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Simple chromatographic methods for determination of cefepime in bulk powder and injection dosage form, and in presence of its hydrolytic degradation products

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ABSTRACT

The present work describes development and validation of stability indicating HPTLC-densitometric and HPLC methods for quantitative analysis of cefepime in bulk and injection dosage form. In HPTLC, separation was performed on silica gel 60 F_{254} using diethylether-ethanol-water-glacial acetic acid (5: 3: 2: 0.05, v/v) as a developing system. The compact band of cefepime at R_f 0.14±0.02 was scanned densitometrically at 257nm and calibration curve was constructed in the range of 0.60-8.00 µg/spot using polynomial regression function. The proposed RP-HPLC method utilizes an isocratic elution on C_{18} column with mobile phase consisting of methanol: water (30:70, v/v) at ambient temperature and a flow rate of 1.2 ml/min. The chromatographic run time was less than 5 min. Quantification was achieved with UV detection at 257nm over concentration range of 0.60 to 20.00 µg/ml. Cefepime was subjected to acid and alkaline induced hydrolytic degradation. The methods distinctly separated it from its degradation products, which infers the specificity of assay methods for estimation of cefepime in the presence of its hydrolytic degradation products. Due to simplicity, rapidity and accuracy of the proposed stability indicating methods, they are effective for quality control analysis.

KEYWORDS

Cefepime;
HPTLC;
RP-HPLC;
Injection;
Hydrolysis.

INTRODUCTION

Cefepime, 1-[[(6R,7R)-7-[2-(2-amino-4-thiazyl) glyoxylamido]-2-carboxy-8-oxo-5 thia -1-azabicyclo [4.2.0] oct-2-en-3-yl] methyl]-1-methyl pyrrolidinium chloride, 7-(Z)-(0-methyloxime) monohydrochloride, monohydrate[1]. It is a fourth-generation cephalosporin with a broad antibiotic spectrum and improved activity against gram-negative bacteria over other commercially available cephalosporins[2].

Cefepime contains a β-lactam ring which is very labile to acid and base[3,4] making it very important to develop specific methods for estimation of this drug in presence of possible hydrolytic degradation products.

Detailed survey of literature of Cefepime revealed several methods for its determination in pharmaceutical formulations and biological matrices like, spectrophotometry[5,6], micellar capillary electrokinetic chromatography[7], polarographic technique[8], bioassay[9], HPLC[10-13], and LC/MS/MS methods[14,15]. However,

these analytical methods do not appear to have widespread utility, especially at the industrial level, where simple, cost-effective, and highly specific methods are needed. Therefore, we attempted to develop rapid, sensitive, accurate, and specific HPTLC-densitometric and HPLC methods for determination of cefepime in bulk powder and injection dosage form, and in presence of its acid and alkaline induced hydrolytic degradation products using ultraviolet detection. The results were validated in accordance with International Conference on Harmonization guidelines[16].

EXPERIMENTAL

Apparatus

- HPLC (BIO-TEK) kontron instrument equipped with a model series 422 pump, knauer injector with a 50 µL loop and a 540+ photodiode array detector. Data acquisition was performed on a model kroma system 2000. Disposable membrane filters, 0.45µm, Phonomenex, Nylon. Syringe filters (Gelman, Sigma-aldrich).
- Desaga densitometer model CD 60 (Germany). AS 30 Desaga applicator. Desaga UV lamp with short wavelength (254nm). HPTLC plates precoated with silica gel 60 F_{254} (20.0 × 10.0 cm) from E. Merck, Germany.

Reagents

Cefepime hydrochloride pure sample was obtained from National Organization for Drug Control & Research (NODCAR), Egypt, 99.9%. Maxipime® vial, 500 mg (Bristol-Myers Squibb Co., Cairo, Egypt) (containing 500 mg cefepime). Methanol and water (HPLC grade) were purchased from (Riedel-de Häen, Sigma-Aldrich, Germany). Ethanol, diethylether and glacial acetic acid were purchased from (E. Merck, Darmastadt, Germany); sodium hydroxide and hydrochloric acid (BDH).

Preparation of standard solution of cefepime

For HPTLC, stock standard solution of cefepime (2.0 mg/ml) was prepared by accurately weighing 200.0 mg of cefepime into 100-ml volumetric flask, dissolved in 10.0 ml water and the volume was completed with methanol. For HPLC, stock standard solution of cefepime (1.0 mg/ml) was prepared by accurately weighing 100.0 mg of cefepime into 100-ml volumetric flask, dissolved in 50.0 ml water and the volume was completed with water. Then, working standard solution (100 µg/ml) was prepared by transferring 10.0 ml of the stock standard solution into 100-ml volumetric flask, then volume was completed with the mobile phase.

Preparation of standard solutions of hydrolytic degradation products

- Stock standard solution of acid-degradation products (1.0 mg/ml) was prepared by accurately weighing 50.00 mg of cefepime, dissolving in 20.0 ml 1 M HCl, heating in water-bath at 80°C for 2.5 hrs, then cooling, neutralizing the media with 1 M NaOH (to give pH 7.0 ± 0.2) and then completing volume to 50.0 ml with methanol (for HPTLC) or water (for HPLC).
- Stock standard solution of alkaline-degradation products (1.0 mg/ml) was prepared by accurately weighing 50.00 mg of cefepime, dissolving in 20.0 ml 0.5 M NaOH, heating in water-bath at 80°C for 1.5 hr, then cooling, neutralizing the media with 0.5 M HCl (to give pH 7.0 ± 0.2) and then completing volume to 50.0 ml with methanol (for HPTLC) or water (for HPLC).
- Working standard solutions (100µg/ml) of the acid- and alkaline-degradation products were prepared separately for HPLC by transferring 10.0 ml of their stock standard solutions into100-ml volumetric flasks, and then volumes were completed with the mobile phase.
- Complete acid and alkaline induced hydrolytic degradation was confirmed by the proposed HPTLC and HPLC methods.

Procedure

(a) Chromatographic conditions

In HPTLC, analysis was performed on 20 x 10 cm HPTLC plates pre-coated with silica gel 60 F_{254} (E. Merck). The plates were spotted 2 cm apart from each other and 1cm apart from the bottom edge. The chromatographic tank was pre-saturated with the developing system for 15 min, then the plates were developed by ascending chromatography using diethylether-ethanol-water-glacial acetic acid (5: 3: 2: 0.05, v/v) as a de-

veloping system to a distance of 9.5 cm. The plates were air dried, detected under UV-lamp and then, scanned at 257 nm under the following experimental conditions of measurements: photo mode=reflectance, scan mode=linear slit scanning, slit width = 0.4mm, slit height = 0.02mm, result output= densitogram and peak list.

The HPLC separation and quantitation were achieved on Targa C_{18} column (5µm, 250 x 4.6 mm, i.d.). A mixture of methanol: water (30:70, v/v) was used as a mobile phase. The mobile phase was prepared daily, filtered by vacuum filtration through 0.45 µm filter, and degassed by ultrasound sonication for 30 minutes just prior to use, and delivered at a flow rate of 1.0 ml/min. The samples were also filtered using 0.45 µm syringe filters. All determinations were performed at ambient temperature. The injected volume was 50 µL. The detector was set at λ 257 nm.

(b) Method validation

(A) Linearity

In HPTLC, accurately measured volumes (2.0 – 20.0 ml) of cefepime stock standard solution (2.0 mg.ml-1) were transferred into a series of 25-ml volumetric flasks, diluted to volume with methanol to obtain a concentration range of 0.16 – 1.6 mg.ml-1. A 5 µl volume of each solution was applied to the plates in triplicates, the chromatographic conditions were adjusted, the plates were developed and the peak areas were measured. The calibration curve representing the relationship between the integrated peak area and its corresponding concentration was constructed and the regression equation was recorded.

In case of HPLC, accurately measured volumes (0.15 – 5.0 ml) of cefepime working standard solution (100.00 µg.ml-1) were transferred into a series of 25-ml volumetric flasks, diluted to volume with the mobile phase to obtain a concentration range of 0.60 - 20.00 µg.ml-1. A 50 µl volume of each solution was injected, in triplicates; separated using the chromatographic conditions described above and the average peak areas were calculated. The calibration curve, representing the relationship between the average peak area and corresponding concentration, was plotted and regression equation was computed.

(B) Sensitivity

The sensitivity of the methods was determined with Limit of detection (LOD) and limit of quantification (LOQ). The LOD and LOQ parameters were determined from regression equations: LOD = 3.3 × SD/s, LOQ = 10 × SD/s, where "SD" is the standard deviation of response and "s" slope of calibration curve.

(C) Accuracy

The previously mentioned procedures under linearity was repeated for determination of different concentrations of cefepime. The concentrations were calculated from the regression equations and the percentage recoveries were then calculated.

(D) Precision

Three concentrations of cefepime were analyzed five times intra-daily and on five successive days using the previously mentioned procedures under linearity. The mean percentage recovery and the relative standard deviation were calculated.

(E) Specificity

The specificity of the proposed methods was established by the analysis of laboratory mixtures, consisting of the intact drug with the acid- and alkaline-degradation products, in triplicate. The peak areas were measured and the concentration of cefepime was then calculated from the regression equations. The mean percentage recovery and the relative standard deviation were calculated.

(F) Robustness

Robustness of HPLC method was evaluated by analyzing a mixture of cefepime with its acid-degradation products after slight but deliberate changes in the analytical conditions - flow rate (± 0.1 ml/min), the proportions of methanol and water (32: 68 and 28: 72, v/v).

(G) System suitability

The system suitability test was performed to confirm that the LC system to be used was suitable for intended application. A standard solution containing 10 µg/ml of cefepime was injected five times. The parameters retention time, resolution, theoretical plates, tailing factor, and %RSD were determined.

(H) Application to the pharmaceutical formulations

The content of one vial Maxipime® (500mg) was transferred into 100 ml volumetric flask containing 50 ml water. The contents of the flask were shaken well

and completed to the mark with water. A suitable aliquot of the obtained solution was diluted quantitatively with methanol (in case of HPTLC) or the mobile phase (in case of HPLC) to obtain a concentration within the linearity range. The suggested procedures stated under linearity were followed for cefepime assay

(I) Validation by standard addition technique

Known amounts of cefepime were added to the drug product, the suggested procedures stated under linearity were carried out. The concentrations, the mean percentage recovery and the relative standard deviation were then calculated.

RESULTS AND DISCUSSION

Method development and optimization

For HPTLC, experimental conditions such as developing system and wavelength of detection were optimized to provide accurate, precise and reproducible results. Different developing systems were tried such as diethylether: ethanol (7.0: 3.0, v/v). With this developing system, the spot of the intact drug remained on the baseline. Thus, the polarity of this system was increased by adding different volumes of water. The best resolution with minimum tailing of cefepime peak from its different degradation products was achieved by using diethylether-ethanol-water-glacial acetic acid (5: 3: 2: 0.05, v/v). The separated drug spots were determined densitometrically on the plates at 257 nm. The tailing factor of cefepime peak was 1.2 and R_f values were 0.14 for cefepime and 0.86 and 0.73 for its acid- and alkaline-degradation products, respectively (Figure 1).

For HPLC, The best chromatographic condition took place on C18 column with mobile phase consisting of methanol: water (30:70, v/v) at flow rate 1.2 ml/min and UV detection at 257 nm (Figure 2). In order to achieve good separation of the intact drug peak from

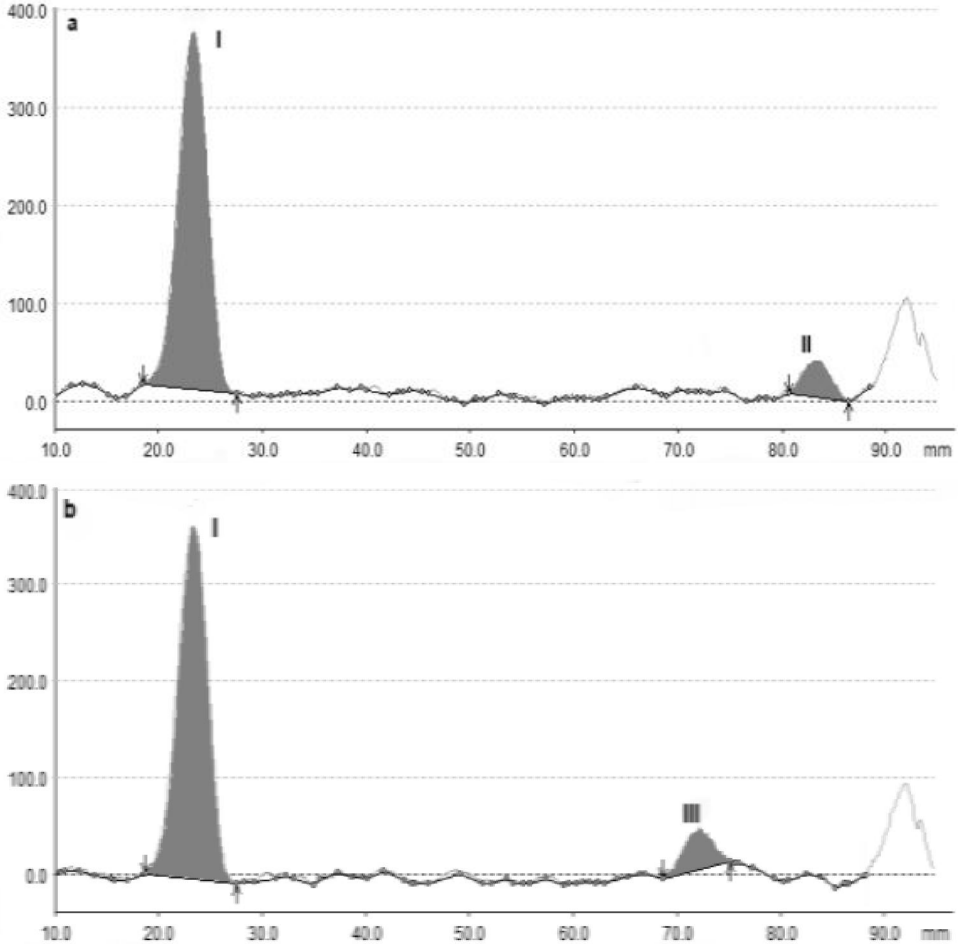

Figure 1 : HPTLC chromatograms of mixture solutions containing cefepime 3.00µg/spot (I) with a) its acid-degradation products 1.00µg/spot (II), b) its alkaline-degradation products 1.00µg/spot (III).

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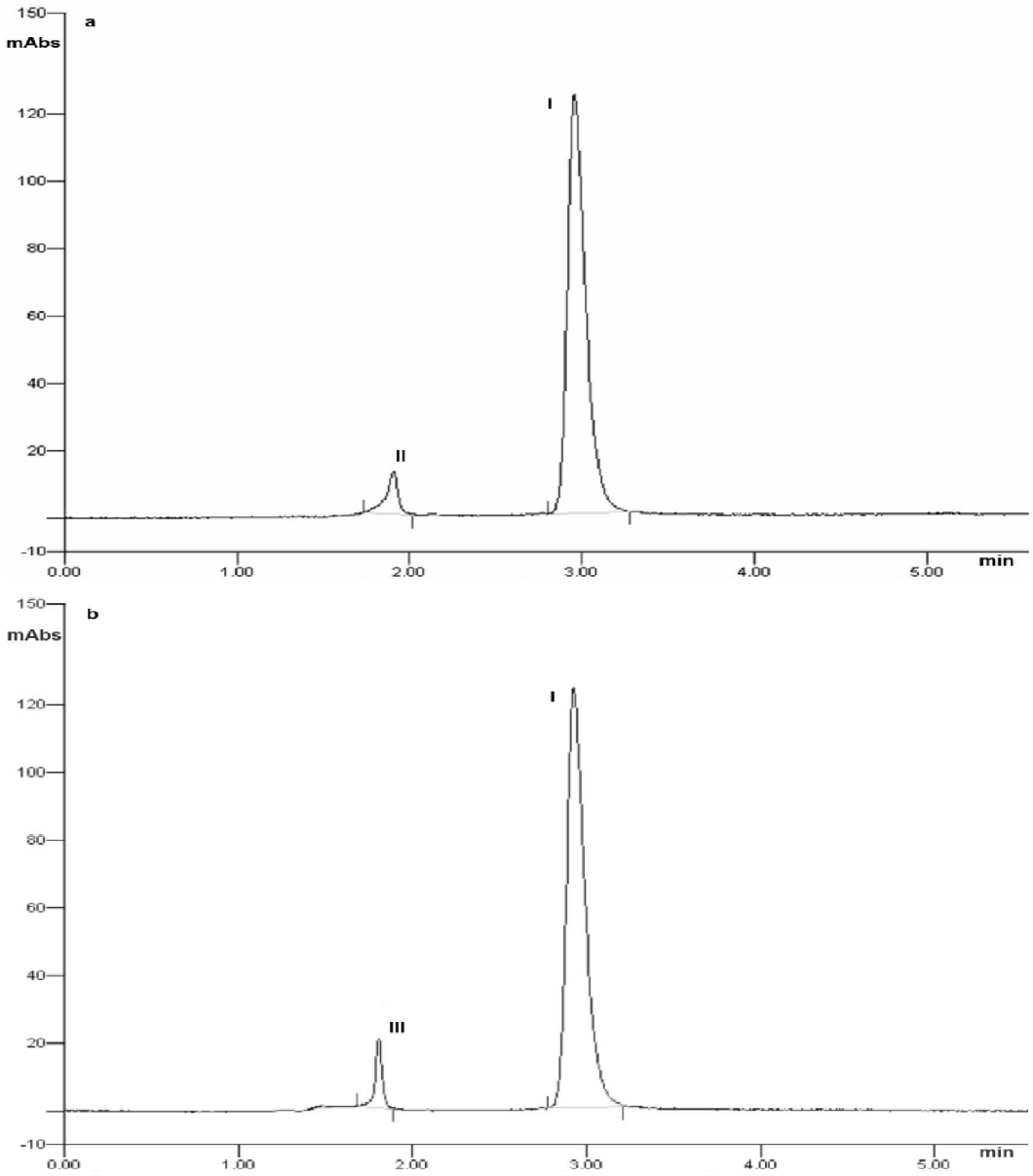

Figure 2 : HPLC chromatograms of mixture solutions containing cefepime (I) with a) its acid-degradation products (II), b) its alkaline-degradation products (III), (each 10.00µg/ml)

the peaks of the acid- and alkaline-degradation products under isocratic conditions, different ratios of methanol and water were tried using C_{18} packing as stationary phase. It was observed that increasing organic modifier Concentration (methanol) not only improves peak shape but also decreasing the run time. Therefore, binary mixture of methanol-water in proportion of 30+70 (v/v) was proved to be the best for the separation since the chromatographic peaks were better defined and resolved, and almost free from tailing. Different flow rates were tested, the rate of 1.2 ml/min was the best with respect to location and peak shape. Using a diode array detector at 257 nm, the above described chromatographic conditions allow a resolution of cefepime from its acid- and alkaline-degradation products with average retention times ± SD, for 10 replicate injections, of (2.92 min ± 0.04) for cefepime and, (1.91 ± 0.01) and (1.80 ± 0.02) for its acid- and alkaline-degradation products, respectively.

System suitability test was applied to a representative chromatogram for cefepime with its acid-degradation products, to check various parameters such as retention time, resolution, injection repeatability, tailing factor and number of theoretical plates (TABLE

1). The data verifies that the resolution and reproducibility of the chromatographic system are adequate for the analysis.

TABLE 1 : System suitability parameters of the proposed HPLC method

Parameters	Cefepime	Acid degradate	Alkaline degradate
Retention time R_t (min)	2.92	1.91	1.80
Resolution R_s (> 2)	-	4.21	5.46
Tailing factor T (≤ 2)	1.58	-	-
Injection repeatability[a] (≤ 1%)	0.579	-	-
Theoretical plates[b] N (>2000)	2578.79	-	-

[a]RSD% for five injections; [b]Measure of column efficiency

For quantitative applications, In case of HPTLC, the calibration curves are generally inherently non-linear due to scattering of light. They generally comprise a pseudo-linear region at low sample concentration and then departure from linearity begins at higher sample concentrations[17]. Moreover, the ICH guidelines[16] mentioned that for some analytical procedures which do not demonstrate linearity, the analytical response should be described by an appropriate function of the concentration of an analyte sample. The relationship between the integrated peak area and the concentration was evaluated with linear and polynomial regression functions. Fitting with linear function gave correlation value, r = 0.9869 while fitting with polynomial function gave better correlation (r = 0.9997) and lower standard deviation values and was therefore used for quantitative analysis. Calibration curves were constructed in the range of 0.60-8.00 µg/spot.

The LOD and LOQ were found to be 0.15 µg/spot and 0.46 µg/spot, respectively, which showed good sensitivity of the proposed HPTLC method. They were obtained by constructing a specific calibration curve including concentrations close to the expected LOD and LOQ. The standard deviation of y-intercepts of the regression lines was used as the standard deviation of response (SD). The mean percent recovery obtained by repeated analysis of five different concentrations was in the range of 99.23-101.64% (standard deviation 1.010). The RSD values of intra-day and inter-day precision were less than 2.0% (TABLE 2).

In case of HPLC, linear calibration graph was obtained with correlation coefficients of 0.9998. The calibration plot was linear from 0.6 to 20 µg/ml. Limit of detection (LOD) and limit of quantification (LOQ) were found to be 0.17 µg/ml and 0.52 µg/ml, respectively, which showed good sensitivity of the proposed HPLC method. The mean percent recovery obtained by repeated analysis of five different concentrations was in the range of 98.93-100.73% (standard deviation 0.680), demonstrating that the proposed HPLC method is highly accurate. The low RSD (<2.0%) values of intra-day and inter-day precision revealed that the proposed method is precise (TABLE 2).

The specificity of the proposed methods is illustrated in Figures 1 and 2, where complete separation of cefepime from its acid- and alkaline-degradation products was noticed, and was also tested by analyzing laboratory prepared mixtures. The results are presented in TABLE 3. The data reveals that cefepime can be determined without any interference from its different degradation products by the proposed methods.

Upon slight variation in the selected parameters, insignificant difference in retention time, resolution and

TABLE 2 : Intra-day and inter-day precision results by the proposed HPTLC and HPLC methods

	HPTLC			HPLC		
	Taken µg/ml	Found* µg/ml	Recovery %	Taken µg/ml	Found* µg/ml	Recovery %
Intra-day precision	1	1.00	100.00	2	1.99	99.50
	4	4.04	101.00	10	10.03	100.30
	8	7.86	98.25	16	16.15	100.94
	Mean±RSD%		99.75±1.39	Mean±RSD%		100.25±0.719
Inter-day precision	1	1.01	101.00	2	2.01	100.50
	4	3.94	98.50	10	9.91	99.10
	8	8.07	100.87	16	15.79	98.69
	Mean±RSD%		100.12±1.40	Mean±RSD%		99.43±0.954

*Mean of five determinations

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TABLE 3 : Determination of cefepime in laboratory prepared mixtures with its acid- and alkaline- degradation products by the proposed HPTLC and HPLC methods

Method	Cefepime µg/ml	Degradation products µg/ml	% Recovery* of cefepime in presence of	
			Acid degradation products	Alkaline degradation products
HPTLC	3.00	1	101.11	99.04
	3.00	1.5	99.21	100.32
	3.00	3	100.55	100.82
	Mean ± RSD%		100.29±0.973	100.06±0.917
HPLC	20.00	2.00	99.52	100.61
	20.00	10.00	98.59	99.53
	20.00	20.00	99.33	99.09
	Mean ± RSD%		99.15± 0.495	99.74±0.784

***Mean of three determinations**

number of theoretical plates was observed indicating robustness of the HPLC method (TABLE 4).

Cefepime containing injection was analyzed by the proposed HPTLC and HPLC methods and satisfactory results were obtained and were in a good agreement with the label claims (TABLE 5). Standard addition technique was also applied and the results obtained are shown in TABLE 5. The results of analysis of the pharmaceutical formulation and the standard addition method suggest that there is no interference from any excipients. TABLE 6 shows a statistical comparison of the results obtained by applying the proposed methods with those obtained by the official USP HPLC method[1]. It is clear that the calculated t ad F values are less than the tabulated ones, indicating that there is no significant difference between the proposed methods and the official method.

TABLE 4 : Results from robustness testing of the proposed HPLC method

Conditions	R_t	N	T	R_S*
Flow rate:				
1.1 ml/min	3.14	2524.06	1.67	4.08
1.3 ml/min	2.74	2481.85	1.75	3.96
Mobile phase composition:				
Methanol : water (28: 72, v/v)	3.29	2561.92	1.83	4.92
Methanol : water (32: 68, v/v)	2.79	2824.16	1.50	3.84

*** Resolution of the acid-degradation product relative to cefepime**

TABLE 5 : Determination of cefepime in pharmaceutical formulation by the proposed HPTLC and HPLC methods and application of standard addition technique

Pharmaceutical formulation	% Found ± SD*		Standard addition technique			
			HPTLC		HPLC	
	HPTLC	HPLC	Added (µg/ml)	% Recovery*	Added (µg/spot)	% Recovery*
Maxipime 500mg vial B.N.:E106019	101.72 ±0.669	99.88 ±0.599	0.60	98.93	4.00	100.33
			1.00	100.63	6.00	99.91
			2.00	100.95	8.00	99.24
			3.00	101.18	10.00	98.69
			4.00	98.92	12.00	99.39
			Mean ± RSD %	100.12 ± 1.108	Mean ± RSD %	99.51 ± 0.634

***Mean of three determinations**

TABLE 6 : Statistical comparison between the proposed HPTLC and HPLC methods, and the official USP HPLC method for determination of cefepime

Parameters	HPTLC	HPLC	Official Method*
Mean	100.69	99.47	100.11
SD	1.010	0.680	0.808
n	5	5	5
Variance	1.020	0.463	0.653
t (1.83)**	0.34	0.46	-
F (6.39)**	1.56	1.41	-

***The official USP HPLC method; 0.288 g% 1-pentanesulfonate: acetonitrile (94: 6, v/v) as mobile phase; **The theoretical values of t and F at 0.05 level of significance**

CONCLUSSION

The proposed HPLC and HPTLC methods are

simple, sensitive, accurate, precise and specific over the specified ranges. The proposed methods are considered as stability indicating methods for the determination of cefepime in presence of its acid and alkaline induced hydrolytic degradation products without prior extraction. Hence, these methods are suitable for stability testing of cefepime and for routine quality control analysis in bulk material and in pharmaceutical formulation where economy and time are essential.

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