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Development and Validation of HPLC and HPTLC Methods for Determination of Cefoperazone and Its Related Impurities

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Abstract

Validated sensitive and highly selective methods were developed for the quantitative determination of cefoperazone sodium (CEF) in the presence of its reported impurities; 7-aminocephalosporanic acid (7-ACA) and 5-mercapto-1-methyl-tetrazole (5-MER). Method A is high-performance liquid chromatography (HPLC), where the mixture of CEF and the reported impurities; 7-ACA and 5-MER were separated on a C8 column (5 µm ps, 250 mm × 4.6 i.d.) using methanol:0.05 M KH_2PO_4 buffer (22.5:77.5 v/v, pH 7.5) as a mobile phase. The three components were detected at 254 nm with a concentration range of 10–90 µg mL^{-1} and the mean percentage recovery 99.67% (SD 1.465). Method B is high-performance thin layer chromatography (HPTLC), where the mixture of CEF and the reported impurities were separated on silica gel HPTLC F_{254} plates using (acetone:methanol:ethyl acetate:2% sodium lauryl sulfate:glacial acetic acid) (3:2:3:0.8:0.2, by volume) as a developing system and scanning at 254 nm over a concentration range of 1–10 µg per band with the mean percentage recovery 99.95% (SD 1.335). The proposed methods were statistically compared with a reported HPLC method with no significant difference regarding accuracy and precision; indicating the ability of the proposed methods to be reliable and suitable for routine analysis of drug product. The proposed HPTLC method proved to be more sensitive, while the HPLC gave more reproducible results besides saving time.

Introduction

Cefoperazone sodium (CEF), (6*R*,7*R*)-7-[[(2*R*)-2-[[(4-ethyl-2,3-dioxopiperazin-1-yl)carbonyl]amino]-2-(4-hydroxyphenyl)acetyl]amino]-3-[[(1-methyl-1*H*-tetrazol-5-yl)sulfanyl]methyl]-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylate (Figure 1A) (1), is a third-generation cephalosporin, which acts by inhibiting biosynthesis of cell wall mucopeptide (2). 7-Amino-cephalosporanic acid (7-ACA) (Figure 1B), (6*R*,7*R*)-3-[(acetyloxy)methyl]-7-amino-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid and 5-mercapto-1-methyl-tetrazole (5-MER) (Figure 1C), 1-methyl-1*H*-tetrazole-5-thiol are considered as specified impurities for CEF according to British Pharmacopoeia (3). 7-ACA is a significant and key intermediate required for the production of semi-synthetic cephalosporin antibiotics in pharmaceutical industries (4). 5-MER is one of the tetrazole derivatives that used as an intermediate of cephalosporin side chains.

A survey of the literature revealed several methods for determination of CEF in its pharmaceutical formulation including spectrophotometric methods for the determination of CEF (5, 6), near infrared reflectance spectroscopy (7) and derivative UV-spectrophotometry for determination of CEF in combination with sulbactam (8). Chromatographic

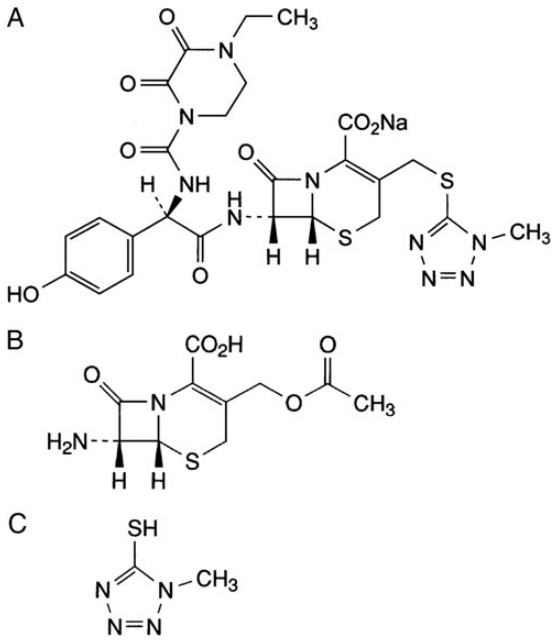

Figure 1. The chemical structure of CEF (A) and its reported impurities 7-ACA (B) and 5-MER (C).

methods were also reported for determination of CEF and sulbactam (9, 10), besides a high-performance liquid chromatography (HPLC) method with β-cyclodextrin stationary phase for determination of CEF, ampicillin and sulbactam was reported (11). Additionally, CEF and sulbactam were determined in plasma as well by the LC–MS-MS method (12). Electrochemical behavior and voltammetric determination of CEF (13, 14) were reported as well.

However, no analytical method has been published for the quantitative determination of CEF in the presence of its reported impurities; 7-ACA and 5-MER in pure form or in pharmaceutical formulation. The aim of this article is to establish and validate two chromatographic methods for the determination of active compound (CEF) in the presence of its reported impurities.

Materials and methods

Apparatus

HPLC (Agilent 1260 Infinity, Germany) instrument was equipped with Agilent 1260 Infinity preparative pump (G1361A), Agilent 1260 Infinity Diode array detector VL (G131SD), Agilent 1260 Infinity Thermostated column compartment (G1316A) and Agilent 1260 Infinity preparative Autosampler (G2260A). Separation and quantitation were performed on a ZORBAX Eclipse Plus C8 column (250 × 4.6 mm i.d., 5 µm particle size, USA).

For high-performance thin layer chromatography (HPTLC), Camag TLC scanner 3 S/N 130319 operated with winCATS software and the following requirements are taken into consideration: slit dimensions: 6 × 0.3 mm; scanning speed: 20 mm s^{-1}; spraying rate: 10 s μL^{-1}; data resolution: 100 µm $step^{-1}$; bandwidth: 6 mm and result output: chromatogram and integrated peak area. Additionally, Linomat IV with a 100-µL syringe (Camag, Muttenz, Switzerland) and HPTLC aluminum plates (20 × 20 cm) coated with 0.25 mm silica gel 60 F_{254} (Merck, Germany) were used. Sonix TV ss-series ultrasonicator (USA) was used as well in preparation of standard solutions for the two methods.

Material and reagents

Pure standard

CEF was kindly supplied by Pharco Pharmaceuticals Co., Egypt. Both 7-ACA (CAS No. 957-68-6) and 5-MER (CAS No. 13183-79-4) were purchased from Sigma-Aldrich through the Egyptian International Center for import and export (EIC, Egypt).

Pharmaceutical formulation

Cefobid® vials (batch no. 2203) were manufactured by Pfizer Pharmaceutical Industries Co. and Cefoperazpne® vials (batch no. 1240395) were manufactured by Sigmatech Pharmaceutical Industries Co. There are three concentrations available in the market for vials; 0.5, 1, and 1.5 g.

Chemicals and reagents

All chemicals and solvents used were of analytical grade. Methanol (E. Merck, Germany), water for injection B.P. 2003 (Egypt Otuska Pharmaceutical Co., S.A.E., 10th of Ramadan city, A.R.E), dipotassium hydrogen phosphate (K_2HPO_4), dihydrogen potassium phosphate (KH_2PO_4), sodium lauryl sulfate, methanol, acetone, hexane, ethyl acetate and glacial acetic acid (El-Nasr Pharmaceutical Chemicals Co., Abu-Zabaal, Cairo, Egypt).

Standard solutions

(a) Stock standard solutions of 1 mg mL^{-1} for each of CEF, 7-ACA and 5-MER were prepared in 3 mL of 0.05 M K_2HPO_4 solution, then the volume was completed with pure methanol.

(b) Working standard solutions of 100 µg mL^{-1} for each of CEF, 7-ACA and 5-MER were prepared in methanol.

All stock standard solutions were freshly prepared on the day of analysis and stored in a refrigerator to be used within 24 h.

Methods

HPLC method

Chromatographic conditions. Chromatographic separation was carried out using isocratic mode on a C_8 column with a mobile phase consisting of methanol:0.05 M KH_2PO_4 buffer (22.5:77.5, v/v). To each 100 mL of 0.05 M KH_2PO_4 buffer, 0.5 mL of KOH was added and then pH was adjusted to 7.5 using a pH meter. The mobile phase flow rate was 2 mL min^{-1}, and the eluate was scanned at 254 nm at room temperature. All the injections were run in three replicates, and the injection volume was 20 µL. The run time was 12 min, and the total peak areas were used to quantify the studied components.

Linearity and construction of calibration curves. Accurately measured aliquots equivalent to 100–900 µg of CEF were transferred from its working solution (100 µg mL^{-1}) into a series of 10-mL volumetric flasks and then the volume was completed with mobile phase. Triplicate injections were carried out for each concentration. The relative peak areas (using 20 µg mL^{-1} as external standard) were used to construct a calibration curve of CEF, and the regression equation was constructed.

Application to pharmaceutical formulation. The contents of each vial of Cefobid® and Cefoperazone® were weighed and mixed well. An accurately weighed portion equivalent to 100 mg of CEF was transferred into a 100-mL volumetric flask. To prepare stock solution, 3 mL of 0.05 M K_2HPO_4 solution was added and then the volume was completed to 100 mL with pure methanol. The solution was diluted to obtain 100 µg mL^{-1} working solution for each method.

The procedure under linearity and construction of calibration curves was followed using Cefobid® and Cefoperazone® vials working solution (100 µg mL^{-1}). Concentrations of CEF were then calculated from the corresponding regression equations, and the percentage recoveries were calculated.

HPTLC method

Linearity and construction of calibration curves. Aliquots equivalent to 1–10 mg of CEF were transferred from its standard solution (1 mg mL^{-1}) into a series of 10-mL measuring flasks, then the volume of each flask was completed with methanol. Ten microliters of each solution were applied in triplicate to HPTLC plates (20 × 11 cm) as bands with 6 mm width using a Camag Linomat IV applicator. The bands were spaced 5 mm from each other and 10 mm apart from the bottom edge of the plate. Linear ascending development was performed in a chromatographic chamber previously saturated with acetone:methanol:ethyl acetate:2% sodium lauryl sulfate:glacial acetic acid (3:2:3:0.8:0.2, by volume) as a developing system for 1 h at room temperature to a distance of 9 cm. The integrated peak areas were recorded using scanning wavelength at 254 nm under the specified instrumental conditions. The calibration curve was constructed by plotting the mean integrated peak area/10^4 versus the corresponding concentration of CEF and then the regression equation was computed.

Application to pharmaceutical formulation. The contents of each vial of Cefobid® and Cefoperazone® were weighed and mixed well. An accurately weighed portion equivalent to 100 mg of CEF was transferred into a 100-mL volumetric flask. To prepare stock solution, 3 mL of 0.05 M K_2HPO_4 solution was added and then the volume was completed to 100 mL with pure methanol. The solution was diluted to obtain 100 µg mL^{-1} working solution for each method. The procedure under linearity and construction of calibration curves was followed using Cefobid® and Cefoperazone® vials working solution (100 µg mL^{-1}). Concentrations of CEF were then calculated from the corresponding regression equations, and the percentage recoveries were calculated.

Results

HPLC results

A sensitive, accurate and highly selective isocratic HPLC method was developed for the analysis of CEF in the presence of its reported impurities; 7-ACA and 5-MER according to ICH guidelines (15), which recommend very restrictive requirements for level of impurities in pharmaceutical products. The separation was carried out using methanol:0.05 M KH_2PO_4/KOH buffer (22.5:77.5, v/v), adjusted to pH 7.5 as a mobile phase and flow rate 2 mL min^{-1}. The retention times were 1.721, 2.047 and 11.808 min for 5-MER, 7-ACA and CEF, respectively, as shown in Figure 2. 7-ACA and 5-MER peaks eluted very close to each other and to the solvent peak, thus could not be quantifiable under the presented conditions. The peak purity figures for the two impurities are presented in Figure 3.

The calibration curve for CEF was constructed by plotting the relative peak area (drug peak area/external standard peak area) versus the corresponding concentration. The regression equations were calculated as follows:

$$Y = 0.0416X + 0.1660 \quad r = 0.9998,$$

where Y is the relative peak area, X is the concentration in µg mL^{-1} and r is the correlation coefficient, as shown in Table I. The ratio chromatogram for different concentrations of CEF and the chosen concentration as external standard are shown in Figure 4.

HPTLC results

HPTLC offers the advantages of automatic application, high sensitivity and selectivity for analysis of CEF in the presence of its reported impurities; 7-ACA and 5-MER in pure form and in pharmaceutical formulation. Accordingly, the HPTLC method was successfully applied for separation of CEF, 7-ACA and 5-MER mixture and for quantification of CEF in the presence of its impurities using acetone: methanol:ethyl acetate:2% sodium lauryl sulfate:glacial acetic acid (3:2:3:0.8:0.2, by volume) as a developing system at 254 nm. Good resolution is shown by the difference in the retention factor (R_f) values of 7-ACA ($R_f = 0.54$), CEF ($R_f = 0.69$) and 5-MER ($R_f = 0.85$) as shown in Figure 5.

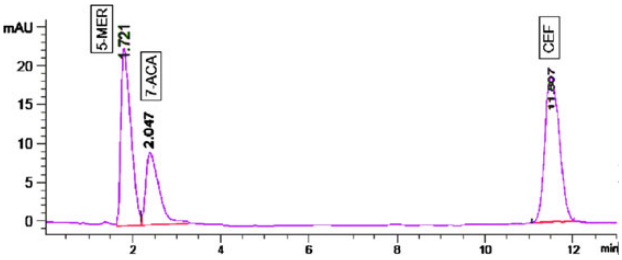

Figure 2. HPLC chromatogram of resolved mixture of 10 µg mL^{-1} of 5-MER, 20 µg mL^{-1} of 7-ACA and 60 µg mL^{-1} of CEF ($R_t = 1.721$, 2.047, and 11.808 min, respectively) using methanol:0.05 M KH_2PO_4 buffer (w/v) (22.5:77.5 by volume) as mobile phase at 254 nm. This figure is available in black and white in print and in color at *JCS* online.

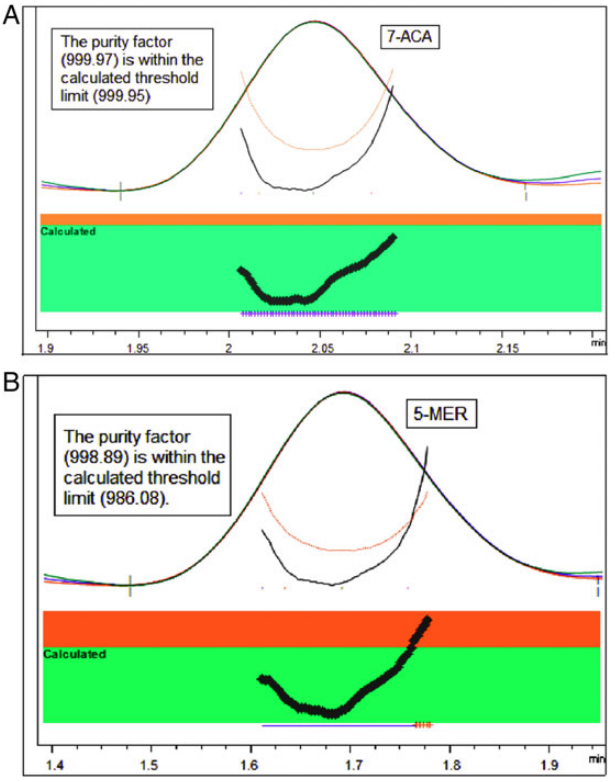

Figure 3. The peak purity figures for the impurities of CEF. This figure is available in black and white in print and in color at *JCS* online.

The calibration curve for CEF was constructed by plotting integrated peak area/10^3 versus the corresponding concentration. The regression equations are calculated as follows:

$$Y = 2.2684X + 0.7806 \quad r = 0.9997,$$

where Y is the integrated peak areas/10^3, X is the concentration in µg per band and r is the correlation coefficient, as shown in Table I.

Optimization of analytical methods

7-ACA is soluble only in slightly alkaline solvent (pH 7–8); hence the solubility was achieved by addition of a small volume of 0.05 M K_2HPO_4 solution first. This step was carried out with all stock solutions of CEF, 7-ACA, 5-MER and dosage form as well. Different parameters were manipulated to obtain an acceptable resolution between CEF and its reported impurities.

Table I. Regression and Analytical Parameters of the Developed HPLC-DAD Method for the Determination of CEF in the Presence of Two Impurities, 7-ACA and 5-MER

Parameter	Cefoperazone, HPLC-DAD method	Cefoperazone, HPTLC method
Calibration range	10–90 µg mL^{-1}	1–10 µg/band
Slope	0.0416	2.2684
Intercept	0.1660	0.7806
Mean %	99.67	99.95
SD	1.465	1.335
Coefficient of variation	0.312	0.490
Correlation coefficient (r)	0.9998	0.9997
LOD*	5	0.8
LOQ*	10	1
RSD%[a**]	1.492–0.941–0.456	1.010–1.058–1.716
RSD%[b**]	1.851–1.322–1.443	1.651–1.561–1.856

*(RSD%)[a]** and (RSD%)[b]*; the intra- and interday RSD of concentrations (20, 40 and 80 µg mL^{-1}) for the HPLC-DAD method and (2, 4, and 7 µg/band) for the HPTLC method.

**Limit of detection and quantitation are determined experimentally by the signal-to-noise ratio (15).

HPLC method optimization

To optimize the developed HPLC method, it was necessary to investigate the effect of different factors to get the desired chromatographic resolution.

Stationary phase. Different stationary phases were tried such as ODS C_{18} column and C_8 column where the best separation was achieved by a C_8 column. The C_8 column provides higher inertness than the ODS column, thus it is a good choice for analyzing compounds which are retained too strongly on ODS columns and elute very slowly.

Mobile phase composition. None of the published HPLC mobile phases was able to achieve good resolution between CEF and its two impurities. Studying the optimum parameters for maximum separation was carried out by trying different mobile phases with different ratios from aqueous and organic solvents. Concerning aqueous phase, water acidified with phosphoric acid or acetic acid, water with triethylamine and with different types of buffer were tested in combination with the organic modifier. It was proved that water adjusted to pH 7.5 with 0.05 M KH_2PO_4/KOH buffer gave better separation of CEF from its impurities and the ratio of aqueous phase gave good separation of the two impurities from each other. Additionally, different organic modifiers (methanol and acetonitrile) were tested to improve chromatographic conditions. Methanol provided better results concerning resolution and peak symmetry. Chromatographic separation was enhanced with increasing polarity.

pH of the mobile phase. Slightly alkaline medium (pH 7.5–8) improves peak shape and resolution. Adjustment the pH value of the mobile phase at 7.5 using 0.05 M KH_2PO_4 buffer, after trying different pH values such as 7, 7.8 and 8, provided sharp and well-separated peaks.

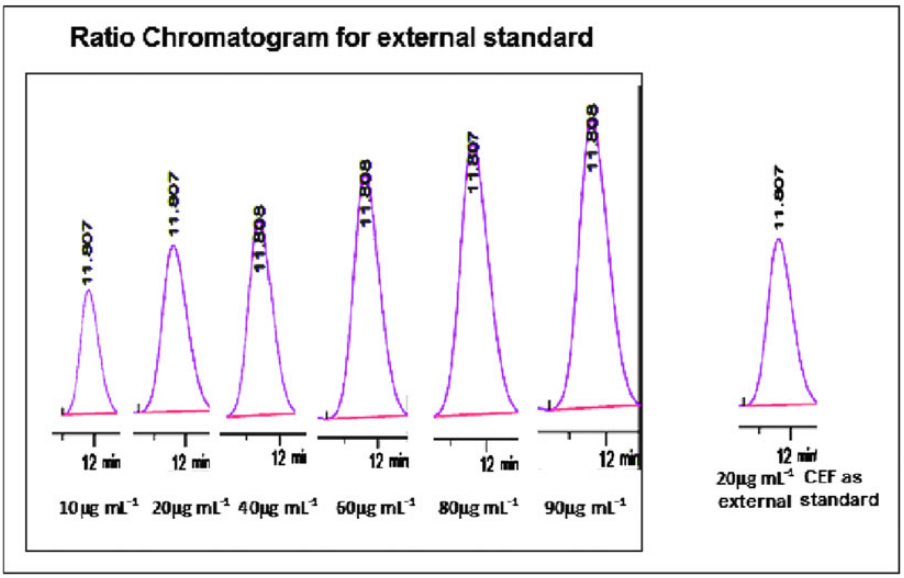

Figure 4. Ratio chromatogram figure. This figure is available in black and white in print and in color at *JCS* online.

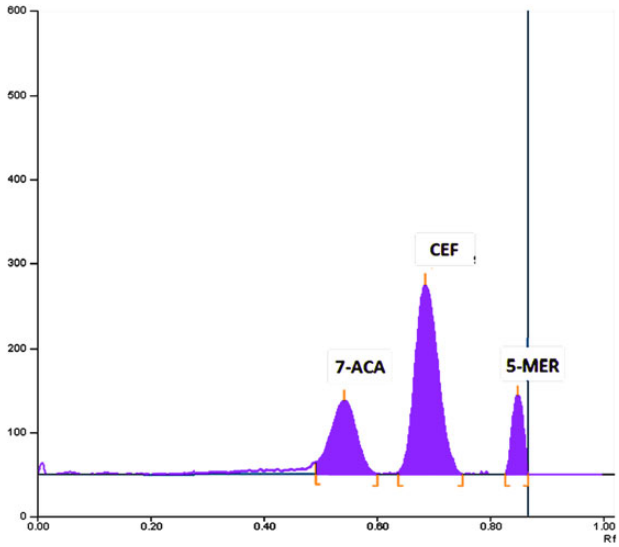

Figure 5. 2D HPTLC densitogram of resolved mixtures of 3 µg/band of 7-ACA (R_f = 0.54), 4 µg/band of CEF (R_f = 0.69) and 3 µg/band of 5-MER (R_f = 0.85) using acetone:methanol:ethyl acetate:2% sodium lauryl sulfate (w/v):glacial acetic acid (3:2:3:0.8:0.2, by volume) as a developing system and scanning at 254 nm. This figure is available in black and white in print and in color at *JCS* online.

Scanning wavelength. Different scanning wavelengths as 230, 254 and 265 nm were tried to obtain maximum sensitivity for CEF. Scanning at 254 nm gave the best sensitivity with minimum noise detected.

Flow rate. The effect of mobile phase flow rate was tested, and a flow rate of 2 mL min^{-1} was proved to give the best resolution within a short analysis time.

After method optimization, chromatographic separation of the four components was achieved using a C_8 column with methanol: 0.05 M KH_2PO_4 buffer (22.5:77.5 v/v, adjusting pH to 7.5) as the mobile phase at a flow rate of 2 mL min^{-1} and with UV detection at 254 nm (Figure 2).

HPTLC method optimization

The following parameters were studied to obtain the maximum chromatographic separation.

Developing system and efficiency. Several developing systems of different compositions and ratios were tried such as chloroform: methanol:glacial acetic acid (8:2:0.2, v/v), chloroform:methanol: (ammonia solution or triethylamine) (8:2:0.2, by volume), chloroform:acetone:methanol:glacial acetic acid (6:2:3:0.2, by volume), acetone:methanol:ethyl acetate:glacial acetic acid (3:3:2:0.2, by volume) and acetone:methanol:ethyl acetate:2% sodium lauryl sulfate: glacial acetic acid (3:2:3:0.8:0.2, by volume) to obtain optimum separation between CEF and its impurities (7-ACA and 5-MER). Acetone was necessary to provide polarity equilibrium with methanol where they can together move the nonpolar components up. The mixture of ethyl acetate and glacial acetic acid remove tailing and make compact spots while the effect of 2% sodium lauryl sulfate solution was enhancing the resolution of CEF from 7-ACA. The best developing system was found to be acetone:methanol:ethyl acetate:2% sodium lauryl sulfate:glacial acetic acid (3:2:3:0.8:0.2, by volume). This selected developing system allowed good separation between CEF and its impurities with satisfactory R_f values without tailing of the separated bands, as shown in Figure 5.

Scanning wavelength. Different scanning wavelengths were tried (230, 254 and 265 nm) in order to obtain good sensitivity of CEF with minimum noise. The wavelength 254 nm was found to be the best wavelength regarding sensitivity of CEF. Peaks were sharp and symmetrical with minimum noise, as shown in Figure 6.

Band dimensions. The bandwidth and interspaces between bands should be chosen carefully to avoid spread of bands outside the scanning tracks and interference between adjacent bands. Different band dimensions were tried to obtain sharp and symmetrical peaks. The optimum bandwidth chosen was 6 mm, and the interspace between bands was 5 mm.

Slit dimensions of scanning light beam. The slit dimensions of the scanning light beam should ensure complete coverage of band dimensions on the scanned track without interference of adjacent bands. Different slit dimensions were tried where 6 × 0.45 mm proved to be the slit dimensions of choice which provided highest sensitivity.

This method offers high sensitivity and selectivity for analysis of CEF in the presence of its impurities using acetone:methanol:ethyl acetate:2% sodium lauryl sulfate:glacial acetic acid (3:2:3:0.8:0.2, by volume) as a developing system and scanning at 254 nm.

Application of the proposed methods to the pharmaceutical formulation

The suggested methods were successfully applied for the determination of CEF in its pharmaceutical formulation [Cefobid® vial (0.5 g) and Cefoperazone® vial (1 g)], showing good percentage recoveries. The validity of the suggested methods was further assessed by applying the standard addition technique as shown in Tables II and III.

Discussion

This work is concerned with determination of CEF in the presence of its related impurities; 7-ACA and 5-MER; Figure 1 besides chromatographic separation. The importance of this work refers to the ability of the presented chromatographic methods to analyze CEF quantitatively in the presence of its impurities. 7-ACA is the core chemical structure of cephalosporins. Chemical compounds contain this core are relatively stable to hydrolysis and tolerance to β-lactamase, the enzyme that hydrolyze cephalosporins. Therefore, 7-ACA is a very important part for the activity of all cephalosporins.

The presented results show high selectivity and good separation of CEF from its impurities (Figures 2 and 5). Application of the suggested methods on pharmaceutical dosage form was also carried on with acceptable recoveries as shown in Tables II and III. Optimization steps for the two methods were implemented to choose the suitable conditions and parameters to apply the methods. Accordingly, selection of the optimum conditions for chromatographic methods was necessary to obtain the best resolution and calibration.

According to the mentioned results, there were two accurate and highly selective methods for quantitative determination of CEF in the presence of its reported impurities; 7-ACA and 5-MER. The HPLC-DAD method has the advantage of being more reproducible

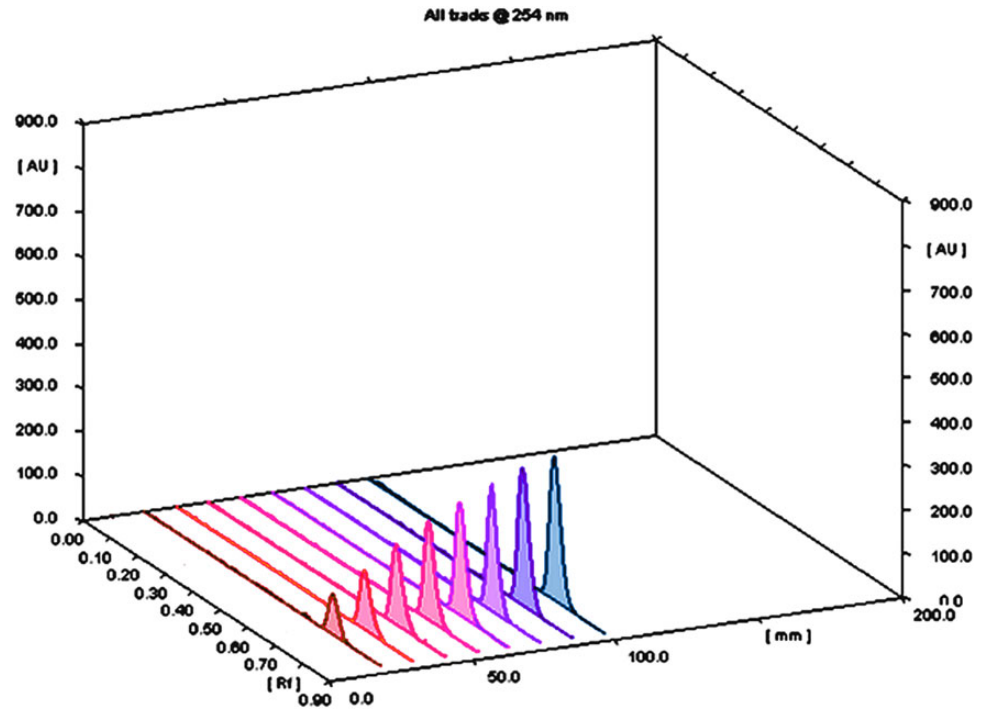

Figure 6. 3D HPTLC densitogram of CEF in the concentration range (1–10 µg/band) using acetone:methanol:ethyl acetate:2% sodium lauryl sulfate (w/v):glacial acetic acid (3:2:3:0.8:0.2, by volume) as a developing system and scanning at 254 nm. This figure is available in black and white in print and in color at *JCS* online.

Table II. Determination of CEF in Pharmaceutical Formulations by the HPLC-DAD Method and Application of the Standard Addition Technique

Cefobid® vial				Cefoperazone® vial			
Taken (µg mL^{-1})	Found %[a] ± SD	Pure added (µg mL^{-1})	Recovery %	Taken (µg mL^{-1})	Found %[a] ± SD	Pure added (µg mL^{-1})	Recovery %
20.00	102.76 ± 1.823	10.00	100.93	20.00	100.07 ± 1.565	10.00	97.12
		20.00	101.74			20.00	97.84
		30.00	98.73			30.00	100.88
Mean ± SD			100.47 ± 1.560	Mean ± SD			98.61 ± 1.999

[a]Average of six determinations.

Table III. Determination of CEF in Pharmaceutical Formulations by the HPTLC Method and Application of the Standard Addition Technique

Cefobid® vial				Cefoperazone® vial			
Taken (µg mL^{-1})	Found %[a] ± SD	Pure added (µg mL^{-1})	Recovery%	Taken (µg mL^{-1})	Found %[a] ± SD	Pure added (µg mL^{-1})	Recovery %
3.00	103.19 ± 1.147	2.00	99.01	3.00	99.04 ± 1.416	2.00	97.29
		3.00	100.98			3.00	100.26
		4.00	98.76			4.00	100.89
Mean ± SD			99.58 ± 1.217	Mean ± SD			99.48 ± 1.922

[a]Average of six determinations.

and short analysis time in addition to providing the peak purity data for the impurities. In HPTLC method, using HPTLC plates with smaller particle size provides higher resolution. It has the advantage of high sensitivity and using small quantity of developing system as well.

Methods validation

Methods validation was performed according to the International Conference on Harmonization (ICH) guidelines (15) for the proposed methods.

Linearity

Under optimum experimental conditions, CEF was determined in triplicates in the range of 10–90 μg mL^{-1} for the HPLC method and in the range of 1–10 μg per band for the HPTLC method (Table I).

Range

The specified range is derived from linearity studies and depends on the application of the analytical procedure. The concentration of CEF present in pharmaceutical formulations gave accurate and precise results with the suggested methods as shown in Table I.

Accuracy

Accuracy was assessed by the standard addition technique and through analysis of market pharmaceutical formulations by the proposed methods. The resulting synthetic mixtures of pure drug portions added to dosage form were assayed, and the results obtained were compared with those expected. The good recoveries of the added pure drug suggest good accuracy of the proposed methods (Tables II and III).

Precision

Repeatability

Two concentrations of CEF (20 and 80 μg mL^{-1}) for the HPLC method and (2 and 7 μg per band) for the HPTLC method were determined in triplicates in the same day to estimate intraday variation. Good results and acceptable relative standard deviation (RSD%) are shown in Table I.

Intermediate precision

The previous procedures were repeated on the same concentrations seven times on different four days to determine the intermediate precision. Good results and acceptable RSD% are shown in Table I.

Specificity

The specificity of the suggested methods was demonstrated by good separation of CEF, 7-ACA and 5-MER. Good separation was evaluated by different retention times in the HPLC method (1.721, 2.047 and 11.808 min for 5-MER, 7-ACA and CEF, respectively, Figure 2). Different R_f values obtained in the HPTLC method (0.54, 0.69 and 0.85 for 7-ACA, CEF and 5-MER, respectively), as shown in Figure 5, proved good resolution in the HPTLC method.

Detection and quantitation limits

Both HPLC and HPTLC methods exhibit baseline noise, so the detection limit is determined by the signal-to-noise ratio. The signal-to-noise ratio is determined by comparing the measured signals of samples with known low concentrations of the analyte with those of blank samples. For detection limits, the minimum concentration at which the analyte can be detected is determined. For quantitation limits, the minimum concentration at which the analyte can be quantified is determined (14). Acceptable detection and quantitation limits are shown in Table I.

Robustness

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small deliberate variations in method parameters and provides an indication of its reliability during normal usage (15).

For the HPLC method, robustness was determined by inducing minor changes in the organic strength (± 0.5%), pH (± 0.1 unit) and flow rate. The %RSD was calculated, where reliable results concerning area under the curve are given in Table IV.

For the HPTLC method, the organic strength of the developing system was deliberately changed by ±1% and it has no significant effect on R_f values or area under the peaks. The %RSD was calculated, where the results are given in Table IV.

System suitability

ICH states that system suitability tests are an integral part of many analytical methods, especially liquid chromatographic methods. They are used to verify that the resolution and reproducibility of the chromatographic system are adequate for the analysis to be done. Parameters including resolution (R_s), capacity factor (k'), peak symmetry and selectivity factor (α) were calculated as shown in Tables V and VI.

Table IV. Experimental Results of Robustness Testing for Determination of CEF in the Presence of Two Impurities, 7-ACA and 5-MER by the Proposed HPLC-DAD and HPTLC Methods

Parameters (% RSD)	HPLC-DAD method CEF (AUC)	Parameters (% RSD)	HPTLC method CEF (AUC)
Methanol/buffer (25:75, v/v)	1.438	Acetone (3 mL ± 1%)	0.517
Methanol/buffer (20:80, v/v)	0.752	Ethyl acetate (3 mL ± 1%)	0.088
Flow rate (1.5 mL/min)	0.613		
Flow rate (1.8 mL/min)	0.518		
pH (7.5 + 0.1)	1.172	Methanol (2 mL ± 1%)	0.507
pH (7.5 – 0.1)	0.429		

Table V. Parameters of System Suitability of the Developed HPLC-DAD Method for the Determination of CEF in the Presence of Two Impurities, 7-ACA and 5-MER

Parameters	5-MER	7-ACA	CEF
Resolution (R_s)	0.724		13.94
Selectivity (α)	1.189		5.768
Capacity factor (K')	0.721	1.047	10.8
Symmetry factor	1	0.4	1
Number of theoretical plates (N)	296.184	268.173	2754.150
HETP (cm/plate)	0.084	0.087	0.009

Table VI. Parameters of System Suitability of the Developed HPTLC Method for the Determination of CEF in the Presence of Two Impurities, 7-ACA and 5-MER

Parameters	7-ACA	CEF	5-MER
Capacity factor (K')	7.3	9.5	12
Symmetry factor	0.944	1	1
Resolution (R_s)	1.517		2.630
Selectivity (α)	1.265		1.238

Table VII. Statistical Analysis of the Two Proposed Methods (HPLC-DAD and HPTLC Methods) and the Reported HPLC Method for Determination of CEF in Pharmaceutical Formulations

Parameters	HPLC-DAD method	HPTLC method	Reported HPLC method[a]
(A) Cefobid® vial			
Mean	102.76	103.19	102.85
SD	1.823	1.147	1.424
Variance	3.324	1.316	2.027
n	6	6	6
Student's *t*-test[b] (2.228)	0.922	0.662	–
F-test[b] (5.050)	1.640	1.540	–
(B) Cefoperazone® vial			
Mean	100.07	99.10	99.40
SD	1.565	1.542	1.317
Variance	2.449	2.378	1.736
n	6	6	6
Student's *t*-test[b] (2.228)	0.442	0.722	–
F-test[b] (5.050)	1.411	1.370	–

[a]Reference method is HPLC (16).

[b]The values between parenthesis are corresponding to the theoretical values of *t* and *F* ($P = 0.05$).

The results obtained by the proposed methods were statistically compared with those obtained by the reference HPLC method (16) using *t*- and *F*-tests. The values obtained are less than the theoretical ones indicating no significant difference between the two proposed methods and the reference HPLC method with respect to accuracy and precision (Table VII).

Conclusion

The presented HPLC and HPTLC methods provide highly selective methods for quantitative determination of CEF in the presence of its reported impurities; 7-ACA and 5-MER. The HPLC method is the first developed one to analyze this ternary mixture at single wavelength in short analysis time and has the advantage of being more reproducible plus providing the peak purity data for the impurities. The HPTLC method has the advantage of high sensitivity and using HPTLC plates with smaller particle size and higher resolution ability besides using a small quantity of developing system. The proposed methods showed high selectivity, accuracy and reproducibility. These merits suggest the use of the proposed methods in routine and quality control analysis without interference of commonly encountered pharmaceutical preparation additives.

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