



NEW RAPID AND VALIDATED ISOCRATIC HPLC METHOD FOR SEPARATION AND ASSAY OF THREE 3rd GENERATION'S CEPHALOSPORINS (CEFTAZIDIME, CEFTRIAXONE, CEFOTAXIME)

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ABSTRACT

The purpose of this research is to find a rapid, sensitive and validated HPLC method for separation and determination of some third-generation cephalosporins (ceftazidime, ceftriaxone, cefotaxime), the three cephalosporins are separated on reversed-phase C18 column using isocratic elution, the mobile phase containing: (30% Methanol and 70% potassium dihydrogen phosphate 40mM) pH is adjusted to 3.5 by the addition of orthophosphoric acid, the samples were detected at 254nm. The qualitative study of the method included the effect of temperature, flow rate, ratio of the components of the mobile phase, pH value and concentration of the buffer on the quality of separation, the linear range of the three compounds is between 32 and 48 µg/ml, the relative standard deviation for precision is not more than (2%). The linearity, selectivity, accuracy and robustness of the developed method show acceptable values. The method was applied to samples of many pharmaceutical preparations of those compounds and to spiked serum samples. Recoveries from serum samples ranged between 91.225 and 95.759 %. Then it was applied to serum samples of patients using ceftazidime, the method is suitable for quality control of ceftazidime, ceftriaxone and cefotaxime in their mixtures and in pharmaceutical preparations. It can be used also to determine their concentration in serum.

Keywords: Cephalosporins 3rd generation, Ceftazidime, Ceftriaxone, Cefotaxime, HPLC, Serum.

INTRODUCTION

Cephalosporins (figure 1) are structurally and pharmacologically related to penicillins. Like penicillins, Cephalosporins have a betalactam ring structure that interferes with the synthesis of the bacterial cell wall^{1,2}. They are used for the treatment of infections caused by Gram (+) and Gram (-) bacteria³. They are among the safest and the most effective broad-spectrum bacteriocidal antimicrobial agents and therefore, they are the most frequently prescribed class of antibiotics^{4,5}.

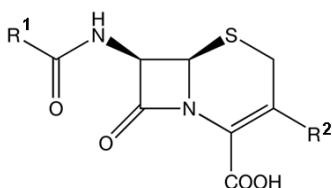
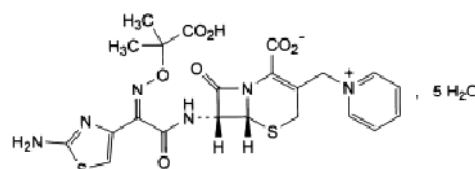


Figure 1: General Common Structure of cephalosporins

The third generation of cephalosporins have more activity and wider spectrum against bacteria especially that are resistant to penicillins and other cephalosporins, and have more stability to bacterial betalactamase^{2,6}.

The importance of this class of cephalosporins in treatment interested the researchers, therefore there are many scientific publications which focused on studying these compounds, their mechanism of action, pharmacokinetics. Ceftazidime, Ceftriaxone, Cefotaxime that are under study in this research (figures 2,3,4) are among the most important cephalosporins used in clinics

and hospitals, let alone the use of Ceftazidime in the treatment of pseudomonas aeruginosa³.



The aim of this study was to find a valid, rapid and sensitive analytical procedure using high performance liquid chromatography (HPLC) for the separation and as well as the assay of a mixture of Ceftazidime, Ceftriaxone, Cefotaxime.

MATERIALS AND METHODS

- Ceftazidime, Ceftriaxone, Cefotaxime as secondary standard materials were obtained from the Syrian Ministry of Health.

- The finished pharmaceutical samples (vials) were obtained from the Community Pharmacy and from several pharmaceutical local factories.

Human serum samples were obtained from a hospital laboratory.

- Solvents especially for HPLC (Methanol, water) were from Merck, Scharlau and Cham Lab companies.

Chemicals

Potassium dihydrogen phosphate from Carlo Erba (Italy).

Ortho phosphoric acid from Prolabo (EEC).

Excipients

Dextrose and sodium bicarbonate from Merck (Germany).

Instruments and equipment

- A Hitachi HPLC chromatography system provided with Hitachi L-2130 HPLC pump, Hitachi diode array detector L-2455(UV\VIS), Hitachi L-2200 auto injector, Hitachi column oven L-2300, laserjet pt005 printer.

- Column C18 (250mm x 4.6mm): knauer (Germany).

- An Ultrasonic device: Selecta (Spain).

- An acidimeter; orion model 310 with epoxy electrode.

- A magnetic stirrer: cole-parmer (Malaysia).

- Filters 0.45 µm from Sartorius stedium biotech.

- HPLC filters 0.45 µm from Whatman (Schleicher & schuell).

- Filtering unit from Sartorius.

- A sensitive balance; Sartorius Analytic Balance CPA225D (sensitivity of 10⁻⁵ g).

- Centrifuge : Hettich EBA20 (Germany).

- Cartridge RP – 18 by Merck, Darmstadt.FR.

Chromatographic conditions

The Mobile phase was: methanol, potassium dihydrogen phosphate 40mM (30:70 v/v), and the pH= 3.5 was adjusted by adding ortho phosphoric acid. The detector wavelength was 254nm. The flow rate was maintained at 0.85 ml/min. The column temperature was set at 30°C. The injection volume was 50 µl.

Preparation of solutions

Preparation of potassium phosphate buffer 40 mM

Weigh 5.4436 g of potassium dihydrogen phosphate and place it in 1 liter volumetric flask dissolve it by sterile water using a magnetic stirrer. Complete volume of the solution by water to 1 liter and adjust pH to 3.5 by ortho phosphoric acid. Run the resulting solution through 0.45 µm filters.

Preparation of mobile phase

To prepare 1 liter take 700 mL of potassium phosphate buffer 40 mM (pH=3.5) and place it in 1 liter volumetric flask. Add 300 mL methanol. Run the resulting solution through 0.45 µm filters.

Preparation of stock solutions

Stock solutions of 200 µg/mL of Ceftazidime, Ceftriaxone and Cefotaxime were prepared. Weigh 100 mg of Ceftazidime and place it in a 500 ml volumetric flask. Dissolve it by water using a magnetic stirrer. Complete volume of the solution by water to get the mentioned concentration. In the same way the Stock solutions of Ceftriaxone and Cefotaxime are prepared.

Preparation of standards

Standard solutions of 40 µg/mL of Ceftazidime, Ceftriaxone and Cefotaxime were prepared. Take 20 mL by calibrated pipette from the stock solution of Ceftazidime 200 µg/mL and place it in a 100 mL volumetric flask. Complete volume of the solution by water to get the mentioned concentration. In the same way the standard solutions of Ceftriaxone and Cefotaxime are prepared.

Preparation of the standard concentrations mixture solution of Ceftazidime, Ceftriaxone and Cefotaxime

Take 20 ml of each of the stock solutions of Ceftazidime, Ceftriaxone and Cefotaxime. Place all in a 100 ml volumetric flask. Complete volume of the solution by water to get a mixture concentration solution of 40 µg/mL of each compound.

Preparation of validity test solutions

Standard Linearity Solutions

Prepare five sequential concentrations from the stock solution containing respectively 80%, 90%, 100%, 110% and 120% of the standard solution concentration. Put in five volumetric flasks -each of a capacity of 100 mL- sequentially and separately 16, 18, 20, 22.24 mL from the stock solution. Complete volume of the solution by water then mix.

Accuracy solutions

Vial excipients (i.e. dextrose for Ceftazidime and sodium bicarbonate for Cefotaxime)¹⁵ are spiked to the standard to obtain analyzed samples. Then nine samples are divided into three groups containing respectively 80%, 100% and 120% of standard solution concentration.



Precision Solutions

Prepare nine samples and divide them into three groups containing respectively 80%, 100% and 120% of standard solution concentration.

Selectivity Solutions

A drug-free sample is prepared from the excipients i.e.(dextrose for Ceftazidime and sodium bicarbonate for Cefotaxime)¹⁵. Three samples containing 100% of standard solution concentration are also analyzed.

Robustness Solutions

Three samples containing 100% of standard solution concentration are analyzed. The first sample is injected after adjusting the column oven temperature at 28°C, second at 30°C and the third at 32°C respectively.

Preparation of vial samples

Ceftazidime vial samples are prepared by weight 10 vials¹⁶ of each pharmaceutical factory individually and then the average weight per vial is calculated. Weigh powder equivalent to 100 mg of Ceftazidime. Place in 500 mL volumetric flask. Dissolve the powder by water using a magnetic stirrer. Complete volume of the solution by water. Run the resulting solution through 0.45 µm filters. The concentration of the resulting solution will be 200 µg/mL. Then take 20 mL of this solution and place it in a 100 mL volumetric flask. Complete volume of the solution by water. The concentration of the resulting solution will be 40 µg/mL. In the same way the vial samples of Ceftriaxone and Cefotaxime are prepared.

Preparation of a series of standard solutions of Ceftazidime to determine the recovery from the serum

Series of Standard Solutions of Ceftazidime were prepared in the following concentrations (10, 20, 40, 80 and 160 µg/mL) by diluting its stock solution (200 µg/mL).

Preparation of a series of serum standard solutions of Ceftazidime

The above-mentioned series of standard solutions were prepared with duplicated concentrations. Add to each 1 ml of series solutions 1 ml drug-free serum to obtain a new series of serum standard solutions with the same concentrations of the said series. Centrifuge for half an hour.

Extraction

The serum solution was extracted in the liquid/solid extraction method using C18 cartridge in the following way:

- Precondition cartridge with 3 ml of methanol, withdraw. Then with 3 ml of water, withdraw.
- Apply the serum solution.
- Ceftazidime was eluted with a mixture of methanol and acetonitrile (1:1). The elutes were left to dry for 15 minutes. Dry residues were reconstituted with water. The

solutions were run through 0.45 µm HPLC filters then injected directly to HPLC.

RESULTS

Method development

Wavelength selection

The study of ultraviolet spectra for Ceftazidime, Ceftriaxone and Cefotaxime to determine maximum absorption wavelength, showed 259 nm for Ceftazidime¹⁷, 261 nm for Ceftriaxone¹⁷ and (262,205) nm for Cefotaxime¹⁸. Therefore, 254 nm wavelength was selected after comparing the spectra to achieve the assay of the studied compounds^{16,19,20}.

Selection of mobile phase and experimental conditions

Many percentages of the mobile phase were tested without any separation until the suitable phase; a mixture of methanol, potassium dihydrogen phosphate 40mM, was used.

Table 1 showed the stadia of finding and development of the method to get the best separation of the studied compounds by changing the percentages of the mobile phase and experimental conditions which are explained in Figures (5 to 9).

(For confirmation of each peak, injecting a solution of each compound separately was done).

Confirmation of the method's validity

Identity of each peak was confirmed by the retention time.

Method compatibility with the requirements of system suitability according to the standards of U.S. Pharmacopeia¹⁶ was performed.

Linearity for the three studied compounds covering the range between 32 to 48 µg/mL was verified.

The calibration curve linearity was examined by studying the correlation coefficient between the concentrations and the response area of each concentration.

Accuracy was assessed by a recovery percentage.

Relative standard deviations for selectivity, repeatability, intermediate precision and robustness were less than 2%.

Table 2 features the most important constitutional standards in the chromatogram of a mixture of Ceftazidime, Ceftriaxone and Cefotaxime. Table -3- summarizes the results of the method validation tests of the three compounds:

Sample test results

Pharmaceutical preparations

Vials containing 1g of Ceftazidime of three pharmaceutical factories were analyzed. The percentage of active substance in each sample was calculated from the peaks areas of samples and standard solutions.



Table 1: The stadia of finding and development of the method

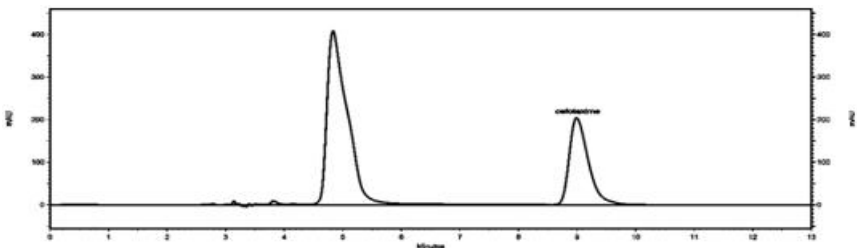
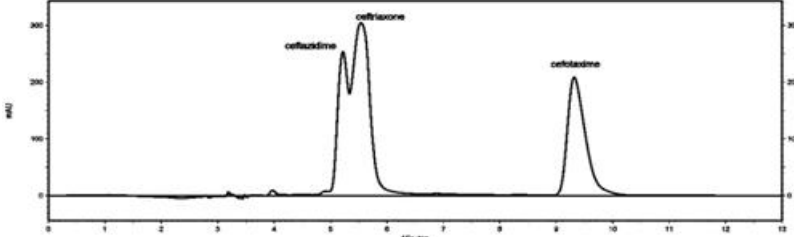
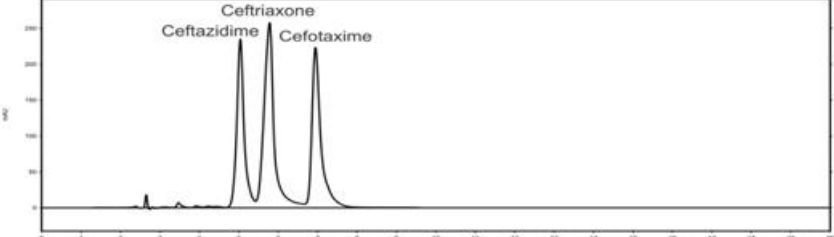
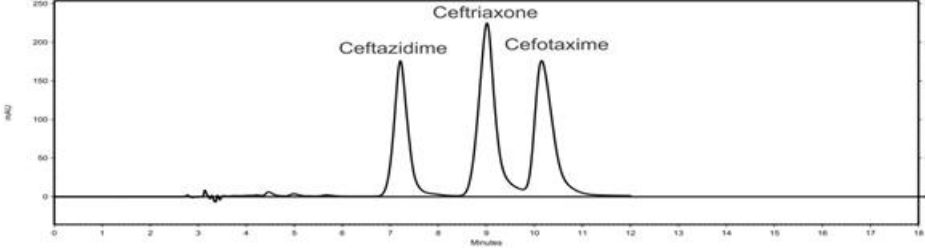
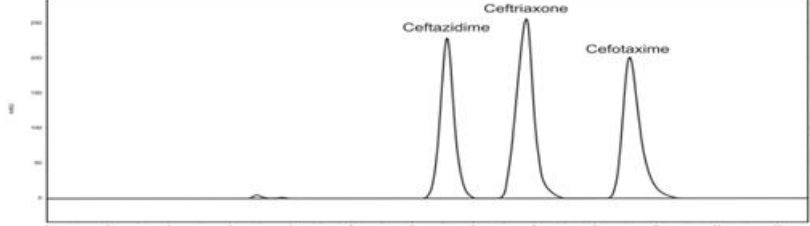
	<p>Figure 5</p>
<p>Experimental conditions: methanol : potassium phosphate 40 mM (35:65) flow rate = 0.85 mL/minute, temperature=25°C. pH = 6</p>	<p>Results: Ceftazidime and Ceftriaxone are appeared in one peak, and then the peak of Cefotaxime is separated alone. All peaks have acceptable symmetry.</p>
	<p>Figure 6</p>
<p>Experimental conditions: methanol : potassium phosphate 40 Mm (35:65) flow rate = 0.85 mL/minute, temperature=25°C. pH = 4.5</p>	<p>Results: The peaks of Ceftazidime and Ceftriaxone are interfering, then the peak of Cefotaxime is separated alone. All peaks have acceptable symmetry. The retention time is increased.</p>
	<p>Figure 7</p>
<p>Experimental conditions: methanol : potassium phosphate 40 mM (35:65) flow rate = 1 mL/minute, temperature = 25°C. pH = 3</p>	<p>Results: The peaks of the three studied compounds are interfering. All peaks have acceptable symmetry. The retention time is decreased.</p>
	<p>Figure 8</p>
<p>Experimental conditions: methanol : potassium phosphate 40 mM (35:65) flow rate = 0.85 mL/minute, temperature = 25°C. pH = 3</p>	<p>Results: The peak of Ceftazidime is separated firstly, the peaks of Ceftriaxone and Cefotaxime are interfering. All peaks have acceptable symmetry. The retention time is increased.</p>
	<p>Figure 9</p>
<p>Experimental conditions: methanol : potassium phosphate 40 mM (30:70) flow rate = 0.85 mL/minute, temperature=30°C. pH = 3.5</p>	<p>Results: Best separation for the three peaks as the resolution is increased and its values are acceptable. All peaks have acceptable symmetry. Therefore these conditions are adopted for the separation of the three studied compounds.</p>

Table 2: The most important constitutional standards in the chromatogram of the mixture

Compound	Area	Theoretical Plates	Tailing Factor	Resolution	Retention Time
Ceftazidime	15012343	4730	1.13106	—	6.587
Ceftriaxone	21854351	2492	1.20656	2.73488	7.900
Cefotaxime	15988756	6585	1.49924	3.20772	9.593

Table 3: Method validation results

Compound	Linearity		Recovery %					Detection Limit µg/ml	Quantification Limit µg/ml
	Equation	Correlation Coefficient	Accuracy	Selectivity	Precision		Robustness		
					Repeatability	Intermediate			
Ceftazidime	y = 40404 x + 94339	0.996	99.320	98.887	99.098	99.088	101.272	0.1	0.33
Ceftriaxone	y = 52396 x + 94283	0.991	99.728	99.473	99.196	98.800	100.068	0.09	0.3
Cefotaxime	y = 40788 x + 41755	0.998	98.949	98.960	99.055	98.816	100.669	0.1	0.33

Table 4: Results of pharmaceutical preparation samples of Ceftazidime.

No.	Factory	Sample Peak Area	Average of Standard Peak Area	Percentage of the Active Substance %
1	A	16164551	15015421	107.653
2	B	14972026	15015421	99.711
3	C	14853855	15015421	98.924

Table 5: Results of pharmaceutical preparation samples of Ceftriaxone

No.	Factory	Sample Peak Area	Average of Standard Peak Area	Percentage of the Active Substance %
1	A	2240544	21868572	102.455
2	B	22802578	21868572	104.271
3	C	22162704	21868572	101.345

Table 6: Results of pharmaceutical preparation samples of Cefotaxime

No.	Factory	Sample Peak Area	Average of Standard Peak Area	Percentage of the Active Substance %
1	A	16529240	15943016	103.677
2	B	16616608	15943016	104.225
3	C	16387507	15943016	102.788

Results of Ceftazidime samples are shown in Table 4.

Vials containing 1g of Ceftriaxone of three pharmaceutical factories were analyzed. The percentage of active substance in each sample was calculated from the peaks areas of samples and standard solutions.

Results of Ceftriaxone samples are shown in Table 5.

Vials containing 1g of Cefotaxime of three pharmaceutical factories were analyzed. The percentage of active substance in each sample was calculated from the peaks areas of samples and standard solutions.

Results of Cefotaxime samples are shown in Table 6.

Serum samples

The method was applied to analyze serum samples.

Standard serological solutions series of Ceftazidime were injected using the adopted Chromatographic conditions of the separation method. Linearity was good in the range of (10 - 160) µg/mL.

Table 7 shows the recovery results of the serum samples of Ceftazidime for each concentration after comparing areas of the standard serological series peaks with areas of standard solution series peaks. Figure 10 shows the

chromatogram of a serum sample of Ceftazidime 20 µg/mL.

Table 7: Recovery results of the serum samples of Ceftazidime

No.	Concentration (µg/mL)	Recovery %
1	10	94.324
2	20	91.360
3	40	95.015
4	80	91.225
5	160	95.759

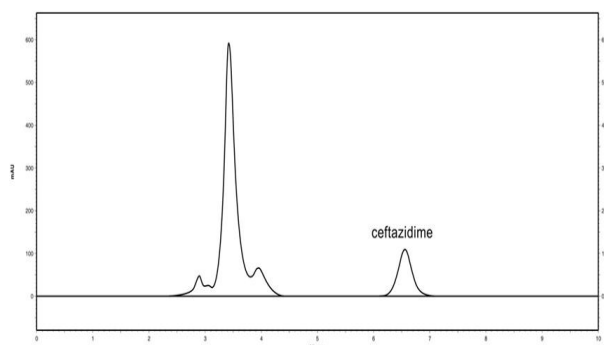


Figure 10: Chromatogram of a serum sample of Ceftazidime 20 µg/mL

DISCUSSION

A new, valid, sensitive, accurate and rapid HPLC analytical method with isocratic elution has been found in this study. It can be used for the assay and separation of a mixture of three cephalosporins; Ceftazidime, Ceftriaxone and Cefotaxime.

International reviews and scientific publications have adopted many HPLC methods with gradient elution for the separation and the assay of several cephalosporin mixtures^{13,14} but the studied mixture was not found in any of them with isocratic elution.

The chromatographic conditions were: reversed phase C18 column, the column temperature was set at 30°C. Mobile phase consisting of: methanol and potassium dihydrogen phosphate 40Mm (30:70 v/v), the pH= 3.5 was adjusted by adding orthophosphoric acid. Flow rate was 0.85 mL/min. UV detector wavelength was 254nm.

Mobile phases containing one solvent or more didn't succeed to achieve the separation of the three compounds. Separation succeeded when a mixture of methanol and potassium dihydrogen phosphate 40mM (30:70 v/v) was used.

The appropriate percentage of methanol for separation of the studied compounds was (30%), increasing methanol above this percentage leads to interfering peaks, while reducing the percentage of methanol leads to an increase in the retention time and bad separation.

Decreasing the flow rate from 1 mL\min to 0.85 mL\min led to an improvement in the separation and prevented interfering of the peaks.

The presence of buffer has great importance for the separation, where separation did not occur without the presence of buffer. There are several factors related to the buffer:

- A. The type of buffer and its concentration: the separation did not occur using several buffers despite the change of other experimental conditions, while it occurred when potassium phosphate buffer 40 mM was used.
- B. pH value of potassium phosphate buffer: pH value influence on the retention time of peaks in general and on the retention time of the peak of ceftriaxone in particular, as in pH=6 the retention time decrease and the peak of Ceftriaxone interferes with the peak of Ceftazidime which appears first, while decrease pH to 3 increases the retention time and the peak of Ceftriaxone interferes with the peak of Cefotaxime which appears last, best separation was when pH=3.5 was used.

Raising column temperature up to 30°C led to best separation and acceptable symmetry.

Examined pharmaceutical preparation samples results of Ceftazidime, Ceftriaxone and Cefotaxime were USP acceptable within the specified range (90-110%)²⁴.

The recovery percentages of serum samples spiked with Ceftazidime ranged between 91.225 to 95.759%.

CONCLUSION

The method described in this study is a simple, rapid, sensitive and accurate assay for the quantitative determination of three cephalosporins: Ceftazidime, Ceftriaxone, Cefotaxime in pharmaceutical preparations. It could be also used for the determination of their concentration in human serum that support studies of bioavailability of these compounds to adjust the concentrations blood therapeutic, or the assay of these compounds especially when used to treat infections in patients who use immunodepressive drugs (organ transplant patients, systemic lupus erythematosus.)^{21,22}

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