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Spectrofluorimetric Protocol for Ceftriaxone in Commercial Formulation and Human Plasma After Condensation with Formaldehyde and Ethyl Acetoacetate

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Abstract A new spectrofluorimetric method has been developed and validated for the quantification of ceftriaxone in bulk powder, pharmaceutical formulations and spiked human plasma. The developed method is reproducible, accurate, sensitive and cost effective. In this method, ceftriaxone was converted into a fluorescent compound by reacting with 0.8 M ethyl acetoacetate and 25% formaldehyde in a buffered medium (pH=4.2) at 90 °C. The excitation and emission wavelengths of the fluorescent reaction product are 316 nm and 388 nm respectively. Optimization of the experimental conditions affecting the condensation reaction were carefully carried out and the optimum experimental conditions were incorporated in the procedure. The developed method has a broad linear range (0.2–20 μg mL^{-1}) with a correlation coefficient of 0.9992. The limit of detection (LOD) and limit of quantification (LOQ) was found to be 1.94×10^{-2} μg mL^{-1} and 6.47×10^{-2} μg mL^{-1} respectively. The common excipients and co-administered drugs were investigated for their interferences effect in the assay. The developed method was validated statistically through recovery studies and successfully applied to ceftriaxone determination in bulk powder, pharmaceutical formulations and spiked human plasma samples. The percent recoveries were found to be in the range of 99.04–100.26% for bulk powder, 98.88–99.92% for pharmaceutical formulations and 94.22–98.48% for spiked human plasma. The results were verified by comparing with reference literature HPLC method and were found in good agreement.

Keywords Ethyl acetoacetate · Formaldehyde · Spectrofluorimetric · Ceftriaxone · Spiked human plasma

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Introduction

The first and second generation cephalosporins are unsuccessful in the treatment of meningitis because they are unable to penetrate the central nervous system. However the third generation cephalosporin not only crosses the blood brain barrier but reaches the therapeutic concentration that are sufficient for the treatment of meningitis caused by aerobic gram-negative bacteria [1].

Ceftriaxone, (6R, 7R)-7-[[(2E)-2-(2-amino-1, 3-thiazol-4-yl)-2-methoxyimino-acetyl] amino]-3-[(2-methyl-5, 6-dioxo-1H-1, 2, 4-triazin-3yl) sulfanylmethyl]-8-oxo-5-thia-1-azabicyclo [4.2.0] oct-2-ene-2-carboxylic acid is third generation semisynthetic cephalosporin antibiotic. It is effective against a wide variety of gram positive and gram negative bacteria including Enterobacteriaceae, H. influenza, N. meningitides, N. gonorrohoeae, and Streptococcuspygenes. It has promising activity against strains of Pseudomonas aeruginosa. [2–4]. It inhibits the growth of microbes by hindering the mucopeptide synthesis in bacterial cell wall [5–7]. 90% decrease in growth was shown by ceftriaxone concentration as low as 0.03 μg mL^{-1} for Escherichia coli, 0.06 μg mL^{-1} for Salmonella spp. and 0.2 μg mL^{-1} for Pateurellamultocida in their isolates from infected Israeli-Friesian male calve [8].

After administering one gram dose of ceftriaxone by intramuscular injection and intravenous infusion, mean peak

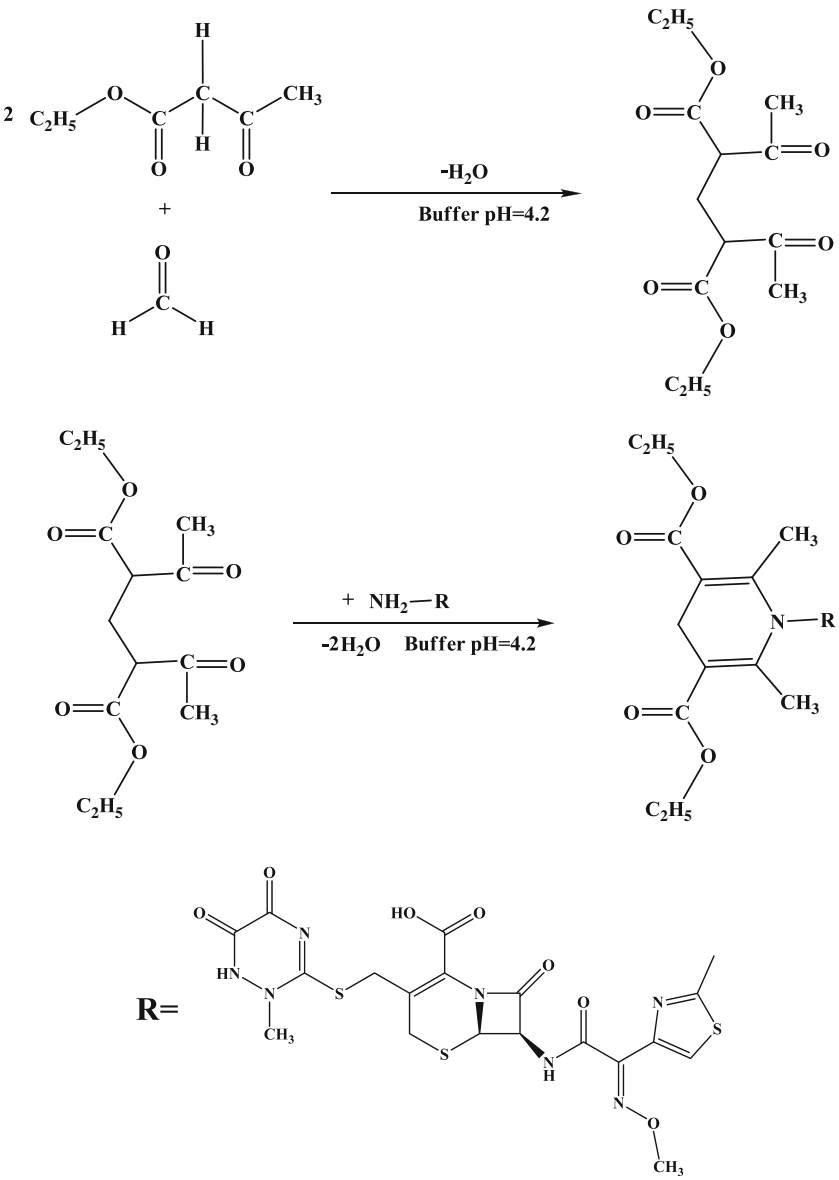
plasma concentration of 79.2 μg mL^{-1} and 123.2 μg mL^{-1}, respectively was reached. Due to prolonged terminal half-life (5.4–8.2 h), ceftriaxone is prescribed on a single administration per day basis in humans [9–13].

The development of a simple, fast and economic method to quantify ceftriaxone in commercial formulations and biological samples is highly desirable. A number of analytical methods have been reported in the literature for quantification of ceftriaxone in pharmaceutical formulations and biological samples. These include HPLC [5, 14–20], ion pair liquid chromatography [21], ion exchange chromatography [22], capillary electrophoresis [23], differential pulse adsorptive stripping polarography [24], chemiluminescence [25] and spectrophotometric [26–29] methods.

Determination of ceftriaxone in biological fluids has been carried out by micro-biological assays [30]. However, these methods are time consuming; require many hours incubation time and also not chemically specific.

Relatively limited number of spectrofluorimetric methods has been described in the literature for the analysis of ceftriaxone in pharmaceutical formulations and biological samples. These methods involve multistep procedures and long heating times, possess narrow linear ranges, utilize expensive reagents and suffer from limitation of application to biological samples [27, 31, 32].

The present work is an attempt to develop a simple, sensitive, inexpensive and efficient spectrofluorimetric method for the quantification of ceftriaxone pharmaceutical formulations and biological samples. The proposed method



Scheme 1 Proposed reaction mechanism for condensation of ceftriaxone with ethyl acetoacetate and formaldehyde

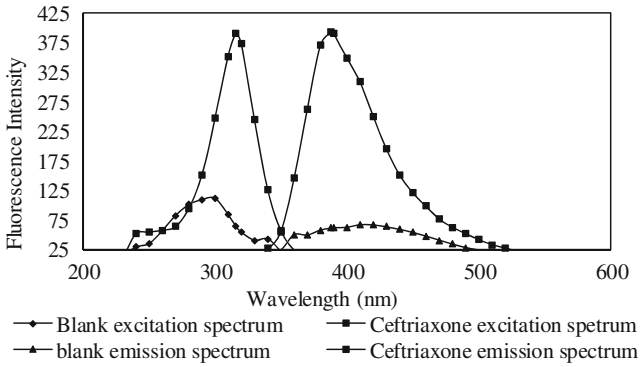


Fig. 1 Excitation and emission spectrum of ceftriaxone after condensation reaction. Reaction condition: 1 mL ceftriaxone (500 μg.mL^{-1}), 1 mL of 37% formaldehyde, 1 mL pH 4 acetate buffer, 1 mL of 1 M ethyl acetoacetate, 100 °C for 15 min, diluted to 25 mL

employs the ethyl acetoacetate and formaldehyde as fluorogenic agent which undergo Hantsch's condensation reaction involving the primary amine group of the target compound.

Experimental

Instruments

RF-5301 PC Spectrofluorophotometer Shimadzu Japan, equipped with 150-watt Xenon discharge lamp, excitation, emission grating monochromators and 1×1 cm quartz cell, was used for measurement of fluorescence intensities. The instrument was operated with excitation and emission slit width set at 5 nm. An electrical thermostatic water bath (YuJia China) with temperature range of 37 °C–100 °C was used for heating purpose.

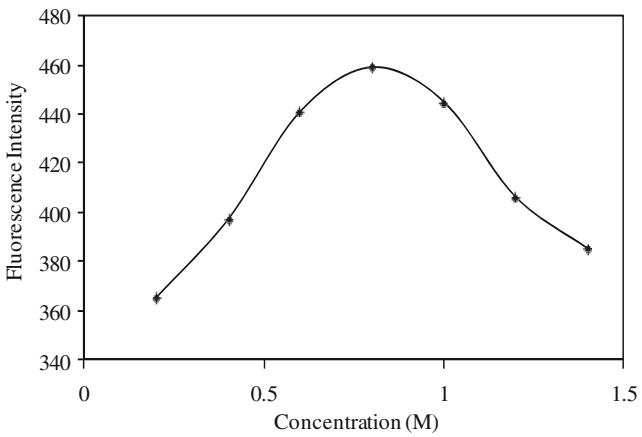


Fig. 2 Effect of concentration of ethyl acetoacetate on condensation reaction. Reaction condition: 1 mL ceftriaxone (500 μg.mL^{-1}), 1 mL of 37% formaldehyde, 1 mL pH 4 acetate buffer, 1 mL of 0.2–1.4 M ethyl acetoacetate, 100 °C for 15 min, diluted to 25 mL

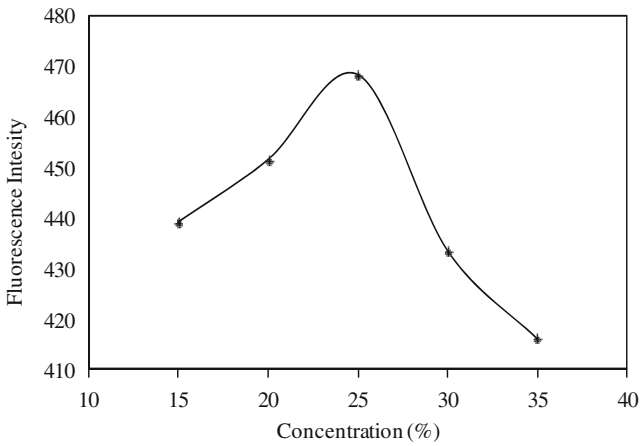


Fig. 3 Effect of concentration of formaldehyde on condensation reaction. Reaction condition: 1 mL ceftriaxone (500 μg.mL^{-1}), 1 mL of 5–35% formaldehyde, 1 mL pH 4 acetate buffer, 1 mL of 0.8 M ethyl acetoacetate, 100 °C for 15 min, diluted to 25 mL

Materials and Reagents

All reagents were of analytical grade purity or high grade purity. Ethyl acetoacetate (Merck Hohenbrunn Germany), formaldehyde 37% (Merck KGaA 64271 Darmstadt, Germany), glacial acetic acid (Merck KGaA 64271 Darmstadt, Germany), sodium acetate trihydrate (Merck KGaA 64271 Darmstadt, Germany), were used in this work. Standard reference ceftriaxone was provided by Cirin Pharmaceutical (Pvt) Ltd., Hattar, Pakistan. Commercial formulations of ceftriaxone (injection Rocephin 250 mg, (manufactured by F. Hoffmann-La Roche ltd, Basel Switzerland), injection Cefexone 250 mg, (manufactured by Bosch Pharmaceuticals (Pvt) Ltd Pakistan) and injection Aventriax 250 mg, (manufactured by Sanofi-Aventis Pakistan limited.) were purchased from local market.

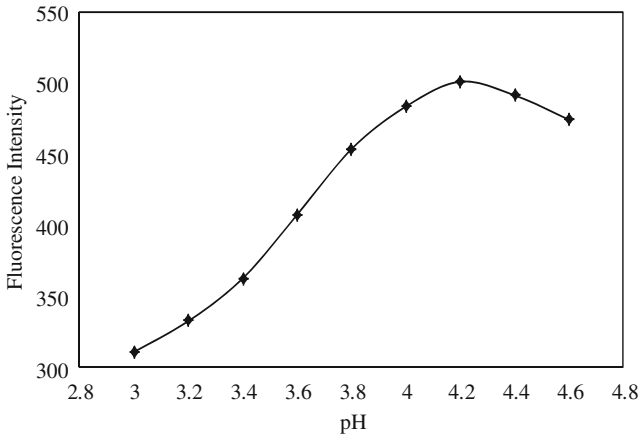


Fig. 4 Effect of pH of buffer on condensation reaction. Reaction condition: 1 mL ceftriaxone (500 μg.mL^{-1}), 1 mL of 25% formaldehyde, pH varied from 3–4.6, 1 mL of 0.8 M ethyl acetoacetate, 100 °C for 15 min, diluted to 25 mL

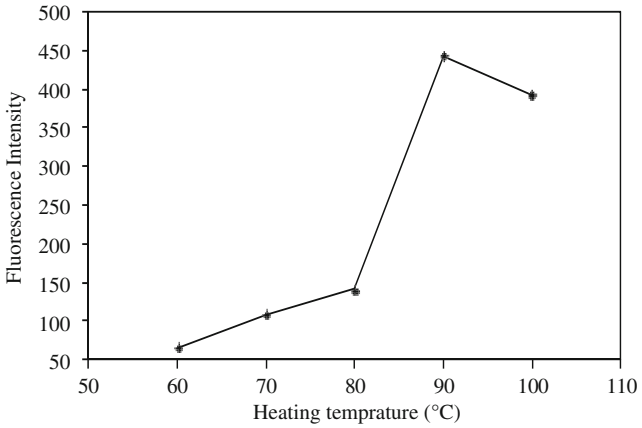


Fig. 5 Effect heating temperature on condensation reaction. Reaction condition: 1 mL ceftriaxone (500 μg.mL^{-1}), 1 mL of 25% formaldehyde, 1 mL pH 4.2 acetate buffer, 1 mL of 0.8 M ethyl acetoacetate, 60–100 °C for 15 min, diluted to 25 mL

Preparation of Reagent Solutions

Ethyl acetoacetate solution (0.8 M) was prepared by diluting 10 mL of the reagent to 100 mL with ethanol. Formaldehyde solution (25%) was prepared by diluting 67.7 mL of 37% to 100 mL with distilled water. Acetic acid-sodium acetate buffer (pH=4.2) was prepared by mixing 0.2 M sodium acetate solution with 0.2 M acetic acid solution and adjusting the pH to 4.2 using pH meter.

Preparation of Standard Solution (250 μg mL^{-1})

Standard stock solution of ceftriaxone (250 μg mL^{-1}) was prepared on daily basis by dissolving 0.0125 g of the authentic standard in distilled water and diluting to 50 mL with distilled water. Working standards were prepared by diluting appropriate volume of the stock solution.

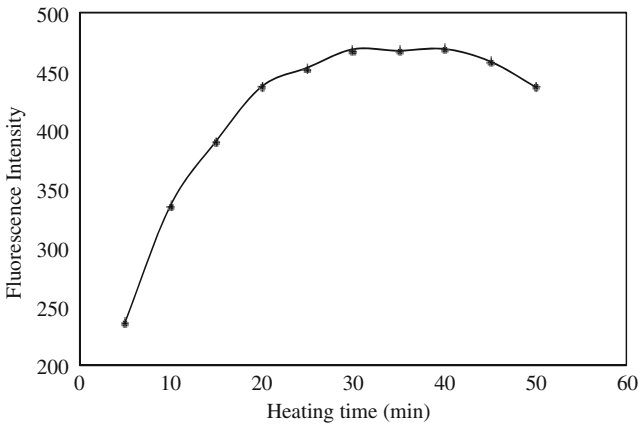


Fig. 6 Effect of heating time on condensation reaction. Reaction condition: 1 mL ceftriaxone (500 μg.mL^{-1}), 1 mL of 25% formaldehyde, 1 mL pH 4.2 acetate buffer, 1 mL of 0.8 M ethyl acetoacetate, 90 °C for 5–125 min, diluted to 25 mL

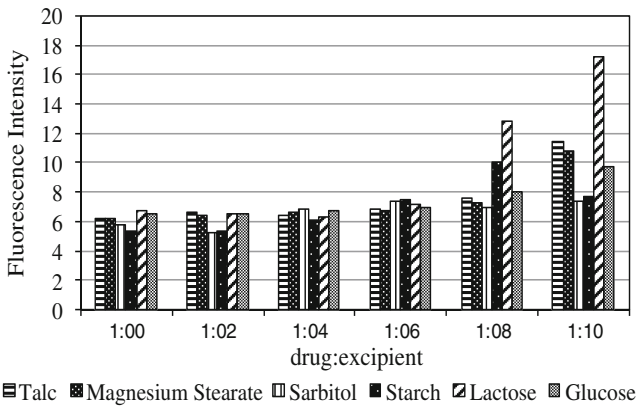


Fig. 7 Effect of excipients on the fluorescence intensity of the fluorescent condensation product

General Procedure for Preparation of Calibration Curve

Appropriate volumes of the stock solution of ceftriaxone, to give final concentration of 0.2–20 μg mL^{-1}, were taken in 20 mL test tubes. 1 mL of 25% formaldehyde and 2 mL of pH 4.2 buffer was added to each test tube. The mixture was gently shaken followed by the addition of 1 mL of ethyl acetoacetate. The components of mixture were, again, gently shaken and heated on a water bath adjusted at 90 °C for 30 min. The contents of the test tubes were cooled by immersing in tap water and then transferred to 25 mL volumetric flasks and diluted to the

Table 1 Percent recoveries of Ceftriaxone (0.2 μg mL^{-1}) in the presence of excipients

Excipients	Excipients added (mg/L)	Drug: Excipient	%Recovery±RSD
Talc	0.4	1:02	99.69±3.96%
	0.8	1:04	100.11±4.98%
	1.2	1:06	100.06±3.28%
Magnesium stearate	0.4	1:02	101.21±2.04%
	0.8	1:04	98.72±2.97%
	1.2	1:06	99.68±3.32%
Sorbitol	0.4	1:02	99.16±2.49%
	0.8	1:04	101.12±1.53%
	1.2	1:06	100.61±2.79%
Starch	0.4	1:02	98.69±2.33%
	0.8	1:04	99.93±1.40%
	1.2	1:06	99.86±3.34%
Lactose	0.4	1:02	100.55±2.34%
	0.8	1:04	100.58±1.55%
	1.2	1:06	100.13±0.44%
Glucose	0.4	1:02	100.3±2.67%
	0.8	1:04	100.23±2.38%
	1.2	1:06	100.3±2.43%

Each result is the average of separate triplicate analysis

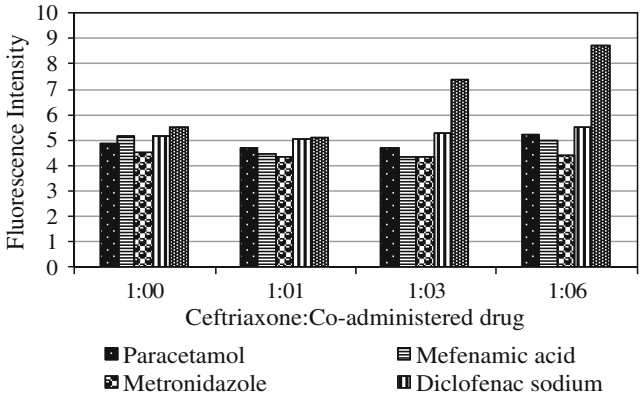


Fig. 8 Interferences effect of Co-administered drugs on the determination of ceftriaxone by the proposed method

mark with distilled water. The fluorescent intensity was measured at λem 388 nm using λex 316 nm against a reagent blank.

Application to Pharmaceutical Formulations

Contents of five vial containing 250 mg active ingredients were mixed, weighed and average mass of the powder in one vial was calculated. The sample of the drug powder claimed to contain 0.0125 g of the active ingredient were dissolved in distilled water and diluted to 50 mL with distilled water. 10 μg mL^{-1} sample solution was prepared from stock sample solution by dilution of the required volume with distilled water. Suitable volume of this solution was analyzed by the procedure described for the preparation of calibration curve

Application to Spiked Human Plasma Sample

5 mL plasma was taken in a centrifuge tube and 250 μg of the drug was added to it. It was then deproteinized by mixing with 15 mL of acetonitrile and centrifuged for 5 min at a rate of 3500 rpm. The supernatant was transferred to 50 mL volumetric flask and diluted with distilled water up

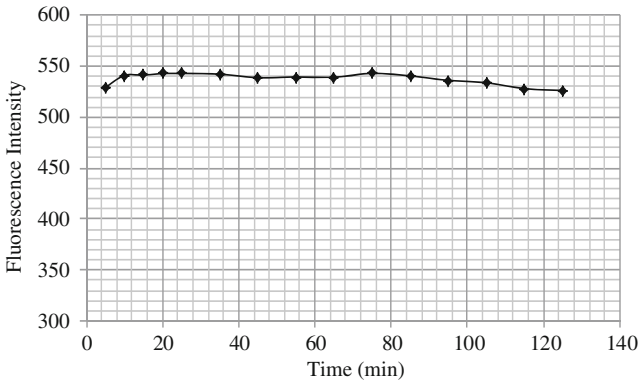


Fig. 9 Effect of time on the stability of fluorescent product. Reaction condition: 1 mL ceftriaxone (500 μg.mL^{-1}), 1 mL of 25% formaldehyde, 1 mL pH 4.2 acetate buffer, 1 mL of 0.8 M ethyl acetoacetate, 90 °C for 30 min, diluted to 25 mL

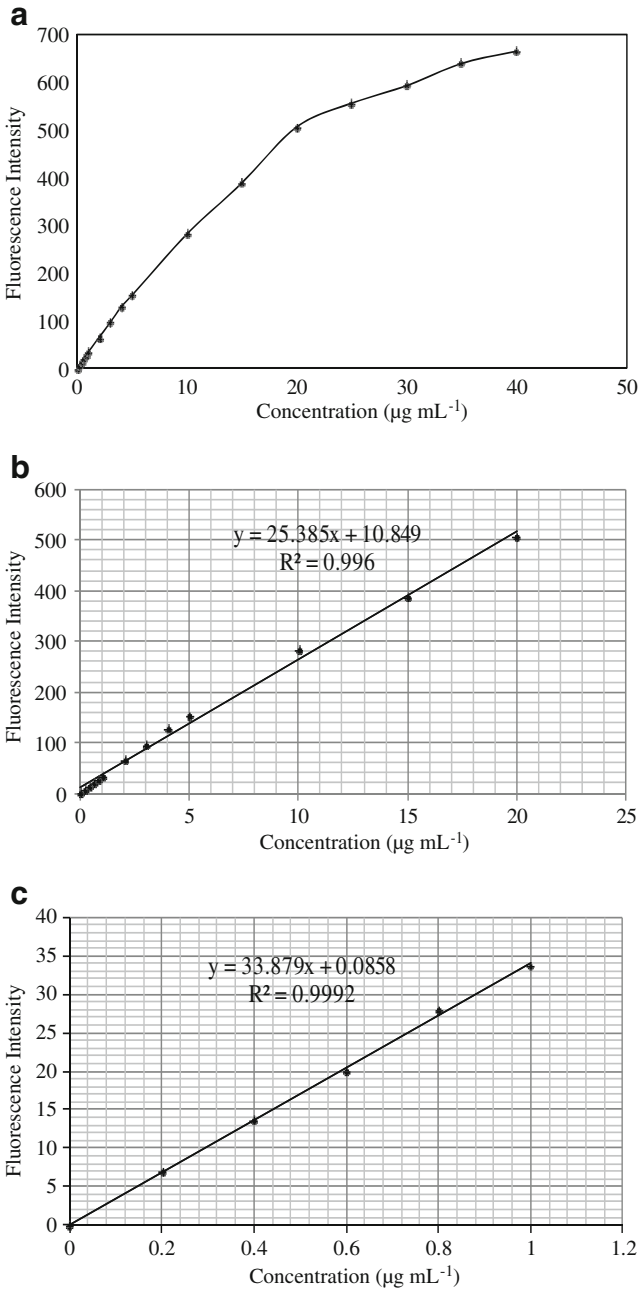


Fig. 10 **a** Effect of concentration of ceftriaxone on fluorescence intensity. Reaction condition: Ceftriaxone (0.2–40 μg.mL^{-1}), 1 mL of 25% formaldehyde, 1 mL pH 4.2 acetate buffer, 1 mL of 0.8 M ethyl acetoacetate, 90 °C for 30 min. **b** Linear range of the fluorescent product of ceftriaxone with ethyl acetoacetate-formaldehyde. Reaction condition: ceftriaxone (0.2–20 μg.mL^{-1}), 1 mL of 25% formaldehyde, 1 mL pH 4.2 acetate buffer, 1 mL of 0.8 M ethyl acetoacetate, 90 °C for 30 min. **c** Calibration curve of the fluorescent product of ceftriaxone with ethyl acetoacetate-formaldehyde. Reaction condition: ceftriaxone (0.2–1 μg.mL^{-1}), 1 mL of 25% formaldehyde, 1 mL pH 4.2 acetate buffer, 1 mL of 0.8 M ethyl acetoacetate, 90 °C for 30 min

Table 2 Analytical parameter for spectrofluorimetric determination of ceftriaxone

Parameter	Value
λ_{ex}(nm)	316
λ_{em} (nm)	388
Linear range (μgmL^{-1})	0.2–20
Limit of detection 3S/b (μg mL^{-1})	1.941×10^{-2}
Limit of quantification 10S/b (μg mL^{-1})	6.47×10^{-2}
Regression equation (y)	Y=33.879X+0.0858
Slope (b)	33.879
Intercept (a)	0.0858
Correlation coefficient (r)	0.9992
Standard deviation (μg mL^{-1})	6.47×10^{-3}
Relative standard deviation (%)	3.18%

to 50 mL. Appropriate volumes of this solution were analyzed by the same procedure as described for the preparation of calibration curve.

Results and Discussion

The ethyl acetoacetate and formaldehyde reacts with amino group of the ceftriaxone in slightly acidic media following the Hantsch's condensation reaction mechanism. In the first step the ethyl acetoacetate reacts with formaldehyde producing diethyl-2, 4-diacetyl pentanedioate. In the second step the diethyl-2, 4-diacetyl pentanedioate undergo condensation with amino group of the ceftriaxone producing fluorescent product (Scheme 1). The fluorescent reaction product showed maximum fluorescence intensity at λem 388 nm when excited at λex 316 nm (Fig. 1).

Effect of Reagent Concentration

The concentration and volume of various reagents affecting the formation of the fluorescent product were carefully studied. The effect of the concentration of ethyl acetoacetate on the condensation reaction was studied in the range of 0.2–1.4 M. It was found that fluorescence intensity increased with increase in concentration up to 0.8 M beyond which the fluorescence intensity decreased due to the formation of water insoluble products (Fig. 2). Then the volume of ethyl acetoacetate was varied keeping the concentration constant. It was found that maximum signal was obtained when 1 mL of 0.8 M of the reagent was used.

The concentration of formaldehyde was varied from 15–35% and its effect on the fluorescent product formation was monitored. An increase in fluorescence intensity was observed up to 25% of formaldehyde concentration after which decrease occurred in the fluorescence intensity mainly due to formation of water insoluble byproducts (Fig. 3). The volume of the formaldehyde was also optimized and 1 mL of the reagent was found sufficient for maximum fluorophore formation.

The reaction was found to be highly pH dependent and the effect of pH on fluorescence intensity was studied in the range of 3.0–4.6. The fluorescence signal increased with

Table 3 Evaluation of accuracy and precision of the proposed method for ceftriaxone determination in pure form

μg taken	μg found	%Recovery±RSD	Confidence limit
0.2	0.2005	100.27±3.62%	100.26±4.14
0.4	0.3962	99.05±2.13%	99.04±5.72
0.6	0.5979	99.32±3.57%	99.65±5.77
X′		99.54%	
±SD		0.645	
t-test		1.23(4.303)	

Each result is the average of separate nine replicate analyses

Table 4 Evaluation of accuracy and precision of the proposed method for ceftriaxone determination in dosage form

Pharmaceutical preparation	Amount taken (μg mL^{-1})	Amount found (μg mL^{-1})	Recovery± RSD
Inj. Cefexone 250 mg	0.2	0.1998	99.92±3.42%
	0.4	0.3976	99.41±2.40%
	0.6	0.5979	99.66±1.04%
Inj. Aventriax 250 mg	0.2	0.1991	99.53±4.77%
	0.4	0.3997	99.92±3.05%
	0.6	0.5981	99.69±0.98%
Inj. Rocephin 250 mg	0.2	0.1990	99.52±6.46%
	0.4	0.3997	99.92±5.72%
	0.6	0.5933	98.88±1.81%

Each result is the average of separate triplicate analysis

Table 5 Evaluation of recovery test of ceftriaxone in injection by the proposed method (Standard addition method)

Pharmaceutical preparation	Amount added (μg mL^{-1})	Amount found (μg mL^{-1})	%Recovery±% RSD
Inj. Cefexone 250 mg	0.2019	0.2	99.05±1.05%
	0.3906	0.3892	99.65±2.84%
	0.5968	0.5896	98.79±1.95%
Inj. Aventriax 250 mg	0.1974	0.1965	99.54±3.087%
	0.4013	0.404	100.66±3.04%
	0.6009	0.6048	100.65±1.63%
Inj. Rocephin 250 mg	0.2023	0.2032	100.38±3.6%
	0.3967	0.4003	100.9±3.99%
	0.5900	0.5944	100.74±1.66%

Each result is the average of separate triplicate analysis

Table 6 Determination of ceftriaxone in commercial formulation and statistical comparison with reference method

S.NO	Name of commercial formulation	Labeled amount	Amount determined	
			Proposed method	Reference method
1	Inj. Cefexone 250 mg	250 mg/inj.	249.15 mg/inj.	–
				–
				–
2	Inj. Aventriax 250 mg	250 mg/inj.	249.27 mg/inj.	248.9 mg/inj.
				F-test=16.81(19)
				t-test=0.306 (4.303)
3	Inj. Rocephen 250 mg	250 mg/inj.	249.4 mg/inj.	249.7 mg/inj.
				F-test=2.36 (19)
				t-test=0.79 (4.303)

Each result is the average of separate triplicate analysis

increase in pH up to 4.2 after which slight decrease was observed in the signal (Fig. 4). The effect of volume of the buffer was also investigated and it was found that 2 mL of pH 4.2 buffer produced maximum fluorescence signal.

Effect of Temperature and Heating Time

The effect temperature (60–100 °C) and heating time (5–50 min) on the fluorophore formation was investigated and it was found that maximum fluorophore formation occurred when the reaction mixture was heated at 90 °C for 30 min (Figs. 5 and 6).

Effect of Interferences

The interferences from the common excipients like talc, magnesium stearate, sorbitol, starch, lactose and glucose in the drug assay were carefully investigated (Fig. 7). Solutions of synthetic mixtures containing the analyte drug and one of the excipients in ratio of 1:2, 1:4, 1:6 were analyzed by the proposed method. No interferences were observed in the determination of ceftriaxone in the presence of these common excipients. Average recoveries obtained were in the range of 98.61% to 100.68% (Table 1). The common co-administered drugs like paracetamol, mefanamic acid, metronidazole and ibuprofen and diclofenac sodium were also studied for their interference effect in the analysis. It was found that these co-administered drugs did not interfere in the determination of ceftriaxone by proposed method (Fig. 8).

Stability of the Fluorescent Reaction Product

The stability of the fluorescent reaction product was investigated by measuring the fluorescence intensity at regular interval up to 125 min (Fig. 9). It was found the no significant change occurred in the fluorescence intensity of the reaction product. Thus the fluorescent reaction product is stable and will not affect the result of analysis even if the fluorescence intensity is measured after 2 h of the dilution.

Analytical Figures of Merit

Under optimum experimental conditions, a linear relationship between concentration of ceftriaxone and fluorescence intensity was observed in the range of 0.2–20 μg mL^{-1}, with a good correlation coefficient of 0.995–0.9992 (Fig. 10a, b, c). The limit of detection (LOD) was calculated with concentration of the ceftriaxone leading to fluorescence intensity which is three times the blank standard deviation (3S/b). The limit of quantification (LOQ) was similarly calculated with concentration of the analyte leading to fluorescence intensity which is ten times the blank standard deviation (10S/b). The LOD and LOQ values were found to be 1.941×10^{-2} μg mL^{-1} and 6.47×10^{-2} μg mL^{-1} respectively. The linear regression equation, slope, intercept, correlation coefficient, and relative standard deviation of the response factor are given in Table 2.

Table 7 Determination of ceftriaxone in spiked plasma sample

μg taken	μg found	% Recovery±RSD
0.2	0.2076	103.8±5.31
0.4	0.4083	102.075±5.24
0.6	0.6148	102.47±2.06

Table 8 % Recovery test of ceftriaxone from plasma (standard addition method)

sample	μg added	μg found	% Recovery±RSD
Plasma	0.2024	0.1907	94.22±2.76%
	0.3935	0.3875	98.48±2.86%
	0.5962	0.5828	97.75±2.63%

Precision and Accuracy

The precision of the proposed method was checked by determining ceftriaxone in pure form, pharmaceutical preparations and spiked plasma samples using three different concentrations in triplicate with in the calibration curve range. The results are summarized in Table 3 for pure form and Table 4 for dosage form and Table 7 for spiked plasma samples. The percent recoveries ranged from 99.04% to 100.26% for pure form, 99.41% to 99.92% for injections and 102.075% to 103.8% for spiked plasma samples, with a good relative standard deviation, indicating better reproducibility of the proposed method. The accuracy of the developed method was ascertained by standard addition method using three different brands of injections (Rocephin 250 mg, Aventriax 250 mg and Cefexone 250 mg) and spiked plasma samples. The percent recoveries were in the range of 99.05% to 100.9% for pharmaceutical preparations (Table 5) and 94.22% to 98.48% for spiked plasma samples (Table 8). This shows high accuracy of the developed method for ceftriaxone determination in both pharmaceutical preparations and spiked plasma samples.

Applicability of the Proposed Method

The developed method was successfully applied to determination of ceftriaxone active ingredient in three different brands of injections. The results were compared with a literature HPLC method [33]. The precision and accuracy were ascertained student's t-test and variance ration F-test, respectively, using statistical analysis. The results obtained from both the methods were in good agreement (Table 6). Due to high specificity of the developed method and absence of interference from common excipients and co-administered drugs, it was applied for the quantification of ceftriaxone in spiked human plasma samples. The results are summarized in the Tables 7 and 8.

Conclusion

A simple, sensitive, precise and accurate spectrofluorimetric method has been developed and validated statistically. The method has been applied for the analysis of active ingredient of ceftriaxone in both pure and dosage form. It has also been successfully applied to the quantification of ceftriaxone in spiked human plasma. The HPLC methods used for this purpose are expensive in terms of instrument

(HPLC system is 2 times more expansive than spectrofluorimeter) as well as solvent system (HPLC grade organic solvents are 100 times more expansive than the reagents and solvent used in the proposed method) are required. Moreover, the HPLC technique needs lengthy operation/maintenance (at least 3 h) and skilled personals for its functioning. The wholesome effect of these factors makes the proposed spectrofluorimetric method 100 times more economic than HPLC technique available for this purpose. The spectrofluorimetric methods are also more sensitive than HPLC methods the in many cases. Therefore, the proposed method can be used as alternative to the HPLC methods for the analysis of ceftriaxone by industrial and research institutional laboratories.

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