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Application of Hantzsch Reaction as a New Method for Spectrofluorimetric Determination of Some Cephalosporins

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ABSTRACT

A selective spectrofluorimetric method was described for determination of some cephalosporins namely, cefadroxil, cefaclor, cefixime, cefpodxime, and cefepime. These drugs were utilized as a source of amine group to react with acetylacetone and formaldehyde reagents via a Hantzsch reaction. The fluorescence intensity of the reaction products were measured at an emission wavelength of482 nm after excitation at a wavelength of 415 nm. The linear calibration graphs were obtained in the concentration ranges of 1-100 µg/mL for either cefadroxil or cefaclor and 1-50 µg/mL for the other three drugs. The limits of detection and quantitation were in the concentration range of 0.31-0.82 and 0.72-2.5 µg/mL, respectively. The percent of relative standard deviation (at 50 µg/mL concentration level, n = 5) were in the range of 0.41-1.70% and 1.51-1.95% for intra-day and inter-day precision, respectively. The method was successfully applied for the assay of some dosage forms of the cited drugs and the results obtained were comparable with those obtained by a reported method.

INTRODUCTION

Cefadroxil, cefaclor, cefixime, cefpodxime, and cefepime (**Table 1**), broad-spectrum cephalosporins *β*-lactam antibiotics, are among the oldest and the most valuable clinical antimicrobial agents used to treat the bacterial infections of skin, soft tissues, and urinary tract. Roughly, cephalosporins can be divided into first, second, third and fourth generation agents based on the time of their discovery and their antimicrobial properties (Sweetman, 2009). As cephalosporins do not absorb in visible region of radiation, many visible spectrophotometric methods were developed for their determination after chemical derivatization as the result of the reactions based on redox reaction for individual determination of cefadroxil (Daabees *et al.,* 1998; Aly *et al.,* 1994; Chilukuri *et al.,* 1997; Salem and Saleh 2002; Gamal 1996) or cefaclor (Issopoulos, 1989; Gamal *et al.,* 2002); or the mixtures of cefadroxil and cefaclor (Issopoulos, 1989; Badawy *et al.,* 1993; Nabi *et al.,* 1997); or cefadroxil, cefaclor and cefixime (El-Shaboury *et al.,* 2010); or cefadroxil and cefepime (Chilukuri *et al.,* 1997; Shah and Pundarikakshudu, 2006; Morelli and Peluso, 1985, Elazazy *et al.,* 2003); cefaclor and cefixime (Shah and Pundarikakshudu, 2006, Al-Momani 2001; Ashok *et al.,* 2011). Visible measurements after complexation of cephalosporins with certain metals were used for individual determination of cefadroxil (Badawy *et al.,* 1993) and cefaclor (Dimitrovska *et al.,* 1996; Salem 2004) or their mixture (Khater and Ibrahim 1993); also for determination of cefixime (Ramadan *et al.,* 2013), cefpodoxime (Rao *et al.,* 2004) or cefepime individually (Hosny, 2014; Elazazy *et al.,* 2004).

The complexation methods based on the charge-tranfer for determination of cefadroxil (Saleh *et al.,* 2001), cefpodoxime

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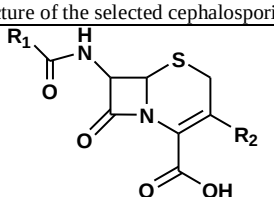
(El-Sattar *et al.,* 2001); or based on formation of ion pairs for determination of cefadroxil (Prasad *et al.,* 2004; Naveed *et al.,* 2014), cefaclor (Avadhanulu *et al.,* 1996), cefadroxil and cefaclor (Salem and Askal 2002), cefixime (Siddalinga *et al.,* 2012; Ivama *et al.,* 1999), cefpodoxime (Siddalinga *et al.,* 2012) were also reported.

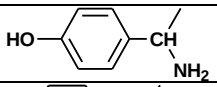
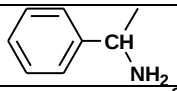
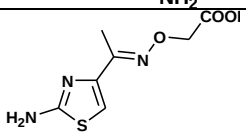
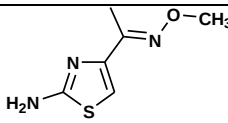
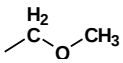
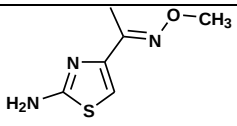
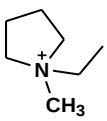
On the another hand, the spectrofluorometric methods for the determination of the investigated cephalosporins could be classified into the following categories (El-Shaboury *et al.,* 2007): methods based on measurement of the fluorescence of the hydrolytic products; as cefadroxil was determined through mixing with sodium hydroxide and heating at 100 ^{O}C (Yang *et al.,* 1998); cefadroxil and cefalexin were determined by using coupling technique of synchronous fluorometry and H-point standard addition methods (Jinghe *et al.,* 1996); cefaclor was determined in formulations by measuring its native fluoresence at 416 nm (Aly *et al.,* 1996). Methods depending on reaction with fluorogenic agents as fluorescamine was described for the determination of cefadroxil, cefixime and cefaclor (Hefnawy *et al.,* 1999, Chaudhari and Patel 2014). Cefaclor alone was determined after derivatization with 4-(2-cyanoisoindolyl) phenylisothiocyanate (Kai *et al.,* 2003). Finally, cefadroxil and cefradine were determined by fluorescence quenching method after mixing each drug with fluorescein-mercuricin in basic medium (Yang *et al.,* 1996).

To our best knowledge and after reviewing the literatures, the spectrophotometric and spectrofluorimetric methods based on redox reactions suffer from lack of suitable selectivity (Daabees *et al.,* 1998; Aly *et al.,* 1994; Chilukuri *et al.,* 1997; Salem and Saleh, 2002; Gamal, 1996; Issopoulos, 1989, Gamal *et al.,* 2002; Issopoulos, 1989; Badawy, *et al.,* 1993; Nabi *et al.,* 1997; El-Shaboury *et al.,* 2010; Shah and Pundarikakshudu, 2006; Morelli and Peluso, 1985; Elazazy *et al.,* 2003; Al-Momani, 2001; Ashok *et al.,* 2011). Although some of the visible measurements, after metal complexation and/or complexation methods based on the charge-tranfer formation with individual cephalosporins, are have highly sensitive (Badawy *et al.,* 1993; Dimitrovska *et al.,* 1996; Salem, 2004; Khater and Ibrahim, 1993; Ramadan *et al.,* 2013; Rao *et al.,* 2004; Hosny, 2014; Elazazy *et al.,* 2004; Saleh *et al.,* 2001; Subbayamma and Rambabu, 2010; El-Sattar *et al.,* 2001; Prasad *et al.,* 2004; Naveed *et al.,* 2014; Avadhanulu *et al.,* 1996; Salem and Askal, 2002; Siddalinga *et al.,* 2012; Ivama *et al.,* 1999) however some cephalosporins need tedious experimental and time consuming procedure such as use of chloroform for extraction of the reaction product (Naveed *et al.,* 2014; Ivama *et al.,* 1999). In addition, the other reported spectrofluorimetric methods may require more expensive reagents such as fluorescamine (Hefnawy *et al.,* 1999; Chaudhari and Patel 2014). Therefore due to the wide use of cephalosporins, there is a still a demand for cost-effective, accurate, applicable, and collective analytical method to be applied during their industrial development and clinical analysis.

The objective of this work is to develop a simple, sensitive, and reliable spectrofluorometric method for determination of the cited drugs in bulk and in dosage forms available in the Egyptian market. The proposed method was designed based on firstly, the presence of free primary amino group in the structure of the studied cephalosporins and secondly, the common approach for the chemical derivatization of primary amino group through Hantzsch condensation reaction (Derker Bratlon and David, 1979; Amin and Zareh, 1996; Hanaa *et al.,* 2014; El-Yazbi *et al.,* 1999; El-Aroud *et al.,* 2007; Ayad *et al.,* 2012) utilizing cheap reagents such as acetylacetone and formaldehyde which are widely available in most quality control laboratories. Thus our target is to apply this reaction for the development of a simple, cost-effective, and selective spectrofluorimetric method for determination of more than one cephalosporins in bulk drugs and in different pharmaceutical dosage forms. Moreover the present fluorimetric method was developed and validated according to ICH (2005) and USP (Washington, 2008) guidelines.

Table 1: Chemical structure of the selected cephalosporins.



S. No.	Generic name	R_1	R_2	Generation
1	Cefadroxil		CH_3	1st
2	Cefaclor		Cl	2nd
3	Cefixime			3rd
4	Cefpodoxime			3rd
5	Cefepime			4th

MATERIAL AND METHODS

All chemicals were of analytical reagent grade and were used without further purification; the distilled water was used throughout. Cephalosporins were generously supplied by their respective manufacturers and were used as supplied: cefaclor monohydrate lot. No: 081120015, potency: 939.99 µg/mg (Pharco Pharmaceuticals Co Amreya, Alexandria, Egypt), cefadroxil monohydrate lot. No: B333184, potency: 941.52 µg/mg (Amoun

Pharmaceutical Industries Co., Cairo, Egypt), cefpodoxime proxetil lot. No: 358927, potency 953.62 µg/mg (Hoechst Marion Roussel, S.A.E., Cairo). cefixime lot. No: B53927, potency 968.93 µg/mg (El-Hekma Co., Cairo,) and cefepime hydrochloride lot. No: 952347, potency 978.59 µg/mg (Bristol Myers Squibb, Cairo,). The following pharmaceutical products containing the studied drugs were purchased from the Egyptian local market; Curafep 1 g vial lot No of 121990 and expiration date in 12/2017 (Delta Pharma Egypt, 10th of Ramadan, El Sharkeya, Egypt) was labeled to contain 1000 mg of cefepime per vial. Ceclor 0.125 g powder for oral suspension lot No of 2048 and expiration date in 02/2017 (Egyptian Co., Pharmaceutical and Chemical Industries, Benisuef, Egypt) was labeled to contain 0.125 g of cefaclor per 5 mL of suspension.

Duricef 0.5 g capsule and powder for oral suspension respectively, with lots No of 106596 and 106488 and expiration dates in 09/2018 and 08/2018 (GlaxoSmithKline El Salam city, Cairo) were labeled to contain 0.5 g of cefadroxil per capsule or per 5 mL of the oral suspension. Ximacef 0.1 and 0.4 g powder for oral suspension and capsules respectively, with lots No of 1540151 and 1540001 and expiration dates in 06/2017 and 01/2018 (Sigmatec Pharmaceutical industries, 6th of October City, Cairo) was labeled to contain 0.1 and 0.4 g of cefixime per 5 mL of suspension or per capsule. Orelox 0.1 g tablet lot No of 3FP9A and expiration date in 07/2016 (Sanofi Winthrop Industry, Compiegne, France) was labeled to contain 0.1 g of cefpodoxime per tablet. Formaldehyde (34% v/v), acetyl acetone (98 % v/v), sodium acetate, and acetic acid were purchased from El-Nasr Chemical Co., (Abu Zaabal, Cairo).

All the measurements were carried out by using a SCINCO spectrafluorimeter (Scinco FS-2, Seoul, Korea) with matched 1 cm thickness quartz cell and the slit width for both monochromators were set at 5 nm. The calibration and linearity of the instrument were checked at frequent intervals with standard quinine sulphate (0.01µg/mL).

The data provided are corrected against standard quinine solution as 0.001mg/mL in 0.05M sulphuric acid at wavelength of emission 445 nm after excitation at 350 nm (Jeffery,Bassett *et al.*, 1989). All fluorescence measurements were recorded at the lower set sensitivity (slit width 5) (Osama 2004). Additionally, MLW type thermostatically controlled water bath (Memmert GmbH, Schwabach, Germany) was used for heating purposes. Digital analytical balance (AG 29, Mettler Toledo, Glattbrugg, Switzerland) and Milwaukee SM 101 pH meter, Portugal.

Reference solution preparation

Cephalosporins solutions

Stock solutions (1mg/mL) of cefadroxil, cefaclor, and cefepime were daily prepared in distilled water while those of cefpodoxime and cefixime were daily prepared in methanol. The working solutions were daily prepared by further dilution allwith distilled water to cover their linearity concentration range.

Formaldehyde solution

A standard solution of formaldehyde (20%, v/v) was daily prepared by mixing 58.8 mL of the reagent to 100 mL distilled water.

Acetyl acetone solution

A standard solution of acetylacetone (10%, v/v) was daily prepared by mixing 10.20 mL of the reagent to 100 mL distilled water.

Sodium acetate buffer solution

Solutions of acetate buffer of different pH in the range of 3.5 -5.5 were prepared by mixing certain volumes of 0.1 M of sodium acetate and acetic acid. The pH of these buffers were checked and adjusted by a pH meter before use.

Preparation of pharmaceutical dosage form samples

Twenty tablets or the contents of twenty capsules were weighed, finely powdered, and mixed thoroughly. Into a 25-mL volumetric flask a weighed amount of the powder equivalent to 25 mg of each drug was treated with about 20 mL of distilled water (or methanol in case of cefpodoxime and cefixime), mixed well and the resultant mixture was completed to volume with the same solvent. The prepared mixture was filtered and the first portion of the filtrate was rejected. Then further dilutions with the same solvent were made to obtain the working standards used for calibration curve.

General procedure

Volumes of 1.0 mL aliquots of sample or standard solution were transferred into a screw capped test tube, 1.0 mL of sodium acetate buffer (0.1 M, pH 4.5), acetylacetone (10%, v/v), and formaldehyde (20%, v/v) were added and the reaction was allowed to stand for about 50 min in a water bath previously heated to 100^{O}C. After cooling, the reaction mixture was transferred quantitatively into10-mL calibrated flasks for each drug under study and then the solution was completed to the mark with distilled water. The fluorescence intensity of the resultant solution was measured at emission wavelength (λ_{em}) of 482 nm after excitation (λ_{ex}) at 415 nm against reagent blank prepared and treated similarly.

RESULTS AND DISCUSSION

Hantzsch reaction is a known condensation reaction which is reported in the literatures as a useful pathway for pyrrole, dihydropyridine and pyridine synthesis (Derker and David, 1979, 2001). Furthermore the reaction was applied for the determination of certain sulpha-drug (Amin and Zareh, 1996), different antibiotics (Gupta *et al.*, 1983), benazepril hydrochloride (El-Yazbi *et al.*, 1999), tranexamic acid (El-Aroud *et al.*, 2007), benoxinate hydrochloride (Al-Farhan and Khalil, 2011), and antihypertensive amlodipine besylate (Ayad *et al.*, 2012) and ant-psychic pregabalin (Hanaa *et al.*, 2014). The selected cephalosporins, being primary amines derivatives, exhibit very

low native fluorescence intensity; consequently, a poor sensitivity could be achieved by the direct spectrofluorimetry. Therefore a derivatized reaction via Hantzsch reaction is required to improve their sensitivity and their selective determination in presence of other ingredients associated with dosage forms. The fluorescence intensity of the product formed is illustrated in **Figure 1**.

Influence of reaction variables

All studied parameters affecting fluorescence intensity are carefully studied using general procedure described in 2.4., where the parameter under study is varied while others are kept constant.

Effect of heating temperature and time

The reaction was carried out at 100^{O}C for different periods of intervals starting from 30 to 70 min. This temperature was selected based on previous reports (Hanaa *et al.*, 2014; Ayad *et al.*, 2012) and was further checked for cefepime as a representative example. Data of such study are summarized in **Figure 2 a and b**. The best results were observed at heating time for about 50 min and the fluorescence intensity of the product formed was found to be stable for more than one hour.

Effect of reagents concentration

Different volumes of either acetylacetone or formaldehyde solutions were investigated. As shown in **Figure 2. c and d**, maximum fluorescence intensities were observed upon using 1 mL from either acetylacetone (10%, v/v) or formaldehyde (20%, v/v) solutions.

Effect of pH value and volume

Volume of one-mL of different sodium acetate buffer (described in2.3.4.) in a pH range 3.5-5.5 were tried. With all the studied drugs, best results were observed at pH 4.5. Next the effect of different volume of the selected pH value was examined; maximum fluorescence intensities were observed upon using 1 mL from acetate buffer pH 4.5, **Figure 2 e and f**.

Effect of diluting solvent

Different diluting solvents were studied such as water, ethanol, methanol, acetonitrile, and acetone. With all the studied drugs, maximum fluorescence intensity was achieved using distilled water as solvent for the reaction. The results of such study can be explained on the fact that distilled water being the most polar solvent used. Distilled water establishes inter-molecular hydrogen bonds, which in turn stabilizes the excited state and production of rigid structured associated molecules. This finding is confirmed by the high intensity of the fluorescence observed and high stokes shift value indicated by the longer emission wavelength.

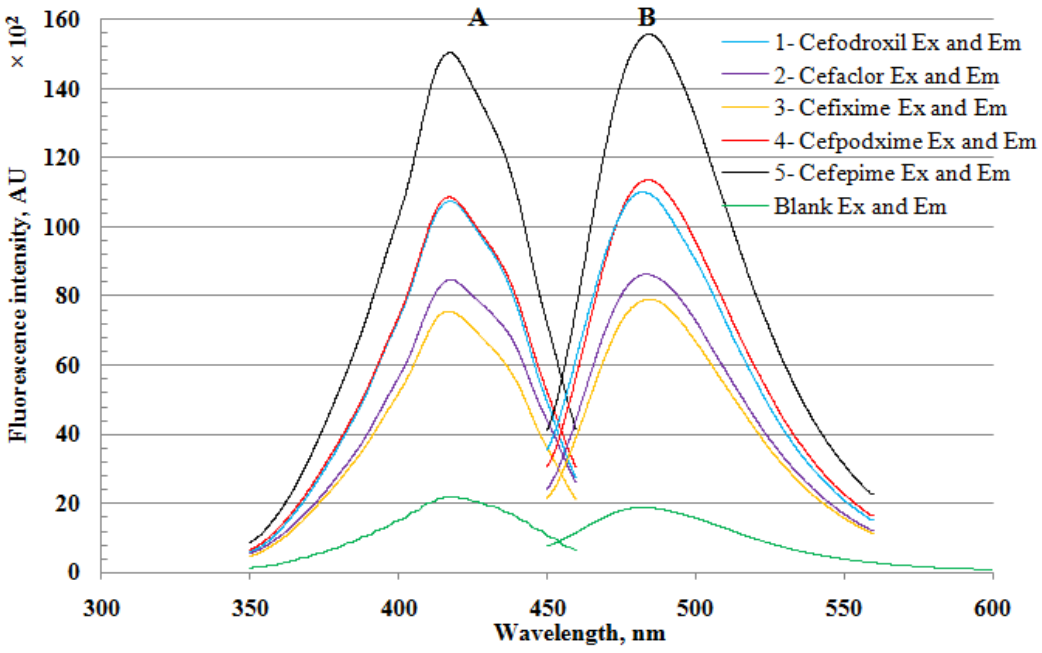


Fig. 1: Excitation and emission spectra, A and B respectively, of reaction products between selected cephalosporins (50 µg/mL) with 1.0 mL of acetylacetone (10%, v/v) and formaldehyde (20%, v/v) reagents against reagent blank prepared and treated similary.

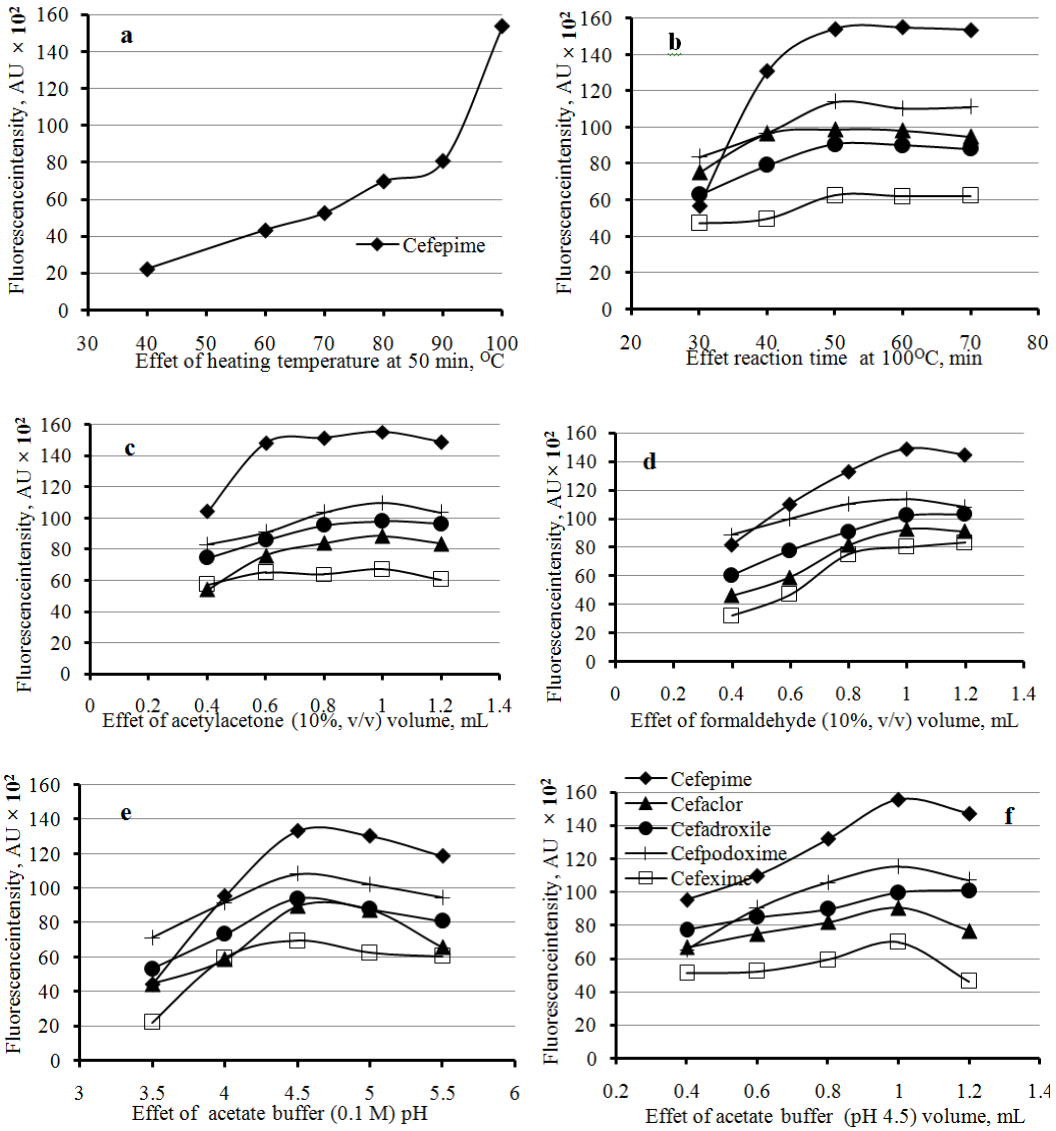
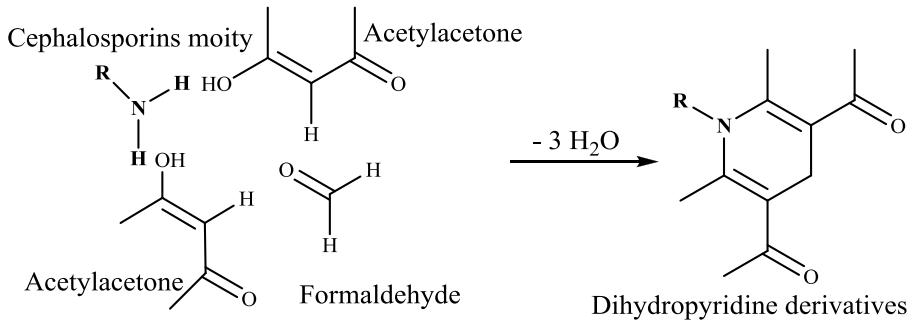


Fig. 2 a-f: The influence of reaction variables on the fluorescence intensity using 1 mLof the selected cephalosporins (50 µg/mL), and different volumes of acetylacetone (10%, v/v), and formaldehyde (20%, v/v) reagents, different pH values as well as different heating time intervals.



Scheme 1: Hantzsch reaction pathwaybetween amine containing cephalosporins, acetylacetone, and formaldehyde reagents

Reported reaction pathway

The studied cephalosporins contain a primary amino group, which could be reacted with acetylacetone and formaldehyde though Hantzsch reaction. The reaction product exhibited strong fluorescence at 482 nm after excitation at 415 nm, Figure 3. A reported (Bartos and Pesez) mechanism pathway was presented in **Scheme 1**.

Table 2: Quantitative parameters for analysis of cephalosporins by the proposed method.

Drug	Concentration, µg/mL	[1]*r*	Regression equation	[2]LOD µg/mL	[3]LOQ µg/mL
Cefadroxil	1-100	0.991	Y = 190x + 70	0.31	0.93
Cefaclor	2-100	0.995	Y = 130x + 83	0.55	1.50
Cefixime	3-50	0.991	Y = 100x – 45	0.82	2.50
Cefpodoxime	1-50	0.995	Y = 150x + 34	0.27	0.82
Cefepime	1-50	0.992	Y = 220x + 90	0.24	0.72

[1]*r*: Correlation coefficient; [2]LOD: Limit of detection; [3]LOQ: Limit of quantitation.

Table 3: Accuracy and precision data of the proposed method.

Drug	Concentration, µg/mL	[1]Accuracy %, ± [2]SD, n=5	Precision as [3]RSD%, n=5	
			Intra-day	Inter-day
Cefadroxil	50	99.65 ± 0.41	0.41	1.82
Cefaclor	50	98.82 ± 0.60	0.61	1.88
Cefixime	50	99.78 ± 0.53	0.53	1.51
Cefpodoxime	50	98.04 ± 0.69	0.70	1.95
Cefepime	50	99.54 ± 0.51	0.51	1.61

[1]Accuracy % = (Found concentration/nominal concentration) × 100; [2]SD; standard division; [3]RSD: relative standard division.

Validation of the developed method

The developed method was validated according to ICH (2005) and USP (Washington, 2008) guidelines to assess the linearity, sensitivity, accuracy, and precision:

Linearity, effective range and selectivity

After study the optimum conditions, series of standard solutions were analyzed by the proposed method and the fluorescence intensity were measured. The calibration plots which represent the relationship between fluorescence intensities and corresponding concentration of the drugs (µg/mL) are constructed. Statistical analysis results of the obtained data by linear least-square method are summarized in **Table 2.** The calculated parameters include; the slopes, intercepts and correlation coefficients (*r*). The plots (n = 5) were linear with acceptable slopes, intercepts and *r*. The limits of detection (LOD) and limits of quantitation (LOQ) were determined using the formula: LOD or LOQ = jS.D.a/b, where j = 3.3 or 10 for LOD and LOQ respectively, S.D.a is the standard deviation of the blank, and b is the slope of the regression line. The LOD and LOQ values were in the range 0.24 - 0.82 and 0.72 - 2.50 µg/mL, respectively as showed in **Table 2.**

Accuracy and precision

Accuracy was assessed with five replicate measurements at one concentration level (Washington, 2008). Results of recovery studies with pure cephalosporins by proposed methods show small values of standard deviation that indicates low scattering of the points around the calibration line **Table 3**. Furthermore, the studies for the validity of the proposed method were checked by applying a standard addition procedure. This procedurewas achieved by adding known amounts of standard to a known concentration of the commercial dosage forms, and then checks the total obtained concentration. The results were expressed as the percentage recovery of the amount added.

The data obtained revealed that an acceptable accuracy was observed for the proposed method and there is no interference from the frequently encountered tablet excipients, **Table 4**.

The proposed method's precision was determined by carrying out replicate analysis of five separated solutions of the working standards at 50 µg/mL. The relative standard deviations (RSD) of the results did not exceed 2%, for intra- and inter-day precision indicating an acceptable repeatability for the developed method, **Table 3**.

Application of the proposed method to analysis of the dosage forms

The proposed method was successfully applied to determine the selected drugs in their dosage forms. The results obtained were statistically compared to the reported method (Saleh *et al.*, 2003) using the student's *t*-test for accuracy and the variance ratio F-test for precision as recorded in **Table 5**. The experimental values of *t* and F did not exceed the theoretical values, revealed the absence of significant difference between the compared methods.

Robustness of the proposed method

The ICH and USP guidelines recommend the evaluation of robustness of the proposed method during the development phase by testing the method parameters susceptible to changes. This study was done by changing the parameters under study while the other parameters remained constant. The involved parameters were the volumes of reagents and buffer used (1 ± 0.1 mL), reaction time (50 ± 5 min).

The observed results compared to control experiments for all the studied drugs proved that there are no significance differences between the control(s) and those experiments carried out after variation of the selected experimental parameters for all drugs studied. Thus the results of such study proved the capacity of the proposed method to remain unaffected by small and deliberate variations in methods parameters.

Table 4: Results of standard addition procedure using the proposed method.

Dosage form	Recovery % ±SD, n=5			
	No variation	50% added	100% added	150% added
Curafep 1g vials	99.65 ± 0.77	95.21 ± 0.10	84.85 ± 0.58	81.67 ± 0.73
Orelox 0.1 g tablet	101.22 ± 0.62	109.54 ± 0.55	99.61 ± 0.74	93.04 ± 0.69
Duricef 0.5 g capsules	99.96 ± 0.82	99.38 ± 0.82	99.86 ± 0.28	98.81 ± 0.66
Duricef 0.5 g suspensions	99.83 ± 0.58	99.97 ± 0.85	100.10 ± 0.69	99.76 ± 0.31
Ceclor 0.125 g suspensions	99.41± 0.24	99.33 ± 0.17	104.22 ± 0.46	94.78 ± 0.55
Ximacef 0.1 g suspensions	97.55 ± 0.10	99.46 ± 0.92	98.27 ± 0.53	99.47 ± 0.42
Ximacef 0.4 g capsules	99.11± 0.57	100.91 ± 0.63	99.34 ± 0.82	99.47 ± 0.11

Table 5: Analysis of certain commercial available pharmaceutical formulations containing cephalosporin using the proposed and reported method.

Dosage forms	Recovery % ± SD (n = 5)		*t*-test*	F value**
	Proposed method	Reported method		
Curafep 1gm vial	99.27 ± 0.40	99.52 ± 0.11	1.41	2.51
Ceclor 125 mg suspension	99.20 ± 0.20	98.51 ± 0.13	1.60	1.59
Duricef 500 mg capsule	99.19 ± 0.84	100.52 ± 0.66	1.47	4.29
Duricef 500 mg suspension	99.15 ± 0.54	99.74 ± 0.11	1.84	1.76
Ximacef 100 mg suspension	99.11 ± 0.50	98.61 ± 0.19	1.14	1.01
Ximacef 400 mg capsule	99.92 ± 0.48	99.24 ± 0.13	1.18	1.28
Orelox 100 mg tablet	99.48 ± 0.42	99.22 ± 0.11	1.35	1.76

*& **Theoretical values for *t*-test and F value at 95% confidence limit (n = 5) were 6.39 and2.78respectively.

CONCLUSION

A validated, sensitive, economic, reliable, and selective method was developed for determination of some selected cephalosporins in bulk and/or in the marketed dosage forms. The methods have been validated in terms of its accuracy, precision, and reliability as well as robustness suggesting its suitability for the routine analysis. Furthermore, the applicability of the developed method has been verified by analyzing different dosage forms, where no interference was observed by these common additives which are co-formulated with drugs as evidenced by the good recoveries obtained.

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