL^{-1} for ceftriaxone and 14.2 ng mL^{-1}, 42.9 ng mL^{-1} for cefpodoxime proxetil respectively. The important analytical characteristics of the proposed method are listed in Table 1.

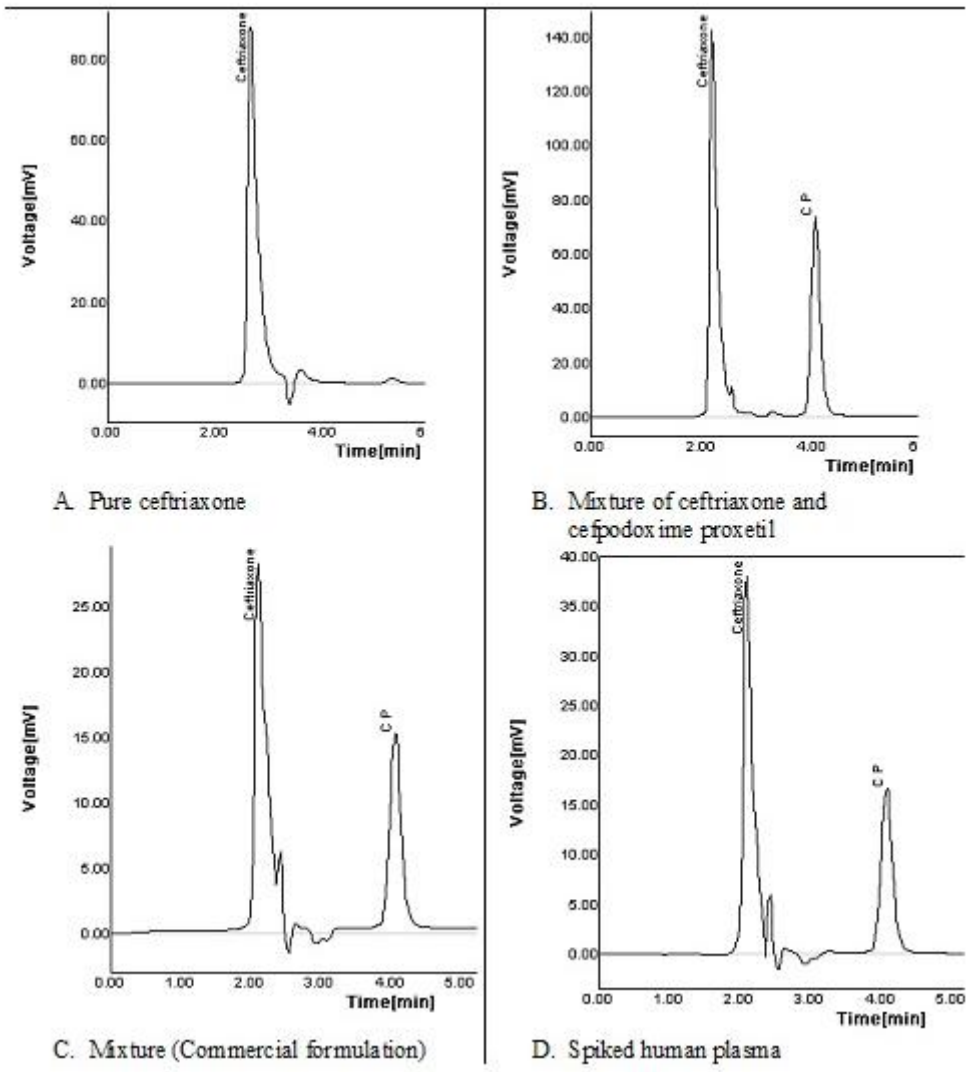

Fig.5. Representative Chromatograms of (A) standard ceftriaxone (B) mixture of the two drugs in pure form (C) mixture of the commercial of the two drugs (D) spiked human plasma

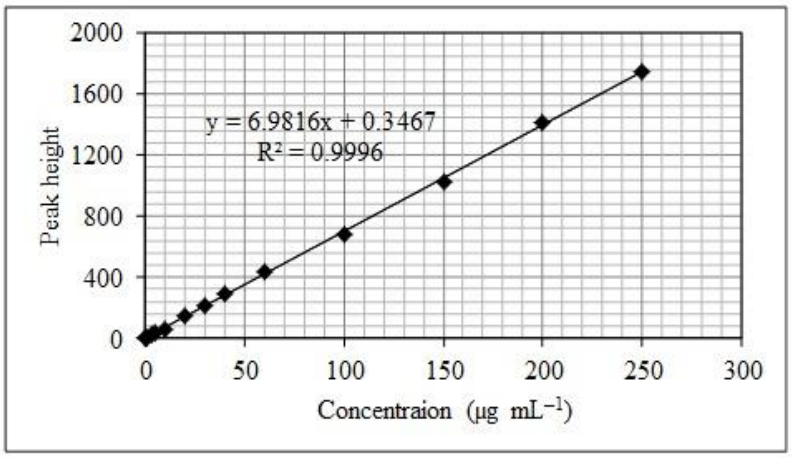

Fig.6a.Calibration curve for ceftriaxone 0.5 to 250 µg mL^{-1}

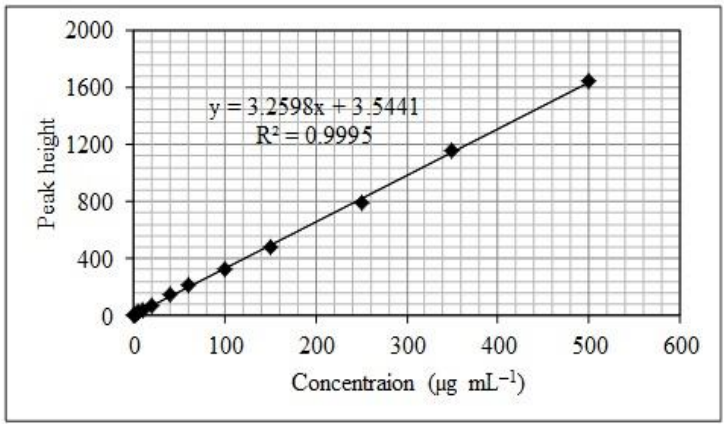

Fig.6b.Calibration curve for Cefpodoximeproxetil 0.5 to 500 µg mL^{-1}

Table 1: Analytical parameters for simultaneous determination of ceftriaxone and cefpodoximeproxetil by RP-HPLC

Parameter	Ceftriaxone	Cefpodoximeproxetil
Measuring Wavelength (nm)	240	240
Linear range (µg mL^{-1})	0.5–250	0.5–500
Limit of detection 3.3σ /b (ng mL^{-1})	6.62	14.2
Limit of quantification 10σ/b (ng mL^{-1})	20	42.9
Regression equation (y)	Y= 6.9816X+0.3467	Y= 3.2598X+3.5441
Slope (b)	6.9816	3.2598
Intercept (a)	0.3467	3.5441
Correlation coefficient (R^2)	0.9996	0.9995
Relative standard deviation (%)	2.68%	2.54%

Table 2: Evaluation of accuracy and precision of the proposed method for ceftriaxone and cefpodoximeproxetildetermination in pure form (n=4)

Ceftriaxone			Cefpodoximeproxetil		
µg taken	µg found	%Recovery ± RSD	µg taken	µg found	%Recovery ± RSD
0.5	0.4931	98.62±2.51	0.5	0.5073	101.47±3.00
2.5	2.5168	100.67±2.20	2.5	2.4996	99.98±2.53
5	5.0598	101.20±2.66	5	5.0142	100.28±2.49
$\overline{X}$		100.16	$\overline{X}$		100.58
±RSD		4.26%	±RSD		4.62%
t-test		0.065 (4.303)	t-test		0.216 (4.303)

Each result is the average of separate three replicates

Precision and accuracy

Precision and accuracy of the proposed chromatographic method was checked by using pure standard drugs, commercial formulations and spiked human plasma. The results are given in Table 2 for pure drugs and in Table 3 for commercial formulations and spiked human plasma. For pure drug percent recovery is in the range of 99.98-101.47% with RSD≤ 3.0. While in case of commercial formulation, the percent recovery for ceftriaxone was found from 99.75–101.92%

(RSD ≤ 2.70) and for cefpodoximeproxetil in the range of 99.64 – 101.72% (RSD ≤ 2.64). With spiked human plasma samples, the percent recoveries were in the range of 98.08 -99.32% (RSD≤272) for both the drugs. The RSD values show good precision of the proposed method (Table 3).

Table.3: Evaluation of accuracy and precision of the proposed method for ceftriaxone and cefpodoxime proxetil determination in mixtures of commercial formulations and spiked human plasma (n=4)

	Ceftriaxone			Cefpodoximeproxetil		
Sample	Amount taken (µg mL^{-1})	Amount found (µg mL^{-1})	%Recovery ± RSD	Amount taken (µg mL^{-1})	Amount found (µg mL^{-1})	%Recovery ± RSD
Commercial formulation # 1	0.5	0.5054	101.08±2.70	0.5	0.4997	99.94±2.64
	2.5	2.5200	100.84±2.23	2.5	2.5106	100.42±2.63
	5	5.0963	101.92±2.25	5	5.0638	101.27±2.23
Commercial formulation# 2	0.5	0.4998	99.96±2.60	0.5	0.5050	101.00±2.48
	2.5	2.4937	99.75±2.40	2.5	2.5316	101.26±2.62
	5	5.0513	101.03±2.67	5	4.9820	99.64±2.27
Human Plasma	0.5	0.4961	99.22±2.48	0.5	0.4907	98.15±2.65
	2.5	2.4829	99.32±2.72	2.5	2.4982	99.93±2.18
	5	4.9539	99.08±2.59	5	4.9154	98.31±2.52

Each result is the average of separate triplicate analysis

Table.4: Evaluation of recovery test of ceftriaxone and cefpodoximeproxetil in artificial mixtures of commercial formulations and spiked human plasmaby the proposed method. (Standard addition method) (n=5)

	Ceftriaxone			Cefpodoximeproxetil		
Pharmaceutical preparation	Amount added (µg mL^{-1})	Amount found (µg mL^{-1})	%Recovery ± RSD	Amount added (µg mL^{-1})	Amount found (µg mL^{-1})	%Recovery ±RSD
Commercial formulation # 1	0.5	0.4973	99.46±2.43	0.5	0.4965	99.31±2.419
	2.5	2.4985	99.94±2.26	2.5	2.545	101.80±2.32
	5.0	4.9730	99.46±2.40	5.0	4.975	99.50±2.22
Commercial formulation # 2	0.5	0.5027	100.55±2.54	0.5	0.4934	98.68±2.516
	2.5	2.4690	98.76±2.37	2.5	2.4977	99.91±2.718
	5.0	4.9650	99.30±2.25	5.0	4.9800	99.60±2.16
Human plasma	0.4961	0.5056	101.12±2.75	0.4907	0.4906	98.11±2.77
	2.4829	2.4746	98.98±2.35	2.4982	2.5619	102.47±2.75
	4.9539	4.9494	98.99±2.21	4.9154	4.9884	99.77±2.12

Each result is the average of separate triplicate analysis

The accuracy of the method was evaluated by spiking commercial formulations and plasma samples. The results are presented in Table 4. The percent recoveries and relative standard deviations, in both cases, are in the acceptable range showing the accuracy of the proposed procedure.

The inter- and intra-day precision of the method was determined by replicate analysis of ceftriaxone and cefpodoxime proxetil. The results are shown in Table 5. The low RSD values showed that the propose procedure exhibit excellent interday and intraday precision.

Table 5: Intraday and interday precision (n=4)

Drug	Type of precision	Amount taken (μgmL^{-1})	Amount found (μgmL^{-1})	%Recovery ± RSD
Ceftriaxone	Interday precision	0.5	0.488	97.69 ± 3.47
		2.5	2.451	98.05 ± 3.22
		5.0	4.976	99.52 ± 2.98
	Intraday precision	0.5	0.484	96.83 ± 3.87
		2.5	2.426	97.05 ± 3.53
		5.0	4.962	99.24 ± 3.21
Cefpodoximeproxetil	Interday precision	0.5	0.481	96.29 ± 3.93
		2.5	2.437	97.49 ± 3.61
		5.0	4.919	98.38 ± 3.10
	Intraday precision	0.5	0.476	95.15 ± 4.04
		2.5	2.407	96.30 ± 3.59
		5.0	4.899	97.98 ± 3.35

Each result is the average of separate triplicate analysis

Method application

The proposed method for simultaneous determination of ceftriaxone and cefpodoximeproxetil in a mixture was applied to commercial formulations. No HPLC method is available for the simultaneous quantification of both drugs in literature; therefore, two separate reference HPLC methods, one for ceftriaxone[23] and another for cefpodoximeproxetil[24] were used for statistical comparison. The results were compared statistically with the two reference HPLC methodsfor precision and accuracy using student's t-test and variance ration F-test at 95 % confidence level (Table 6). No significant difference in precision and accuracy was found between the proposed method and reference methods, as the calculated t-values and F-values were lower than the theoretical values.

The results obtained from these investigations show that the proposed procedure is suitable for the simultaneous quantification of both the studied drugs in commercial formulation and biological samples.

Table 6: Determination of ceftriaxone in commercial formulation and statistical comparison with reference method (n=4)

			Amount found	
Drug	Sample	Labeled amount mg/inj.	Proposed method mg/inj.	Reference method[25,26] mg/inj.
Ceftriaxone	Sample # 1	250	247.975± 2.61	246.525± 2.35
				F-test 1.133 (19)
				t-test= 1.162 (4.303)
	Sample # 2	250	246.05 ± 2.46	245.175 ± 2.79
				F-test= 1.398 (19)
				t-test=0.611 (4.303)
Cefpodoximeproxetil	Sample # 1	100	98.61 ± 2.77	98.15 ± 2.97
				F-test=1.156 (19)
				t-test=0.487 (4.303)
	Sample # 2	100	98.64 ±2.27	97.82 ±2.55
				F-test=1.633 (19)
				t-test=0.895 (4.303)

Each result is the average of separate triplicate analysis

CONCLUSION

HPLC methods are always the first choice for the drug analysis in a variety of sample matrices requiring high degree of specificity and selectivity. The running cost and speed of the HPLC method plays important role in deciding the suitability of the method for a particular analysis. In the present work, a simple, reproducible, specific HPLC procedure,with a single and low cost solvent system for two different third generation cephalosporins, has been developed. The simultaneous quantification of the two important third generation cephalosporins with single solvent system in a single run not only saves the solvent but also the time of analysis.It can be concluded that the developed HPLC method will provide the researcher a better choice for the analysis of these drugs in quality control and research laboratories with single solvent system leads to saving the time and cost of analysis as compared to reference methods where separate solvent system is required for each drug.

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