Validated Chromatographic Methods for Determination of Hydrochlorothiazide and Spironolactone in Pharmaceutical Formulation in Presence of Impurities and Degradants

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Abstract

Two specific, sensitive, and precise stability indicating chromatographic methods have been developed, optimized, and validated for Hydrochlorothiazide (HCT) and Spironolactone (SPR) determination in their mixtures and in presence of their impurities and degradation products. The first method was based on thin layer chromatographic (TLC) combined with densitometric determination of the separated spots. The separation was achieved using silica gel 60 F_{254} TLC plates and ethyl acetate–chloroform–formic acid–triethyl amine (7:3:0.1:0.1, by volume) as a developing system. Good correlations were obtained between the integrated peak area of the studied drugs and their corresponding concentrations in different ranges. The second method was based on the high-performance liquid chromatography with ultraviolet detection, by which the proposed components were separated on a reversed phase C_{18} analytical column using gradient elution system with deionized water–acetonitrile (97:3, v/v) for 8 min. Then acetonitrile was successively increased to 35% in the next 2 min, and kept constant in the following 10 min, finally 3% acetonitrile was regained again to stabilize the chromatographic system. The flow rate was maintained at 2 mL/min and the detection wavelength was at 230 nm. Linear regressions were obtained in the range of 4.0–50 µg/mL and 5.0–50 µg/mL for both HCT and SPR, respectively. Different parameters affecting the suggested methods were optimized for maximum separation of the cited components. System suitability parameters of the two developed methods were also tested. The suggested methods were validated in compliance with the ICH guidelines and were successfully applied for determination of HCT and SPR in their commercial tablets. Both methods were also statistically compared to each other and to the reported method with no significant difference in performance.

Introduction

Hydrochlorothiazide (HCT) (Figure 1A) is chemically designated as (6-Chloro-3,4-di hydro-2H-1,2,4-benzothiadiazine-7-sulfonamide1,1-dioxide) (1). It is one of the benzothiadizines diuretics that are widely used in antihypertensive formulations either alone or in combination with other drugs acting by decreasing active sodium absorption and reducing the peripheral vascular resistance (2). Spironolactone (SPR) (Figure 1B), is chemically designated as (17-hydroxyl-7α–mercapto-3-oxo-17 α pregn-4ene-21carboxylic acidγ-lactone acetate) (3). It is a synthetic steroid and an aldosterone competitive antagonist, which has been used as an effective diuretic agent, especially in patients of heart failure or liver cirrhosis (4). A combination of both HCT and SPR is used to improve urine output and lung function in infants with broncho-pulmonary dysplasia (5).

Several high-performance liquid chromatography (HPLC) techniques were described for the determination of HCT and SPR in their binary mixture, either as official methods (3), or non-official reported methods (6–15), alone or in combination with other diuretics, in pharmaceutical formulations, or in biological fluids. A gas chromatography-mass spectrometry method was also described for the determination of a number of drugs

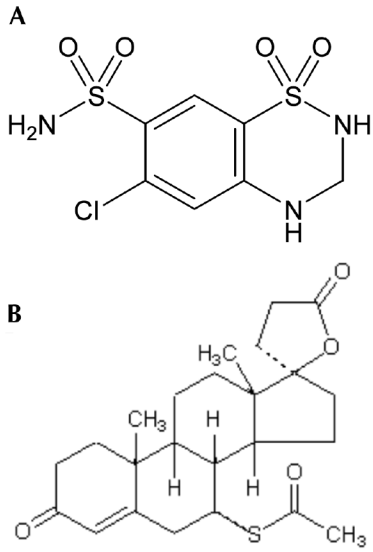

Figure 1. Chemical structure of hydrochlorothiazide (A) and spironolactone (B).

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including the proposed compounds (16). Also the chemiluminescent behaviors of both diuretics and other diuretics were studied (17).

Several spectrophotometric methods were also reported for determination of the cited drugs in their binary mixture, including ratio spectra (18,19) and chemometric techniques along with carenone (20,21). Derivative and indirect spectrophotometric methods were also used for their determination (22,23). Colorimetric determination was described (24).

A comprehensive literature search revealed the lack of a suitable stability-indicating method for determination of the studied drugs in pharmaceutical formulations without interference from drug impurities or degradation products. All the reported chromatographic methods failed to separate the parent drugs from their impurities and degradation products.

So, the present work deals with the determination of both active compounds and assessment of stability of the bulk drugs and of pharmaceutical dosage forms using a simple TLC and a gradient RP-HPLC method.

Experimental

Instruments

For TLC, the samples were spotted with a sample applicator for TLC linomat V with 100 µL syringe (Camage, Muttenz, Switzerland) on TLC plates (20 × 10 cm) coated with silica gel 60 F_{254} (Merck KgaA, Darmstad, Germany) with 200 µm thickness. Scanning was performed on TLC Scanner 3 densitometer (Camage) controlled by winCATS software (V 3.15, Camage). A UV lamp with short wavelength 254 nm (Germany) was used for scanning until optimization of the proposed method. The LC system (Perkin-Elmer, Waltham, MA) consisted of Series 200 Vacuum degasser, Series 200 LC pump, Series 200 variable-wavelength UV–Vis detector and Series 200 autosampler fitted with a 200 µL sample loop. LC separations were performed on Phenomenex C_{18} analytical column (22 × 4.6 mm, i.d, 5 µm p.s.) (Torrance, CA) and data were processed using TotalChrom Workstation Chromatography Software. Mass spectra (MS) were run on JEOL JMS- AX 500 mass spectrometer (Tokyo, Japan) at 70 ev, and IR spectra were recorded using Bruker Vector 22 spectrometer (Tokyo, Japan), using KBr discs and values were represented in cm^{-1}.

Samples

Authentic samples

Hydrochlorothiazid (HCT, 98.50%) was kindly supplied by El Ameria Pharm. Ind., Alexandria, Egypt. SPR (SPR, 99.00%) was kindly supplied by El Kahira Pharm. and Chem. Ind. Co. (Cairo, Egypt). Chlorothiazide (CT, 99.50%) and DSA (DSA, 99.25%) were supplied by Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany.

Commercial products

Aldactazide tablets (Batch No. 0611108) labeled to contain 25 mg of each of HCT and SPR manufactured by El Kahira Pharm. and Chem. Ind. Co., (under license of Searle Pharmaceuticals, Division of G.D Searle and Co., Ltd., England) were purchased from the local market.

Degraded samples

Preparation of SPR degradation product (SPR Deg). 0.5 gm of powdered SPR was refluxed with 75 mL of 0.1N NaOH for 2 h. The degradation process was followed using TLC, where chloroform–ethyl acetate–formic acid–tri ethyl amine (5:5:0.10:0.15, by volume) as a developing system. The solution was cooled, extracted with chloroform to remove any traces of SPR that may be present, and the pH was adjusted to pH 6. A yellow precipitate of SPR Deg was then obtained, filtered, and washed three times each with 10 mL of distilled water and left to dry at 60°C for 5 h. The obtained degradation product was subjected to IR and MS analyses for subsequent structure elucidation.

Chemicals and solvents

All chemicals and solvents used throughout this work were of analytical grade and were purchased from El-NASR Pharmaceutical Chemicals Co., Abu-Zabaal, Cairo, Egypt. Acetonitrile was HPLC grade (SDS, France).

Solutions

Stock solutions

Standard stock solutions of both HCT and SPR of 1 mg/mL concentration were prepared in either methanol (for TLC method) or in acetonitrile (for HPLC method). Stock solutions containing 1 mg/mL of each of CT, DSA, and SPR degradate were prepared in methanol (for TLC method) or acetonitrile (for HPLC method).

Working solutions

Working standard solutions were made of HCT and SPR (0.1 mg/mL). Appropriate dilutions were made from the stock standard solutions of both HCT and SPR to prepare their corresponding working standard solutions. Working solutions were made of CT, DSA, and SPR degradate (0.1 mg/mL).

Appropriate dilutions were made from their stock solutions to prepare the corresponding working solutions.

Procedure

Chromatographic conditions

TLC-densitometry

TLC was performed on 20 × 10 cm TLC aluminum sheets precoated with 0.25 mm silica gel 60 F_{254}, the plates were prewashed with methanol and activated at 100°C for 5 min. The samples were applied as bands (bandwidth: 6 mm; spacing: 8.9 mm; 15 mm from bottom edge of the plate). Linear ascending development was done in a chromatographic tank previously saturated with ethyl acetate–chloroform–formic acid–triethyl amine (7:3:0.1:0.1, by volume) for 1 h at room temperature to a distance of approximately 80 mm from the lower edge. The developed plates were air dried and scanned at 235 nm on Camage TLC scanner 3 operated in the absorbance mode; with deuterium lamp as a source of radiation; the slit dimension was

kept at 3 mm × 0.45 mm and 20 mms^{-1} scanning speed was employed.

RP-HPLC method

HPLC was carried out at ambient temperature on RP-C_{18} column (220 × 4.6mm i.d., 5 µm p.s.). The mobile phase consisted of water–acetonitrile. The initial proportion of the mobile phase was 97:3 (v/v). A gradient elution was used during the analysis (from 97:3 to 65:35, v/v). The mobile phase was filtered using 0.45 µm Millipore membrane filter (Billerica, MA) and was delivered at rate of 2 mL/min. The injection volume was 15 µL and the effluent was detected at 230 nm.

Linearity

TLC method

Accurate aliquots (4–18 µL) and (3–15 µL) of HCT and SPR working standard solutions, respectively, were separately applied in triplicate to TLC plates, chromatographed, and scanned. The calibration curves relating the integrated area under the peak to the corresponding concentrations of HCT and SPR, as µg/band, were respectively constructed.

RP-HPLC method

Working standard solutions (0.1 mg/mL) of HCT and SPR were further diluted with acetonitrile to obtain dilutions in the range of (4–50 and 5–50 µg/mL) of each of HCT and SPR respectively. Triplicate 15 µL injections were made for each prepared solution and chromatographed. The peak areas $*10^{-6}$ were plotted against the corresponding concentrations to obtain the calibration graph for each component.

Application to commercial products

Twenty tablets of Aldactazide were separately weighed and finely powdered. An accurately weighted portion of the powder equivalent to 0.1 gm of each of HCT and SPR was separately transferred into 100-mL volumetric flask and then 75 mL methanol (or acetonitrile for HPLC method) was added. The prepared solution was sonicated for 45 min, the volume was completed with the same solvent to get 1 mg/mL of each drug, and the prepared solution was filtered. An appropriate dilution was made to prepare the working solution.

Application of standard addition technique

When carrying out the standard addition technique, different known concentrations of pure standard HCT and SPR were added to the pharmaceutical formulation before proceeding in the previously mentioned methods.

Results and Discussion

The ideal stability-indicating method was one that quantified the standard drug alone and also resolved its degradation products. The British Pharmacopeia (25) reported that, both CT and DSA were considered to be HCT process impurities. In another report (26), it was cited that HCT had one primary degradation pathway which yielded 4-amino-6-chlorobenzen-1,3 disulphonamide, DSA, and formaldehyde by hydrolysis. CT was found to be pharmacologically less active than HCT (27,28). Also, it was reported that HCT was fairly rapidly absorbed from gastrointestinal tract, and CT was incompletely and variably absorbed (29). SPR was subjected to acid and alkaline hydrolysis, oxidation, and photo degradation. The drug underwent degradation only under acidic and basic conditions with the same degradation product and NaOH was the hydrolyzing agent of choice according to ICH guidelines (30). And so, the analysis of the two suggested diuretics became of great importance especially in quality control laboratories.

After preparation, complete separation and purification of SPR degradation product, the drug, and the degradation product were subjected to IR and MS analyses for comparison. The assignment of the SPR degradation product was based on comparison of IR spectral data for the separated compound with that of the intact drug. The IR spectrum of the degradation product showed disappearance of thiol ester group peak at 1690 cm^{-1} in the parent compound and appearance of a new peak of SH group at 2607 cm^{-1} which indicates the breaking of thiol ester linkage. The carbonyl groups at 1679.2 and 1767.9 cm^{-1} in the parent drug, shifted to 1654.5 and 1723.2 cm^{-1} and a new broad band at 3289.9 cm^{-1} appeared indicating hydrolysis of the cyclic ester to its acid derivative. Structure elucidation using MS showed molecular ion peak at *m/z* 416.85 corresponding to the intact drug, and the molecular ion peak of the degradation product was at *m/z* 392.

TLC method

A sensitive, stability-indicating TLC method was suggested for the determination of HCT and SPR in presence of their degradation products. The method was based on the difference in the migration rates of the five compounds using ethyl acetate–chloroform–formic acid–triethyl amine (7:3:0.1:0.1, by volume) as a developing system. It was necessary to test the effect of different variables to optimize this method. Different developing

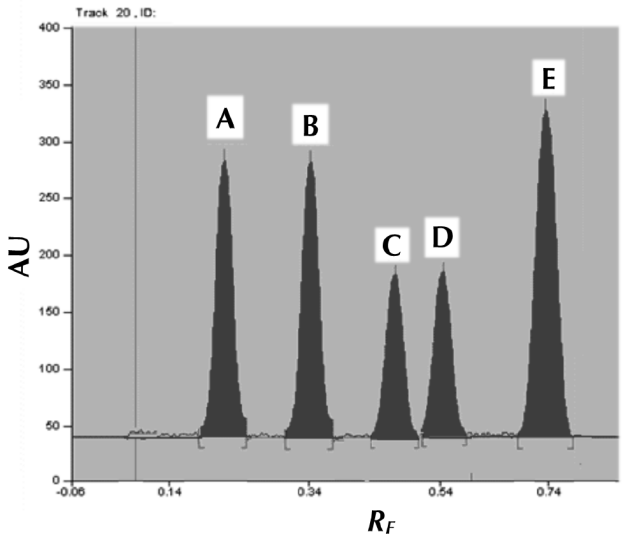

Figure 2. Thin layer chromatogram of chlorothiazide (A), hydrochlorothiazide (B), spironolactone degradate (C), salamide (D), and spironolactone (E), using ethyl acetate–chloroform–formic acid-triethylamine (7:3:0.1:0.1 by volume).

systems of different composition were tested in order to obtain optimum separation. Satisfactory separation was achieved upon using chloroform–ethyl acetate–formic acid–tri ethyl amine (7:3:0.1:0.1, by volume). This system was found to give compact sharp symmetrical spots for the five cited compounds with suitable R_f values at 235 nm. Figure 2 shows a typical chromatogram of the five components.

In order to minimize band diffusion, the optimum bandwidth chosen was 6 mm and the interspaces between bands were 8.9 mm. Different scanning wavelengths were tested, where scanning at 235 nm was suitable providing good sensitivity for both HCT and SPR with a single plate scan.

The time the sample is left to stand on the solvent prior to chromatographic development can influence the stability of separated spots which are required to be investigated for validation (31,32). Two dimensional chromatography was applied to find any decomposition occurring during spotting and development. Because no additional spots were detected, this indicated the stability of drugs in the solutions. In order to detect any related impurities, large volumes of the two drugs standard stock solutions were applied on TLC plate and the chromatogram was run as described previously. No additional spots were observed.

RP-HPLC method

A simple, accurate, and selective RP-HPLC had been investigated and validated for quantitative analysis of HCT and SPR. The LC procedure was optimized with a view to develop a quantitative and stability-indicating method in a convenient time analysis and with high quality separation of the five proposed components. The chromatographic operational conditions were selected by considering the peak resolution and retention times of the first and the last eluted components.

Parameters affecting the efficiency of the chromatographic separation had been tested and optimized in a trial to obtain the maximum separation of the cited components. At the first attempt, an isocratic mobile phase of deionized water–acetonitrile (97:3, v/v) was used to separate the five compounds, unfortunately, SPR and its degradation product eluted after very long time (> 45 min) with tailed peak. However, increasing the organic modifier (acetonitrile) more than 10% resulted in very bad resolution of HCT, CT, and DSA (eluted together as one peak without separation) and also with very long time of analysis for elution of SPR and its degradation product. Several trials were tried by addition of different surface active agents, tetra hydro furan, tri ethyl amine, and different combinations of different mobile phases. None of these trials succeeded in separation of the proposed components.

An elution gradient had been investigated to improve the resolution of the five cited components and decrease the time of analysis. Several trials for optimization of the elution program had been carried out to enhance the resolution. Acetonitrile in the mobile phase was set at 3% until 8 min to allow sufficient resolution of CT, DSA, and HCT, and then subsequently increased to 35% to separate both SPR and its degradation product in suitable time analysis. In the gradient program the organic modifier (acetonitrile) was kept at 3% for 8 min, followed by successive increasing up to 35% within the next 2 min and kept constant in this ratio for 10 min and after that the mobile phase of 3% was regained within 2 min and still at 3% for the last 2 min to stabilize the chromatographic system prior to the next injection.

The pH values had no effect on the separation efficiency. Scanning the effluent at 230 nm gave reasonable sensitivity for the active drugs, but with poor sensitivity for SPR degradation product which had maximum absorption at 280 nm. And so the mixtures were scanned at both wavelengths and the two chromatograms were compared to detect SPR degradation product

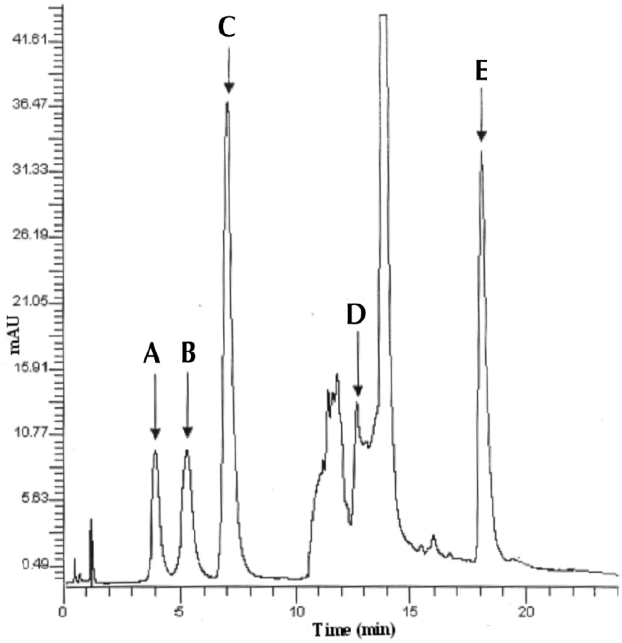

Figure 3. HPLC chromatogram of the mixture of chlorothiazide (A), salamide (B), hydrochlorothiazde (C), spironolactone degradate (D), and spironolactone (E) at 230 nm.

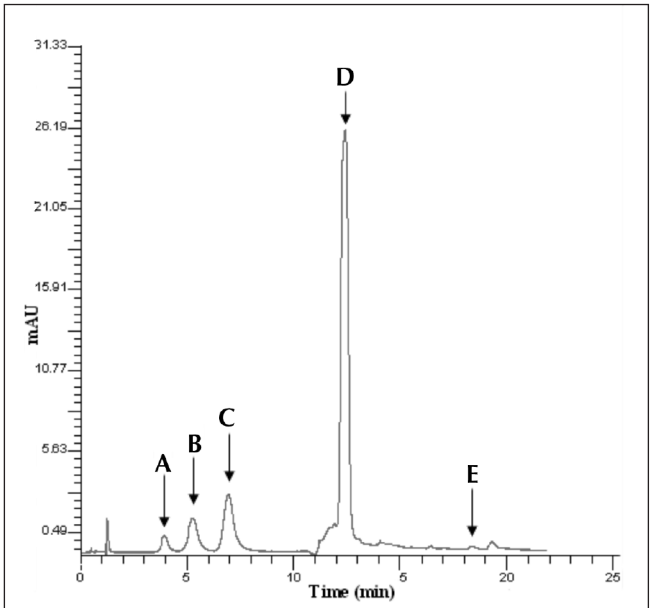

Figure 4. Chlorothiazide (A), salamide (B), hydrochlorothiazde (C), spironolactone degradate (D), and spironolactone (E) at 280 nm.

(Figures 3 and 4). Type of column (C_8 and C_{18}) had no effect on HCT and its impurities but using C_{18} column enhanced the shape of SPR peak. Reasonable separation with good resolution and suitable analysis time was obtained upon using flow rate of 2 mL/min. Under the optimum chromatographic conditions, CT, DSA, HCT, SPR degradation product, and SPR were eluted at 4.1, 5.3, 7.1, 11.7, and 18.25 min, respectively, as shown in Figure 3.

Method Validation

ICH guidelines for method validation (30) were followed for validation of the suggested methods.

Linearity and ranges

Under the previously described experimental conditions, linear relationships were obtained by plotting the drug concentrations against peak areas for each drug, for both chromatographic methods. The corresponding concentration ranges, calibration equations, LOD and LOQ and other statistical parameters are listed in Table I.

Accuracy

The accuracy of the investigated methods was validated by analyzing pure samples of both HCT and SPR in triplicate. The concentrations of the active drugs were calculated from the calculated regression equations or by comparison with standard applied on the same plate (in TLC method). Good results are shown in Table I.

Precision

Precision was evaluated by calculating intra- and inter-day precision by repeating the assay of three different concentrations three times in the same day and assaying the same samples in triplicate on three successive days, using the developed chromatographic methods and calculating the recovery % and RSD %. Results in Table I indicate satisfactory precision of the proposed methods.

Specificity

Specificity was ascertained by analyzing different mixtures containing the five proposed components in different ratios. The spots of the active drugs in the prepared mixtures were confirmed by comparing their R_f values and spectra with those of a standard solutions (in TLC method). Other parameters such as resolution, capacity factor and selectivity for the separated spots and peaks were then calculated.

Application to commercial tablets

The suggested methods were successfully applied for determination of both diuretics in Aldactazide tablets. The results shown in Table II were satisfactory and with good agreement with the labeled amounts. Applying the standard addition technique, no interference due to excipients was observed as shown from the results in Table II.

In order to validate the suggested chromatographic methods, an overall system suitability testing was done to determine if

Table I. Results of Regression and Assay Validation Parameters of the Proposed Chromatographic Methods for Determination of Hydrochlorothiazide and Spironolactone

	TLC method		RP-HPLC	
Parameters	**HCT**	**SPR**	**HCT**	**SPR**
Range	0.4–1.8 µg /band	0.3–1.5 µg/band	4–50 µg/mL	5–50 µg/mL
Linearity				
Slope	0.4349	0.6147	0.0390	0.0273
Intercept	0.1294	0.0354	–0.0215	–0.0359
*r**	0.9997	0.9997	0.9999	0.9999
Standard error†	0.0040	0.0058	0.0002	0.0002
Confidence limit†	0.4251-0.4448	0.6007–0.6288	0.0385–0.0396	0.0269–0.0276
Standard error‡	0.0046	0.0049	0.0069	0.0052
Confidence limit‡	0.1182–0.1405	0.00350.0473	–0.0382–0.0047	–0.0486 to –0.0238
Accuracy	99.86 ± 0.720	99.88 ± 1.536	100.16 ± 1.417	99.90 ± 1.332
Specificity and selectivity	The method showed good separation of the two drugs and the degradates.			
Precision (RSD %)				
Repeatability§	1.752–1.213–0.979	1.193–1.810–1.105	1.527–1.803–1.010	1.6.98–1.582–1.299
Intermediate precision*	1.862–1.359–1.901	1.916–0.894–1.560	1.664–1.107–1.302	1.899–0.653–0.952
LOD**	0.13 µg/ band	0.09 µg /band	0.93 µg/ mL	0.85 µg /mL
LOQ**	0.39 µg /band	0.28 µg/ band	2.82 µg /mL	2.56 µg/ mL

* r = Correlation coefficient. † of the slope ‡ of the intercept
§ The intra- day and inter- day relative standard deviations of 0.5, 1, and 1; 20, 30 and 40 µg /mL of both drugs.
** Limit of detection and quantitation are determined via calculations:
LOD = (SD of the response/ slope) × 3.3, LOQ = (SD of the response/ slope) × 10

Table II. Determination of the Studied Drugs in Tablets by the Proposed Chromatographic Methods

	RP-HPLC method		TLC method	
Sample	**HCT**	**SPR**	**HCT**	**SPR**
Aldactazide* (B.No. 0611108)	99.13 ± 1.056	101.43 ± 1.724	98.59 ± 1.727	98.75 ± 1.782
Standard addition†	100.62 ± 1.459	99.21 ± 1.827	99.73 ± 1.634	99.32 ± 1.752

* Average of 6 determinations.
† Average of 3 determinations.

Table III. Statistical Analysis of Parameters Required for System Suitability Testing of TLC Method

	Obtained value				
Parameters	**CT**	**HCT**	**SPR Deg**	**DSA**	**SPR**
Symmetry factor	1.17	1	1	1	1
Resolution (Rs)	2.67	3.14	1.71	4	
Capacity factor (k')	0.6	1.6	2.7	3.3	4.5
Selectivity (α)	1.63	1.42	1.16	1.28	

the operating systems are performing properly. Good results were obtained and shown in Tables III and IV.

When results obtained by applying the proposed methods for analysis of pure HCT and SPR compared to those obtained by applying the reported method (19), they showed no significant difference regarding accuracy and precision, and results were given in Table V.

In order to compare the ability of the proposed methods to determine pure HCT and SPR, the obtained results were subjected to statistical analysis using one-way ANOVA test, there was no significant difference between all of the proposed methods (Table VI).

Table IV. Statistical Analysis of Parameters Required for System Suitability

Parameters	Obtained value HCT	Obtained value SPR	Reference value
Resolution (Rs)	2.12	12.22	> 0.8
Relative retention (α)	1.44	1.48	> 1
Tailing factor (T)	1.03	1.17	T = 1*
Capacity factor (k′)	4.92	14.21	1–10 acceptable
No of theoretical plates	3103.02	29522.66	Increase with the efficiency of the separation
HETP*	17.09×10^{-3} cm^{-1}	7.45×10^{-4} cm^{-1}	The smaller the value the higher the column efficiency

* for a typical symmetrical peak
† HETP = height equivalent to theoretical plates (cm/ plate).

Table V. Statistical Comparison of the Results Obtained by the Proposed Methods and the Reported One for Determination of HCT and SPR in Pure Form

Items	RP- HPLC HCT	RP- HPLC SPR	TLC method HCT	TLC method SPR	Reported method* HCT	Reported method* SPR
Mean	100.16	99.90	99.86	99.88	100.09	99.76
SD	1.417	1.332	0.720	1.536	1.047	1.128
RSD%	1.415	1.333	0.721	1.538	1.046	1.131
n	8	8	8	8	6	8
Student's t- test	0.092 (2.179)†	0.235 (2.145)†	0.489 (2.179)†	0.178 (2.145)†		
F- value	1.829 (4.876)†	1.394 (3.787)†	2.116 (3.972)†	1.855 (3.787)†		

* Reference 19. First derivative of ratio spectra spectrophotometric determination of HCT at 270.7 nm and SPR at 237nm using (0.1N HCl / MeOH) as a solvent.
† Figures between parenthesis represent the corresponding tabulated values of t and F at P = 0.05.

Table VI. Results of ANOVA (Single Factor) for Comparison of the Proposed Methods and the Reported One for Determination of HCT and SPR

Source of Variation	SS*	df†	MS‡	F§	P-value	F crit
Hydrochlorothiazide						
Between groups	0.386	2	0.193	0.158	0.855	3.522
Within groups	23.167	19	1.219			
Total	23.553	21				
Spironolactone						
Between groups	0.097	2	0.048	0.027	0.974	3.467
Within groups	37.845	21	1.802			
Total	37.942	23				

* SS = Sum of squares. † df = degree of freedom. ‡ MS = Mean square.
§ F = critical corresponding to theoretical value.

Conclusion

HCT and SPR are coformulated together in antihypertensive formulations and being widely used drugs, it is important to find simple, rapid, and inexpensive methods of their analysis especially in quality control laboratories. The suggested chromatographic methods provide simple, accurate, and reproducible stability-indicating methods for their quantitative analysis in presence of their impurities and degradantion products. The developed TLC method is highly sensitive, and it may be used for analysis of the suggested drugs in biological fluids. It has the advantages of short run time, large sample capacity, and use of minimal volume of solvents. The HPLC method gives a good resolution between the five proposed components within suitable analysis time. It is highly specific but more expensive. The proposed methods have advantage over other published methods of analyzing the binary mixture in presence of their impurities and degradation products. So the applied methods could be useful for stability investigation of the active drug and checking the extent of degradation in pharmaceutical formulations.

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