Selective Densitometric Determination of Four α-Aminocephalosporins Using Ninhydrin Reagent

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Abstract

A simple, selective, and precise densitometric method for analysis of four α-aminocephalosporins, namely cefaclor monohydrate, cefadroxil monohydrate, cefalexin anhydrous, and cefradine anhydrous, both in bulk drugs and in formulations was developed and validated. The method employed thin-layer chromatography (TLC) aluminium sheets precoated with silica gel G 60 F_{254} as the stationary phase. The solvent system consists of ethyl acetate–methanol–water with different ratios for all studied drugs (R_f values of 0.40–0.60). The separated spots were visualized as blue to violet color after spraying with ninhydrin reagent. The linear regression analysis data for the calibration plots of all studied drugs produced a good linear relationship with correlation coefficients ranging from 0.9990 to 0.9996 and coefficients of determination ranging from 0.9986 to 0.9992 over the concentration range 2–10 µg/spot. The limits of detection and quantitation for all studied drugs ranged from 0.09 to 0.23 and from 0.27 to 0.84 µg/spot, respectively. The developed method was applied successfully for the determination of the studied drugs in their pharmaceutical dosage forms with good precision and accuracy. Also, the method can be employed as a promising stability-indicating assay.

Introduction

Cefaclor monohydrate, cefadroxil monohydrate, cefalexin anhydrous, and cefradine anhydrous are semi-synthetic α-amino-β-lactam antibiotics widely used in clinical chemotherapy. They are useful for serious infections caused by susceptible strains of micro-organisms in lower respiratory infections, genito-urinary infections, gynecologic infections, skin infections, and central nervous system infections. Cephalosporins operate by inhibiting bacterial cell wall biosynthesis, which grows actively against a wide range of both gram-positive and gram-negative bacteria. The positive results of these drugs include the resistance of penicillinases and ability to treat infections that are resistant to penicillin derivatives. The official methods for analyzing α-aminocephalosporins are mostly chromatographic methods (1). Most of the reported methods involve the cleavage of the β-lactam moiety of the cephalosporin structure. These methods include spectrophotometric (2–6), spectrofluorimetric (7–10), and electrochemical methods (11–13). A direct chemical analysis based on the reactivity of the intact molecule is not frequently encountered. Thin-layer chromatography (TLC) is one of the most widely used techniques for the separation and identification of drugs, and it is an ideal technique because of its simplicity, low cost, selectivity, sensitivity, and ability to be performed without a remote area with limited volumes of solvents (14). Few TLC methods (15–19) have been reported for the determination of α-aminocephalosprins and these methods utilized UV at 270 nm (15), 265 nm (16), 254 nm (17), 263 nm (18), and 260 nm (19) for detection and quantitiation of the separated spots, different mobile phase systems for the spot development ,and mostly silica gel G 60F_{254} as a stationary phase.

Ninhydrin has been reported as a detecting reagent for quantitation of primary amines and amino acids on high-performance TLC plates (20–22). It has been used for the identification of 30 cephalosporins on TLC plates after heating at 105°C for 15 min (23). The aim of this work is to develop a simple, rapid, sensitive, selective, and promising stability-indicating densitometric method that can be applied at quality-control laboratories for the estimation of four α-aminocephalosprins in pure form and in different pharmaceutical dosage forms. This analytical procedure is based on the separation of the studied drugs without degradation on TLC aluminium sheets and subsequent detection and quantitation of the separated spots with ninhydrin reagent.

Experimental

Apparatus

UVP scanner (300 dpi, scan mode: gray or color) and software GelWorks 1D Advanced version 3.01 (Cambridge, UK), UV lamp short wavelength 254 nm (Vilber lournate 220V 50 Hz, Marnelavallee Cedex, France), and hot air oven (Heating incubator) (WTB

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binder 7200 Tübingen, Schwabach, Germany) were used. A test tube atomizer (12 mL) from Desaga GmbH (Wiesloch, Germany) was connected to a positive-pressure outlet valve of a membrane pump (Cole-Parmer, Chicago, IL). A thin-layer chromatographic spotting syringe (25-µL) was obtained from Hamilton (LKB, Bromma, Sweden). A TLC tank (standard type) (27.0 cm W× 26.5 cm H × 7.0 cm D) was from Sigma Aldrich (St. Louis, MO).

Materials and reagents

All solvents used were of analytical-reagent grade. TLC aluminum sheets precoated with silica gel G F_{254} plates (20 × 20 cm, 0.25 mm layer thickness) were obtained from E. Merck (Darmastadt, Germany). Ninhydrin (E. Merck) 0.5% ethanolic solution, sodium hydroxide (El-Nasr Chemical Co. Cairo, Egypt), 0.01M aqueous solution, Hydro-chloric acid (El-Nasr Chemical Co.), 0.5M solution, L-arginine (Sigma, Seelze, Germany), Cefaclor monohydrate, cefradine anhydrous (Sigma), cefadroxil monohydrate (Amoun Pharmaceutical Industries Co., APIC, Cairo, Egypt), and Cefalexin anhydrous (GalaxoWellcome, S.A.E., El Salam City, Cairo, Egypt) were obtained as gifts and were used as supplied. Pharmaceutical formulations containing the studied drugs were purchased from local market.

Standard preparations

Stock solutions containing 50 mg/mL of each α-amino-cephalosporin were prepared in methanol. Working standard solutions containing 1–5 mg/mL of each α-amino-cephalosporin were prepared by suitable dilution of the stock solutions with methanol.

Chromatographic conditions

Trials for selecting the best solvent system are given in Table I using cefalexin as a representative example for these trials. The mobile phases selected for the studied drugs are given in Table II.

Table I. Trials for Selection of Mobile Phase System for Cefalexin

Mobile system components	Ratio (v/v) or (v/v/v)	R_f*
Ethyl acetate–methanol	1:3	0.10
	1:5	0.20
	1:7	0.23
	1:9	0.30
	1:10	0.35†
Ethyl acetate–methanol–acetic acid	1:3:1	0.75
	1:3:3	0.85
	1:5:3	0.87†
	3:5:3	0.77†
Ethyl acetate–methanol–water	1:3:3	0.91
	3:3:3	0.83
	4:1:3	0.35
	4:3:3	0.71
	5:3:3	0.62
	6:3:3‡	0.52
Chloroform–ethanol–acetic acid	6:7:1	0.04
	5:7:3	0.10
	2:6:5	0.15†
	2:6:7	0.23†
	2:7:8	0.28†
Ethyl acetate–methanol–ammonia	1:3:1	0.22
	1:3:3	0.32
	1:5:3	0.45†
	3:5:3	0.55

* The retardation factor. † Tailed spots. ‡ Selected for further work.

Fifty milliliters of the mobile system chosen for cefalexin was poured into the TLC tank that was lined with a thick filter paper on few sides to help with the chamber saturation. The tank was then covered with a lid and pre-saturated with the mobile system vapor for at least 30 min at room temperature (25°C ± 5°C) before use. Size of the plate used directly for analysis was 20 cm × 5 cm. The sample-loaded TLC plate was transferred to the TLC tank, and the plate was then developed until the solvent front moved about three-fourths of the length of the plate (~10 min).

Other conditions were as follows: air-drying time, 5 min; oven-temperature, 110°C; heating time, 10 min; amount of spraying agent, 10.0 mL; and distance between the sprayer and the plate, 30 cm. The same conditions were employed for the remaining cephalo-sporins. For analysis, 2 µL was applied onto the TLC plate.

Procedures

General procedure

Two microliters of the working standard or sample solutions were spotted on the marked start edge of the TLC plate at 1 cm apart from the lower edge of the plate using the specified TLC-Hamilton glass syringe. The plate was then allowed to be air-dried for 5 min before its transfer to the TLC tank for the development. The plate was developed with the corresponding mobile phase for each drug until the ascended solvent front moved about three-fourths of the lenght of the plate. The plate was removed, air-dried for ~ 5 min, viewed under UV lamp, sprayed with ninhydrin reagent, and then heated in air oven for 10 min at 110°C. During this time, a blue to violet spot on a faint pink background corresponding to the cited cephalosporin persisted on the plate. The TLC chromatogram was captured by the scanner and the image was then loaded into the GelWorks software.

Procedure for calibration curves

Two microliters of the working standard solution containing 2–10 µg/spot of each of the studied drugs was spotted in triplicate on TLC plate, and then the densitometric analysis was performed as described under the General procedure section. The calibration curve was established by plotting the average optical density versus the corresponding concentration.

Procedure for tablets and capsules

An accurately weighed amount of powder obtained from 20 tablets or capsules equivalent to 250 mg was transferred into a 50-mL volumetric flask, dissolved in ~ 25 mL methanol, sonicated for 15 min, diluted to the mark with methanol mixed well, and filtered; the first portion of the filtrate was rejected. Further

Table II. Mobile Phase Composition and Retardation Factors of the Investigated α-Aminocephalosprin Antibiotics

Drug	Ethyl acetate–methanol–water ratios (v/v/v)	R_f*
Cefaclor	7:3:3	0.45
Cefadroxil	11:6:2	0.60
Cefalexin	6:3:3	0.52
Cefradine	7:5:3	0.57

* Retardation factor.

dilutions were made to obtain sample solution (3 mg/mL) and then the general procedure was followed.

Procedure for vials and powder for oral suspension

Twenty tablets were weighed, finely powdered and mixed thoroughly. An accurately weighed amount of powder equivalent to 250 mg of each drug was transferred into a 50-mL volumetric flask, and then the procedure was followed as under the Procedure section for tablets and capsules section, beginning from dissolved in ~ 25 mL methanol.

Procedure for preparation of acid- and base-induced degradation products (forced drug degradation)

Thirty milligrams of each drug were transferred into 10-mL volumetric flask, dissolved in 5 mL 0.01M NaOH. The flask was heated at 100°C for 1, 5, 10, 15, 25, or 30 min, cooled to room temperature, neutralized, and completed to volume with methanol. Two microliters of the resulting solution of each drug (containing 6 µg/spot) corresponding to each time interval were spotted in triplicate on TLC plate, and then the general procedure was followed. The same procedure was employed using 0.5M HCl instead of 0.01M NaOH.

Data processing and treatment

The TLC chromatogram, as an image, was captured by the scanner, and the image was then loaded into the Gel Works software. In the software, the following operations were performed: The series of spots to be manipulated were selected as a "lane by lane" creation function. Once the lane was created, a chromatogram was generated. The generated chromatogram was a function of spot position with the corresponding optical density, represented as pixel intensity.

After background subtraction, the signals of the chromatogram were assigned with numbers according to the sequence of the corresponding spots in the previously selected lane.

Quantity calibration was then performed by pre-assignment of the concentration of the active material of each spot. Once the authentic known concentrations were assigned, the calibration graph was automatically generated. The generated graph correlates the concentration with the corresponding signal intensity (represented as raw volume or area under the peak).

Once the described operations were performed, a report for all operations was given by the software. Figure 1 illustrates an example of 5-point calibration curve.

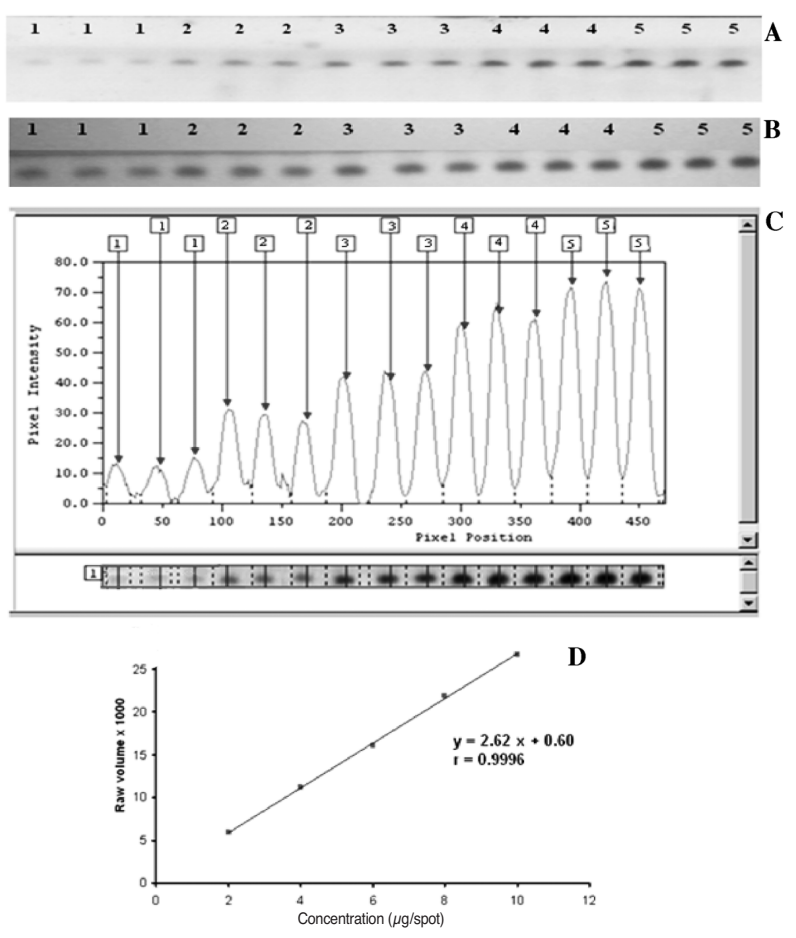


Figure 1. Thin layer chromatogram after spraying with ninhydrin indicating cefalexin concentration of 2, 4, 6, 8, and 10 µg/spot (spots 1–5; respectively) (A), TLC plate under UV lamp indicating cefalexin concentration of 2, 4, 6, 8, and 10 µg/spot (spots 1–5; respectively) (B), The treated chromatogram representing the pixel intensity for the corresponding spot (C) and The calibration curve for densitometric analysis of cefalexin correlating the concentration of the drug per spot with the corresponding raw volume calculated by the software (D).

Validation

The method was validated according to the International Conference on Harmonization (ICH) guidelines on the validation of analytical methods (24). All results were expressed as percentages, where *n* represents the number of values. For the statistical analysis, Excel 2003 (Microsoft Office) was used. A 5% significance level was selected. The TLC method developed was validated for the following parameters:

Accuracy

Three concentration levels (4, 6, and 8 µg/spot) covering the low, medium, and higher ranges of the calibration curve were spotted on the TLC plate. These spots were analyzed (n = 6) by using the described TLC method and calculated from the calibration curve carried out simultaneously on the same TLC plate. Accuracy was expressed as a recovery percentage (observed concentration × 100/theoretical concentration).

Precision

Repeatability of sample application and measurement of the optical density, expressed as (raw volume) were carried out using six replicates of the same spot at three concentration levels (4, 6, and 8 µg/spot) covering the low, medium, and higher ranges of the calibration curve (intra-day variation). The inter-day variation was evaluated by analyzing each one of the studied drugs repeatedly at a concentration range of 2–10 µg/spot (n = 6) over a period of seven days. Precision was expressed as the % RSD.

Linearity

Reference solutions were prepared at five concentration levels and were analyzed in triplicate. The concentration levels were within the range of 2–10 µg/spot.

Sensitivity

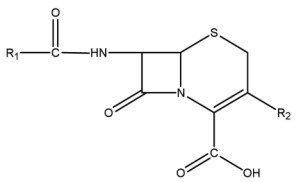
The sensitivity of the method was determined in terms of limit of detection (LOD) and limit of quantitation (LOQ). The LOD of the studied drugs as well as the LOQ were calculated for all studied drugs as follow (24):

LOD or LOQ = K.SDa / b

where K is a numerical constant, K = 3.3 for LOD, K = 10 for LOQ, SDa = is the standard deviation of intercept and b = is the slope.

A graph was obtained by plotting the optical density, expressed as raw volume against the concentration of studied drug (µg/spot) to determine the linearity range and correlation coefficient.

Table III. Chemical Structures of the Investigated α-Aminocephalosprin Antibiotics



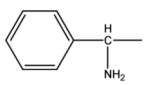
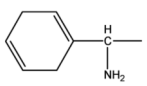
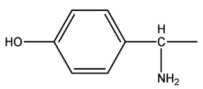
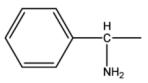
Drug	R_1	R_2	Generation
Cefalexin		-CH3	First
Cefradine		-CH3	First
Cefadroxil		-CH3	First
Cefaclor		-Cl	Second

Table IV. Summary of Quantitative Parameters and Statistical Data Using the Proposed Procedure*

Drug	Intercept (a) ± SD	Slope (b) ± SD	r†	r^2‡	LOD§ (µg/spot)	LOQ** (µg/spot)
Cefaclor	2.76 ± 0.10	3.77 ± 0.02	0.9996	0.9992	0.09	0.27
Cefadroxil	1.48 ± 0.15	3.29 ± 0.07	0.9993	0.9986	0.15	0.46
Cefalexin	0.60 ± 0.22	2.62 ± 0.03	0.9996	0.9992	0.28	0.84
Cefradine	1.34 ± 0.17	2.40 ± 0.05	0.9995	0.9990	0.23	0.71

* Calibration range: 2–10 µg/spot, (n = 3); † r = Correlation coefficient.
‡ r^2 = Determination coefficient. § Limit of detection. ** Limit of quantitation.

Results and Discussion

Different solvent systems were tried for the separation of each of the studied drugs from its impurities and/or degradation products. Table I shows the effect of using different solvent systems for separation of cefalexin, which was chosen as a representative example. Compact spots as well as complete separation of all studied drugs were obtained using different ratios of ethyl acetate–methanol–water (Table II). This could be explained on the basis that these compounds are structurally related. The chemical structures of the investigated csephalosporins are given in Table III.

Ninhydrin (1,2,3-indantrione monohydrate) when heated with α-amino acids, a blue violet colored product is obtained (25). The reaction pathway can be presented as following (26).

This reaction was used as a basis for the determination of the cited drugs. As the investigated drugs contain a-amino group in theri moleties, this reaction was used as a basis for their densitometric analysis. L-arginine reacts with ninhydrin via the same mechanism (27). Ninhydrin has been also reported for colorimetric determination of cephalosporins (28,29). TLC with ninhydrin detection is employed as an identification test in USP *31* (1).

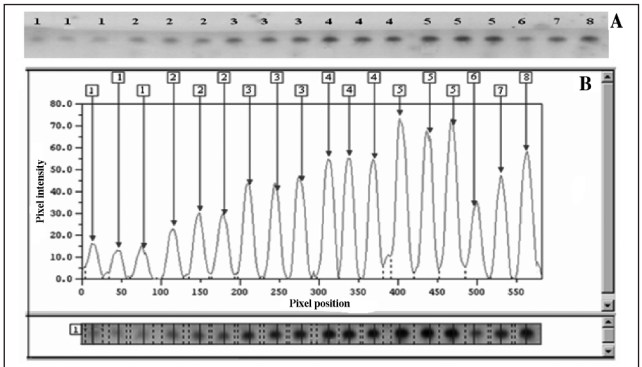


Figure 2. Assessment of accuracy of the proposed TLC method for analysis of cefalexin at three concentration levels: 4, 6, and 8 µg/spot (spots 6, 7, and 8; respectively). The calibration concentration were 2, 4, 6, 8, and 10 µg/spot (spots 1–5; respectively). The image for the developed TLC plate (A) and the data processed by the software (B).

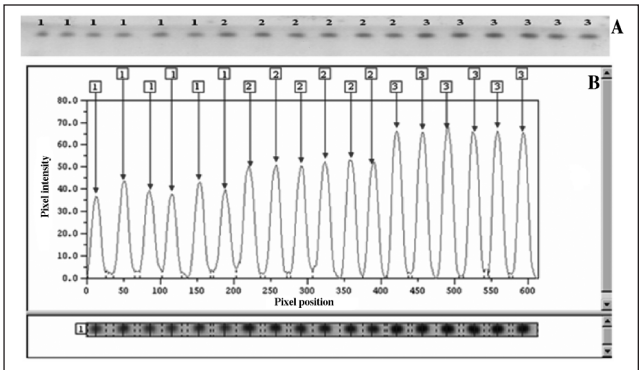


Figure 3. Assessment of precision of the proposed TLC method for analysis of cefalexin at three concentration levels: 4, 6, and 8 µg/spot (spots 1,2 and 3; respectively). The image for the developed TLC plate (A) and The data processed by the software (B).

Calibration curves

The linear regression data for the calibration curves shows a good linear relationship over the concentration range of 2–10 µg/spot with respect to raw volume. Calibration curves had correlation coefficients ranging from 0.9990 to 0.9996 and coefficients of determination ranging from 0.9980 to 0.9992 (Table IV). Figure 1 shows the developed TLC plate for cefalexin either under UV lamp or after spraying with ninhydrin reagent and it also shows the data processed by software and the calibration curve.

Table V. Intra- and Inter-Day Precision of the TLC Method

Drug	Drug Conc. (µg/spot)	Intra-day precision Mean* ± SD	Intra-day precision % RSD	Inter-day precision Mean* ± SD	Inter-day precision % RSD
Cefaclor	4	97.9 ± 2.92	2.99	96.7 ± 3.10	3.21
	6	99.5 ± 2.13	2.14	101.5 ± 2.32	2.29
	8	100.6 ± 1.72	1.71	98.5 ± 1.64	1.67
Cefadroxil	4	103.0 ± 2.78	2.70	97.4 ± 2.97	3.04
	6	102.1 ± 2.35	2.30	98.0 ± 2.15	2.20
	8	99.6 ± 1.54	1.5	98.9 ± 1.12	1.14
Cefalexin	4	97.6 ± 3.01	3.08	97.8 ± 2.90	2.96
	6	98.0 ± 2.51	2.56	99.5 ± 2.31	2.32
	8	101.8 ± 1.92	1.90	100.3 ± 1.35	1.34
Cefradine	4	97.0 ± 2.99	3.08	97.0 ± 3.11	3.21
	6	98.5 ± 2.11	2.15	98.2 ± 2.52	2.57
	8	99.9 ± 1.22	1.22	100.5 ± 1.65	1.64

* Average of six determinations.

Table VI. Robustness of the Proposed TLC Method

Exp. parameter variation	Recovery (%) ± SD* Cefaclor	Cefadroxil	Cefalexin	Cefradine
No variation†	100.9 ± 2.58	99.4 ± 2.21	99.8 ± 2.31	99.5 ± 1.82
1-Volume of spraying reagent				
9.0 mL	101.8 ± 2.11	98.3 ± 0.85	99.5 ± 1.47	101.5 ± 2.32
11.0 mL	97.5 ± 2.54	98.6 ± 1.19	98.5 ± 1.83	98.2 ± 2.52
2-Ninhydrin concentration				
0.4%	99.4 ± 2.31	102.0 ± 0.25	100.3 ± 1.35	98.2 ± 1.80
0.6%	102.1 ± 2.35	98.5 ± 2.11	100.9 ± 0.92	98.0 ± 2.15
3-Heating temperature				
105°C	99.5 ± 2.13	98.0 ± 2.15	97.9 ± 2.20	102.4 ± 2.56
115°C	102.7 ± 2.21	100.9 ± 2.15	100.8 ± 1.99	100.4 ± 1.90
4-Heating time				
5 min	97.5 ± 1.98	100.5 ± 1.23	98.3 ± 2.34	97.4 ± 2.12
15 min	101.4 ± 2.04	102.0 ± 1.88	99.2 ± 1.56	98.5 ± 1.57

Experimental parameter variation	Recovery (%) ± SD*
Mobile phase: Ethyl acetate–methanol–water	Cefalexin 99.8 ± 2.311
No variation†	
(7:3:3)	100.3 ± 2.22
(5:3:3)	97.7 ± 2.21
(6:4:3)	102.2 ± 1.78
(6:2:3)	98.7 ± 1.45
(6:3:4)	101.9 ± 1.00
(6:3:2)	99.5 ± 2.31

* Average of three determinations and drug concentration used; 6 µg/spot.
† Following the general assay procedure conditions.

Method validation study

The developed procedure was validated according to ICH (24) and complied with USP 31 validation guidelines (1).

Accuracy

The accuracy of the method was determined by investigating the recovery percentage of each of the studied drugs at three concentration levels covering the specified range (six replicates of each concentration) (Figure 2). The recovery percentages ranged from 98.3 to 102.2%, which indicate good accuracy of the proposed method.

Precision

The precision of the method was expressed as the agreement between the results of analyses carried out repeatedly (Figure 3). The obtained results reveal good precision of the assay method (Table V), and % RSD values were ≤ 3.21 %.

LOD and LOQ

The LOD and LOQ were also determined. The obtained values indicate high sensitivity of the proposed method. The LOD and LOQ for all studied drugs ranged from 0.09 to 0.28 and from 0.27 to 0.84 µg/spot, respectively (Table IV).

Robustness

Robustness was examined by evaluating the influence of small variation in the experimental parameters on the analytical performance of the method (30). The most critical parameters were interchanged while keeping other parameters unchanged and the chromatographic behavior was observed and recorded, and the recovery percentage was calculated each time. The studied parameters were: the composition of the mobile phase (mobile phases having different composition of ethyl acetate–methanol–water were tried at one concentration level, 6 µg/spot, 3 times), ninhydrin concentration (± 0.1%), volume of spraying reagent (± 1.0 mL), heating temperature (± 5°C), and heating time (± 5 min). It was found that none of these variables significantly affect the performance of the method. The obtained results are shown in Table VI.

Forced-degradation study

The ICH guideline mentioned that stability testing of drug substances and products requires the stress testing to be carried out to elucidate the inherent stability characteristics of the active substance and provide a rapid identification of differences that

Table VII. Recovery Percentages for Forced Degradation of Cefalexin

Time (min)	0.01M NaOH CR* (µg/6 µg) ± SD†	0.01M NaOH Recovery (%)	0.5M HCl CR (µg/6 µg) ± SD†	0.5M HCl Recovery (%)
1	4.3 ± 0.10	71.7	3.2 ± 0.23	53.3
5	2.5 ± 0.22	41.7	2.2 ± 0.12	36.7
10	1.1± 0.13	18.3	1.4 ± 0.10	23.3
15	0.5 ± 0.18	8.3	0.9 ± 0.08	15.0
25	0	0	0	0
30	0	0	0	0

* CR = Content remained; † Average of three determinations.

might result from changes in the manufacturing processes or source sample (31). Susceptibility to oxidation, acid/base hydrolysis, and photolytic stability are the required tests. An ideal stability-indicating method is one that quantifies the standard drug alone and also resolves its degradation products. Acid–base hydrolysis at different time intervals was chosen to illustrate the ability of the developed method to determine the studied drugs in the presence of their forced degradation products. The proposed concentrations of NaOH (0.01M) and HCl (0.5M) were chosen to analyze the intact drug in the presence of its degradation products. Higher concentrations induced rapid degradation, and the spot of the intact drug was completely disappeared. The developed chromatograms of the samples degraded with acid and base showed well-separated spots of pure drugs as well as some additional spots at different R_f values (0.8 and 0.01). The spots of degraded products were well resolved from the drug spot. The content of the drug remained and recovery percentage were calculated and listed in Table VII. Figure 4 shows the data processed for the developed TLC plate taking cefalexin as an example for acidic degradation.

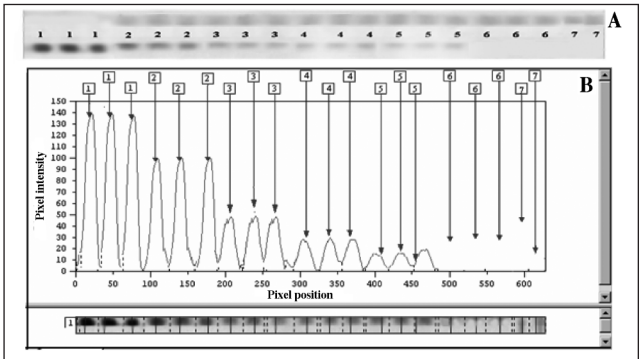


Figure 4. Degradation of 6 µg/spot of cefalexin with 0.5M HCl. Spot 1 represents intact cefalexin (6 µg/spot) whereas spots 2, 3, 4, 5, 6 and 7 represent cefalexin after 1, 5, 10, 15, 25, and 30 min; respectively of its contact with 0.5M HCl. The image for the developed TLC plate (A) and the data processed by the software (B).

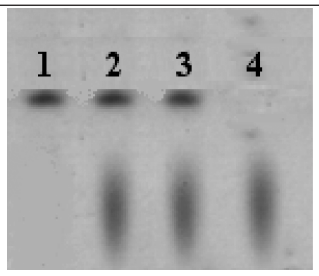


Figure 5. The developed TLC plate after spraying with ninhydrin reagent. Spot 1 is pure cefradine (6 µg/spot), spot 2 and 3 are Velosef suspension and Velosef vials; respectively (6 µg/spot) and spot 4 is pure L-arginine (0.5 µg/spot).

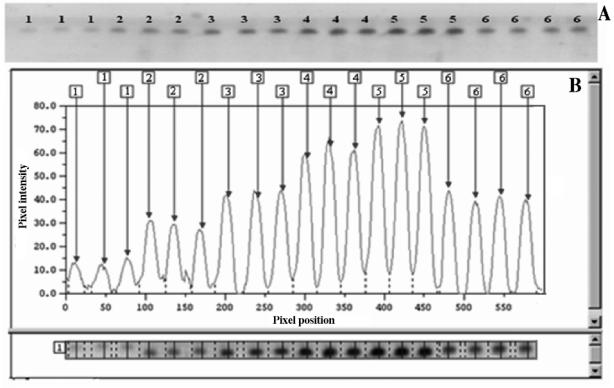


Figure 6. Thin layer chromatographic analysis of Duricef® tablets containing cefalexin. Spots 6 are the sample of the test tablet solution and spots 1-5 are the calibration standard solutions of concentration 2, 4, 6, 8, and 10 µg/spot; respectively. Part A is the image for the developed TLC plate; and part B is the data processed by the software.

Selectivity

The selectivity of an assay is a measure of the extent to which the method can determine a particular compound in the analyzed matrices without interference from matrix components. Figure 5 shows the developed TLC plate for two pharmaceutical products, Velosef suspension and Velosef vials, containing L-arginine plus cefradine, which was completely separated from the spot due to L-arginine. The proposed method could be considered selective and can be used to determine the studied drugs in

Table VIII. Determination of the Studied Drugs in their Pharmaceutical Formulations by the Proposed and the Reported Method at 95% Confidence Level

Drug	Pharmaceutical formulation	Recovery (%) ± SD PM* (n = 4)	Recovery (%) ± SD RM*,† (n = 4)
Cefaclor	Ceclor® suspension§ 250 mg of cefaclor monohydrate/5 mL	98.7 ± 0.30 t = 1.664‡, F =1.193‡	99.0 ± 0.20
	Bacticlor® suspension** 250 mg of cefaclor anhydrous/5 mL	97.9 ± 1.30 t = 1.089, F = 1.032	98.9 ± 1.30
Cefadroxil	Duricef® tablets†† 1 g of cefadroxil monohydrate/tablet	97.8 ± 0.50 t = 0.312, F = 1.641	97.7 ± 0.40
	Duricef® suspension†† 250 mg of cefadroxil monohydrate/5 mL	99.3 ± 1.60 t = 0.600, F = 1.770	98.7 ± 1.20
	Duricef® capsules†† 500 mg of cefadroxil monohydrate/capsule	97.2 ± 0.50 t = 1.562, F = 1.441	96.7 ± 0.40
	Biodroxil® capsules‡‡ 500 mg of cefadroxil monohydrate/capsule	102.3 ± 1.40 t = 1.405, F = 4.498	101.2 ± 0.70
	Biodroxil® suspension‡‡ 250 mg of cefadroxil monohydrate/5 mL	103.5 ± 1.50 t = 0.524, F = 3.357	103.1 ± 0.80
Cefalexin	Ceporex® tablets§§ 500 mg of cefalexin anhydrous/tablet	96.6 ± 1.30 t = 1.310, F = 2.879	97.6 ± 0.80
	Ceporex® suspension§§ 250 mg of cefalexin anhydrous/5 mL	98.9 ± 1.30 t = 1.219, F = 3.626	98.0 ± 0.70
	Ospexin® suspension*** 250 mg of cefalexin anhydrous/5 mL	103.1 ± 0.60 t = 1.109, F = 2.497	102.7 ± 0.40
Cefradine	Velosef® capsules** 250 mg of cefradine anhydrous/capsule	98.2 ± 1.80 t = 0.298, F = 3.932	97.9 ± 0.90
	Velosef® tablets** 1 g of cefradine anhydrous/tablet	102.1 ± 1.40 t = 0.650, F = 1.381	101.5 ± 1.20

* PM = Proposed method and RM = Reported method; † Reference 32. ‡ theoretical value for t and F at 95% confidence limit, t = 2.447 and F = 9.280; § Egyptian Pharmaceuticals and Chemicals Industries, S.A.E., Bayad El-Arab, Beni Suef, Egypt; ** Pharco Pharmaceuticals, Alexandria under license from Ranbaxy UK; †† Bristol-Myers Squibb Pharmaceutica, Cairo, Egypt; ‡‡ Kahira Pharm. & Chem. under license from Novartis Pharma S.A.E., Cairo, Egypt; §§ GlaxoSmithKline, S.A.E., El Salam City, Cairo, Egypt; *** Pharco Pharmaceuticals, Alexandria under license from Biochemie GmbH., Vienna, Austria.

Table IX. Standard Addition Method for the Assay of the Studied Drugs in Their Pharmaceutical Dosage Forms by the Proposed Method

Pharmaceutical formulation	Authentic drug added to* (µg/spot)	Authentic drug found (µg/spot)	Recovery (%) ± SD†
Ceclor	0.00	0.00	98.7 ± 0.30
suspension	2.00	1.94	97.0 ± 1.20
	4.00	4.12	103.0 ± 0.90
Bacticlor	0.00	0.00	97.9 ± 1.30
suspension	2.00	1.95	97.5 ± 1.20
	4.00	4.09	102.3 ± 0.80
Duricef	0.00	0.00	97.8 ± 0.50
tablets	2.00	2.01	100.5 ± 0.70
	4.00	3.95	98.8 ± 1.40
Duricef	0.00	0.00	99.3 ± 1.60
suspension	2.00	1.94	97.0 ± 1.20
	4.00	3.97	99.3 ± 1.10
Duricef	0.00	0.00	97.2 ± 0.50
capsules	2.00	1.97	98.5 ± 0.40
	4.00	4.13	103.3 ± 1.00
Biodroxil	0.00	0.00	102.3 ± 1.40
capsules	2.00	2.04	102.0 ± 1.20
	4.00	4.10	102.5 ± 0.70
Biodroxil	0.00	0.00	103.5 ± 1.50
suspension	2.00	1.98	99.0 ± 0.60
	4.00	4.09	102.3 ± 1.30
Ceporex	0.00	0.00	96.6 ± 1.30
tablets	2.00	2.05	102.5 ± 0.70
	4.00	4.06	101.5 ± 0.60
Ceporex	0.00	0.00	98.0 ± 0.70
suspension	2.00	1.96	98.0 ± 1.00
	4.00	3.97	99.3 ± 1.20
Ospexin	0.00	0.00	103.1 ± 0.60
suspension	2.00	1.98	99.0 ± 0.80
	4.00	4.03	100.8 ± 0.90
Velosef capsules	0.00	0.00	98.2 ± 1.80
	2.00	2.06	103.0 ± 0.50
	4.00	4.09	102.3 ± 0.70
Velosef	0.00	0.00	102.1 ± 1.40
tablets	2.00	1.97	98.5 ± 1.20
	4.00	3.99	99.8 ± 0.80
Velosef	0.00	0.00	97.1 ± 0.70
suspension‡	2.00	1.93	96.5 ± 0.70
	4.00	3.90	97.5 ± 1.30
Velosef	0.00	0.00	96.7 ± 0.90
vials†	2.00	1.94	97.0 ± 0.60
	4.00	3.89	97.3 ± 1.10

* Drug concentration used is 6 µg/spot; † Average of four determinations; ‡ Bristol-Myers Squibb Pharmaceutical Co., Cairo, Egypt (Velosef suspension contains 250 mg of cefradine anhydrous/5 mL and Velosef vials contains 1 g of cefradine anhydrous/vial).

the presence of their degradation products as well as marketed formulations containing the studied drugs plus other ninhydrin-positive inactive ingredients (e.g., L-arginine).

Applications

The proposed method was applied successfully for determination of the studied drugs in their pharmaceutical dosage forms. Four replicate measurements were made in each case, the results obtained were validated by comparison with a previously reported method (32) by means of t- and F-tests at 95% confidence level (Table VIII). No significant difference was found, indicating good accuracy and precision. Figure 6 shows the developed TLC after spraying with ninhydrin reagent and it also shows the data processed by software. Recovery studies were also carried out by standard addition method (33,34) through addition of different amounts of authentic cephalosporin antibiotic to the corresponding sample antibiotic (pharmaceutical formulation) and the proposed method was then applied. Results obtained in Table IX indicate good recoveries (96.5% to 103.5%) and confirm the absence of interference due to common excipients.

Conclusion

The developed densitometric technique is precise, selective, accurate, and sensitive. The processing of samples and standards together at the same time (in-system calibration) leads to improved reproducibility and accuracy. Statistical analysis proves that the method is repeatable and selective for the analysis of cefaclor monohydrate, cafalexin anhydrous, cefadroxil monohydrate, and cefradine anhydrous in bulk drugs and in pharmaceutical formulations. The proposed method could be considered more selective than visible spectrophotometric analysis with ninhydrin reagent as the latter could not be used for products containing the studied drugs plus other ninhydrin-positive inactive ingredients (L-arginine). In addition, the method can be used to determine the purity of the drug available from various sources by detecting the related impurities. As the method separates the drugs from their degradation products, it can be employed as a promising stability indicating assay. This method is worth recommending because of a short time of analysis, little usage of reagents and simplicity.

References

1. United States Pharmacopeia 31 and NF 26, American Pharmaceutical Association, Washington, DC, 2008.
2. G.A. Saleh. Two selective spectrophotometric methods for the determination of amoxicillin and cefadroxil. *Analyst* **121:** 641–645 (1996).
3. C.S.P. Sastry, K.R. Rao, and D.S. Prasad. Determination of cefadroxil by three simple spectrophotometric methods using oxidative coupling reactions. *Microchim. Acta* **126:** 167–172 (1997).
4. L.G. Martinez, P.C. Falco, and A.S. Cabeza. Comparison of several methods used for the determination of cephalosporins. Analysis of cefalexin in pharmaceutical samples. *J. Pharm. Biomed. Anal.* **29:** 405–423 (2002).
5. I.F. Al-Momani. Flow-Injection spectrophotometric determination of amoxycillin, cefalexin, ampicillin, and cefradine in pharmaceutical formulations. *Anal. Lett.* **37:** 2099–2110 (2004).
6. H.A. El-Obeid, E.A. Gad-Kariem, K.A. Al-Rashood, H.A. Al-Khames, F.S. El-Shafie, and G.A.M. Bawaseer. A selective colorimetric method for the determination of penicillins and cephalosporins with α-aminoacyl functions. *Anal. Lett.* **32:** 2809–2823 (1999).
7. J.H. Yang, G.J. Zhou, N.Q. Jie, R.J. Han, C.G. Lin, and J.T. Hu. Simultaneous determination of cefalexin and cefadroxil by using the coupling technique of synchronous fluorimetry and H-point standard additions method. *Anal. Chim. Acta* **325:** 195–200 (1996).
8. W.P. Cai, Y.G. Ouyang, X.Y. Lin, and J.G. Xu. Phtochemical fluorimetric determination of cefradine. *Anal. Lett.* **31:** 439–450 (1998).
9. J. Yang, G.J. Zhou, X.H. Cao, Q.L. Ma, and J. Dong. Study on the fluorescence characteristics of alkaline degradation of cefadroxil, cefradine, cefotaxime sodium and amoxicillin. *Anal. Lett.* **31:** 1047–1060 (1998).
10. F.A. Aly, M.M. Hefnawy, and F. Belal. A selective spectrofluorimetric method for the determination of some α-aminocephalosporins in formulations and biological fluids. *Anal. Lett.* **29:** 117–130 (1996).
11. Q.L. Li and S. Chen. Studies on electrochemical behavior of cefalexin. *Anal. Chim. Acta* **282:** 145–152 (1993).
12. O. Chailapakul, P. Aksharanandana, T. Frelink, Y. Einaga, and A. Fujishima. The electrooxidation of sulfur-containing compounds at boron-doped diamond elec-

trode. *Sens. Actuators, B* **80:** 193–201 (2001).
13. O. Chailapakul, A. Fujishima, P. Tipthara, and H. Siriwongchai. Electroanalysis of glutathione and cefalexin using the boron-doped diamond thin-film electrode applied to flow-injection analysis. Anal. Sci. 17: i417–i422 (supplement 2001).
14. J.G. Kirchner, Techniques of Chemistry, 2nd ed., vol. XIV: Thin Layer Chromatography, 1978.
15. S. Eric-Jovanovic, D. Agbaba, D. Zivanov-Stakic, and S. Vladimirov. HPTLC determination of ceftriaxone, cefixime and cefotaxime in dosage forms. *J. Pharm. Biomed. Anal.* **18:** 893–898 (1998).
16. D. Agbaba, S. Eric, D.Z. Stakic, and S. Vladimirov. HPTLC assay of cefalexin and cefaclor in pharmaceuticals. *Biomed. Chromatogr.* **12:** 133–135 (1998).
17. C.V. Shabadi, B.A. Shelar, A.R. Shelar. Simultaneous determination of cefadroxil and cefalexin in pharmaceutical preparations by quantitative TLC. *Indian Drugs* **35:** 766–770 (1998).
18. S.A. Coran, M. Bambagiotti-Alberti, V. Giannellini, A. Baldi, G. Picchioni, and F. Paoli. Development of a densitometric method for the determination of cefalexin as an alternative to the standard HPLC procedure. *J. Pharm. Biomed. Anal.* **18:** 271–274 (1998).
19. U.P. Halkar and N.P. Bhandari. Simultaneous determination of cefalexin and probenecid in pharmaceutical preparations by HPTLC. *Indian Drugs* **35:** 382–383 (1998).
20. C. Sullivan and J. Sherma. Development and validation of an HPTLC-densitometry method for assay of glucosamine of different forms in dietary supplement tablets and capsules. *Acta Chromatographia* **15:** 119–130 (2005).
21. J. Pachuski, B. Fried, and J. Sherma. HPTLC analysis of amino acids in Biomphalaria glabrata infected with Schistosoma mansoni. *J. Liq. Chromatogr. Relat. Technol.* **25:** 2345–2349 (2002).
22. R.A. Steiner, B. Fried, and J. Sherma. HPTLC determination of amino acids in snail-conditioned water from Biomphalaria glabrata, two strains of Helisoma trivolvis, and Lymnaea elodes. *J. Liq. Chromatogr. Relat. Technol.* **21:** 427–432 (1998).
23. I. Quintens, J. Eykens, E. Roets, and J. Hoogmartens. Identification of cephalosporins by thin-layer chromatography and color reactions. *J. Planar Chromatogr. - Mod. TlC* **6:** 181–186 (1993).
24. Topic Q2 (R1): Validation of Analytical Procedures: Text and Methodology, International Conference on Harmonisation (ICH), Nov 2005. Through www.emea.europa.eu/pdfs/human/ich/038195en.pdf
25. R.L. Shirner, R.C. Fuson, D.Y. Curtn, and T.C. Morill. The systemic identification of organic compounds, 6th ed., John Wiely & Sons, New York, 1979, p. 242.
26. I.L. Finar, Organic Chemistry, 5th ed., vol. 2: Stereochemistry and the chemistry of natural products, Longman Group LTD, 1986, p. 654
27. http://antoine.frostburg.edu/chem/senese/101/organic/faq/amino-acid-test.shtml <http://antoine.frostburg.edu/chem/senese/101/organic/faq/amino-acid-test.shtml>
28. O.I. Abd El-Sattar, N.M. El-Abasawy, S.A. Abd El-Razeq, M.M.F. Ismail, and N.S. Rashed. Spectrophotometric determination of cefepime and cefprozil by three different methods. *Saudi Pharma. J.* **9:** 186–192 (2001).
29. M.M. Mahrous and M.M. Abdelkhalek. Spectrophotometric determination of certain cephalosporins with ninhydrin. *Analyst* **109:** 611–613 (1984).
30. Y.V. Heyden, A. Nijhuis, J. Smeyers-Verbeke, B.G.M. Vandeginste, and D.L. Massart. Guidance for robustness: ruggedness tests in method validation. *J. Pharm. Biomed. Anal.* **24:** 723–753 (2004).
31. International Conference on Harmonisation (ICH), Guidance for Industry, Q1A (R2): Stability Testing of New Drug Substances and Products, IFPMA, Geneva, 2000. Through http://www.fda.gov/cber/gdlns/ichstab.htm
32. G.A. Saleh, H. Askal, I. Darwish, and A. El-Shorbagi. Spectroscopic analytical study for the charge-transfer complexation of certain cephalosporins with cholranilic acid. *Anal. Sci.* **19:** 281–287 (2003).
33. L.C. Rodriguez, L.G. Gracia, E.M.A. Loèpez, and J.M.B. Sendra. Calibration in chemical measurement processes. II. A methodological approach. *Trends Anal. Chem.* **20:** 620–636 (2001).
34. D. Harvey, Modern Analytical Chemistry, McGraw-Hill, Boston, MA, 2000, p. 108.

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