Comparative Study of Reversed-Phase High-Performance Liquid Chromatography *versus* Thin-Layer Chromatography–Densitometry for Determination of Citicoline Sodium in Presence of Its Alkaline Degradation Products

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Key Words

Citicoline
Degradation products
Thin-layer chromatography–densitometry
Reversed-phase high-performance liquid chromatography

Summary

Simple, sensitive, selective, and precise stability-indicating thin-layer chromatography (TLC)–densitometric and reversed-phase high-performance liquid chromatography (RP-HPLC) methods were developed and validated for the determination of citicoline sodium (CT) in the presence of its alkaline degradation products (CT Deg.) and in pharmaceutical oral solution. TLC–densitometry employs aluminum TLC plates precoated with silica gel 60 F_{254} as the stationary phase and ammonia–ethyl acetate–triethylamine (6:3.5:0.5, *v/v*) as the mobile phase to give compact spots for citicoline sodium (R_F = 0.35) and its degradation product (R_F = 0.1); the chromatogram was scanned at 272 nm. RP-HPLC utilizes a C18 column and a mobile phase consisting of methanol–water–acetic acid (60:40:0.1, *v/v*); the pH level was adjusted to 4 using 0.1% orthophosphoric acid, at a flow rate of 1 mL min^{-1} for the separation of citicoline sodium (t_R = 1.801) and its degradation product (t_R = 3.422). Quantitation was achieved by ultraviolet (UV) detection at 272 nm. Citicoline sodium was exposed to alkaline hydrolysis, and a comparative study was carried out to show the advantages of the proposed chromatographic methods in determination of citicoline sodium in the presence of its alkaline degradation products. The chromatographic methods were developed and validated as per the International Conference on Harmonization guidelines. As the methods could effectively separate the drug from their degradation products, these techniques can be employed as stability-indicating methods that have been successively applied to pharmaceutical formulations without interference from the excipients.

1 Introduction

Instability of pharmaceuticals affects both the safety and efficacy of drug therapy. Therefore, appropriate analytical stability-indicating methods which allow accurate and precise quantitation of a drug in the presence of its degradation products are needed in order to assess the stability of pharmaceuticals. Recently, the development of stability-indicating methods has increased enormously and is even being extended to drug combinations [1].

Citicoline sodium (CT) **(Figure 1),** chemically cytidine 5′-(trihydrogen diphosphate) p′-[2-(trimethylammonio)ethyl], designated as ester inner salt, is the generic name of the synthetic CDP choline (cytidinediphosphate choline). Citicoline prevents memory impairment resulting from poor environmental conditions [2]. Citicoline supplements help improve focus and mental energy and may possibly be useful in the treatment of attention deficit disorder [3]. It seems to increase a brain chemical called phosphatidylcholine. This brain chemical is important for brain functions [4].

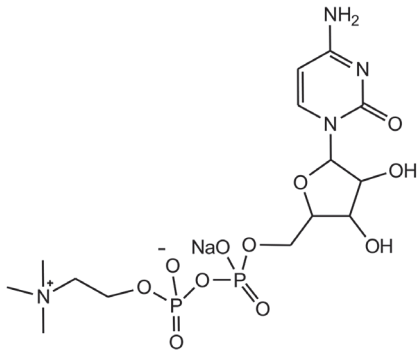

Figure 1

The chemical structure of citicoline.

A literature review shows that a stability study has been carried out for the degradation of CT under different stress conditions as acidic, alkali, and oxidative degradation; the reported method shows that CT was more liable to alkaline degradation [5]. A survey of the literature revealed that there are no stability-indicating thin-layer chromatographic (TLC) methods for the determination of CT in the presence of its alkaline degradation products. Few methods have been reported for the determination of CT such as ultraviolet (UV) spectrophotometry [6–12] or CT combined with piracetam [13–16], high-performance liquid chromatography (HPLC), and liquid chromatography (LC) [17–34].

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The aim of this work was to develop a comparative study for the efficiency of the two stability-indicating chromatographic techniques (RP-HPLC and TLC–densitometry) to analyze CT in the presence of up to 90% of its alkaline degradation products. A comparative study was carried out to show the advantages of the proposed chromatographic methods over the reported one.

2 Experimental

2.1 Instruments

2.1.1 TLC–Densitometry System

CAMAG TLC Scanner 3 S/N 130319 operated with winCATS software, Linomat 5 autosampler (CAMAG, Muttenz, Switzerland), CAMAG microsyringe (100 µL), and TLC aluminum sheet (20 × 20 cm) precoated with silica gel 60 F_{254} (Merck KgaA, Darmstadt, Germany) were used**.**

2.1.2 RP-HPLC System

Agilent 1200 series chromatographic system equipped with quaternary pump, microvacuum degasser, thermostatted column compartment, and variable wavelength UV–visible detector was used. Sample injections were made through an Agilent 1200 series autosampler. Data collection and processing were performed using Agilent ChemStation software, version A.10.01. Column Agilent Zorbax SB-C18 (150 mm × 4.6 mm, 5-µm particle size i.d.) was from Agilent Technologies (Polo Alto, CA, USA). A "Jenway 3505" pH meter (Jenway, UK) equipped with combined glass electrode was used for pH adjustment.

2.2 Chemicals and Reagents

2.2.1 Pure Sample

Citicoline sodium was kindly supplied by Kyowa Hakko Bio Co., Ltd. (Tokoya, Japan). The purity of the sample was found to be 100.12% according to the reported RP-HPLC method [35].

2.2.2 Pharmaceutical Dosage Form

Somazina® oral solution bottles, batch No. A0260111, manufactured by October Pharma, 6th October City, Egypt, under license of Ferrer International, Spain, were purchased from Egyptian market and labeled to contain 100 mg citicoline sodium/mL.

2.2.3 Reagents

All chemicals used throughout the work were of analytical grade, and solvents were of spectroscopic and HPLC grade: methanol HPLC grade and triethylamine (S.D. Fine-Chem Limited, Industrial State, Mumbai); aqueous hydrochloric acid solution and sodium hydroxide, glacial acetic acid, ammonia solution 33%, ethyl acetate, and orthophosphoric (El-Nasr Pharmaceutical Chemicals Co., Abu-Zabaal, Cairo, Egypt); double-distilled deionized water (Otsuka, Cairo, Egypt).

2.2.4 Standard Solutions

– CT stock standard solution: A stock solution containing 1 mg mL^{-1} in distilled water was prepared by dissolving 100 mg of CT in sufficient amount of distilled water and completed to 100 mL with the same solvent.

– CT working standard solution (for TLC): A volume of 40 mL of CT stock standard solution (1 mg mL^{-1}) was transferred into a 100 mL volumetric flask and the volume was completed to the mark with distilled water to obtain a final concentration of 400 µg mL^{-1}.

– CT working standard solution (for RP-HPLC): A volume of 20 mL of CT stock standard solution (1 mg mL^{-1}) was transferred into a 100 mL volumetric flask and the volume was completed to the mark with distilled water to obtain a final concentration of 200 µg mL^{-1}.

– CT alkaline induced degradation: A stock solution of degraded CT was prepared as described under degraded samples [2.3.1].

– Laboratory prepared mixtures: For TLC, solutions containing different ratios of drugs and their degradation products in distilled water were prepared from their working solutions. For RP-HPLC, solutions containing different ratios of drugs and their degradation products were prepared from the working solutions and diluted with the mobile phase.

2.3 Procedures

2.3.1 Degraded Samples

Degraded CT sample was prepared by accurately weighing 50 mg of pure CT, refluxed with 30 mL 4 N NaOH for 3 h. Then, the solution was neutralized using 10 N HCl (to pH 7) and quantitatively transferred to a 50-mL volumetric flask. The volume was then completed using distilled water to produce concentration equivalent to 1 mg mL^{-1}. Degradation was checked every half hour by using TLC and ammonia–acetic acid (6.0:0.3, *v/v*) as the developing solvent. Complete degradation was also confirmed by applying a reported RP-HPLC method [35] using mobile phase constituted of phosphate buffer–methanol (95:5, *v/v*, pH 4.5) and detection at 272 nm by flow rate 1 mL min^{-1}.

2.3.2 Construction of TLC Calibration Curves

Aliquots (1.5–5.5 mL) of CT standard solution (400 µg mL^{-1}) equivalent to 600–2200 µg of CT were accurately transferred into a series of 10-mL volumetric flasks, and the volumes were completed to the mark with distilled water to give final concentrations of 60–220 µg mL^{-1} of CT. Aliquots (10 µL) of each concentration equivalent to 0.60–2.20 µg of CT were applied to the TLC plates; the plates were developed using the developing mobile phase (ammonia–ethyl acetate–triethylamine [6:3.5:0.5, *v/v*]). The plates were then removed, air dried, visualized, and scanned under UV lamp at 272 nm. The calibration curve representing the relationship between the relative peak area (calculated following the external standard technique using an external standard of 1 µg $band^{-1}$ of CT) and the corresponding concentration were plotted, and the regression equation was calculated.

2.3.3 Construction of RP-HPLC Calibration Curves

Aliquots (0.15–10 mL) of CT were accurately transferred from its working standard solutions (200 µg mL^{-1}) equivalent to 30–2000 µg into separate series of 10-mL volumetric flasks; the volumes were completed to the mark with the mobile phase of

methanol–water–acetic acid (60:40:0.1, *v/v*), the pH level was adjusted to 4 using 0.1% orthophosphoric acid. A 20-µL aliquot of each solution was injected onto a ZorbaxSB-C18 column (150 × 4.6 mm, 5-µm particle size i.d.), using the mobile phase, at flow rate 1 mL min^{-1} and detection at 272 nm. The calibration curves were constructed by plotting the peak ratio, using 20 µg mL^{-1} of CT as the external standard, and the corresponding concentration in micrograms per milliliter, and the regression equation were computed.

2.3.4 System Suitability

In order to validate the suggested chromatographic methods, an overall system suitability testing was done to determine if the operating systems are performing properly.

For TLC–densitometry: The system suitability parameters including resolution (*R*s) and peak symmetry were calculated for both mixtures according to United States Pharmacopeia (USP) guidelines [36].

For RP-HPLC: The system suitability parameters including retention time, tailing factor, theoretical plate count (*N*), height of theoretical plate (HETP), and resolution were calculated according to USP guidelines [36].

2.3.5 Assay of Laboratory-Prepared Mixtures

The peak area ratios of the laboratory-prepared mixture were scanned and processed as described for the calibration for each of the proposed TLC–densitometry or RP-HPLC methods, respectively. The concentration of CT in each mixture was calculated using the specified regression equations.

2.3.6 Application to Pharmaceutical Formulation

A stock solution containing 1 mg mL^{-1} in distilled water was prepared by transferring 1 mL of the Somazina® oral solution in sufficient amount of distilled water and completed to 100 mL with the same solvent. A volume of 20 mL of previous stock solution (1 mg mL^{-1}) was transferred into a 100-mL volumetric flask, and the volume was completed to the mark with distilled water to obtain a final concentration of 200-µg mL^{-1}. For TLC, 10 µL was applied onto TLC plates, whereas for RP-HPLC analysis, the last solution was further diluted by transferring 0.15–10 mL aliquots of it to 10-mL volumetric flasks, and the volumes were completed with the RP-HPLC mobile phase. The general procedures described above for each method were followed to determine the concentration of CT in the prepared dosage form solutions.

3 Results and Discussion

The development of analytical methods for the determination of compounds in the presence of their degradation products without previous chemical separation is always a matter of interest. A stability-indicating method has been studied and validated for the determination of CT under different stress conditions as acidic, alkali, and oxidative degradation; the reported method showed that CT was more liable to alkaline degradation [5]. The aim of this work was to perform a complete degradation of CT; this can be obtained by refluxing CT with 4 N NaOH; alkali degradation was obtained. The structure of the alkaline degradates was elucidated by mass spectrometry. The mass ion peak of the degradation products was at 325 and 184 *m/z*. The main degradation route is *via* hydrolysis of the P–O bond giving two degradation products, which are 5′-cytidylic acid (cytidine monophosphate) and 2-((hydroxyhydrophosphoryl)oxy)-*N*,*N*,*N*-trimethylethanaminium (phosphocholine), as shown in **Figure 2;** this result was also obtained by *Patel* et al. [5]; this can explain that one band was detected on TLC plate and one peak was found in RP-HPLC chromatogram as a result of one of the formed degradation products which is cytidylic acid.

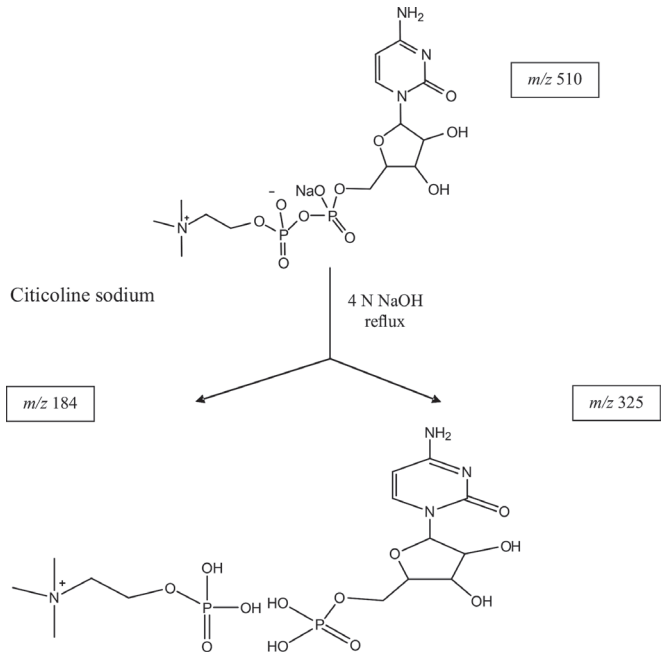

Figure 2

Schematic diagram showing the identified citicoline degradation products.

TLC–densitometry and RP-HPLC were successfully used in the determination of CT in the presence of its alkaline degradation products. These methods could effectively separate CT from its degradation products and have been successively applied to pharmaceutical formulation without interference from the excipients.

3.1 TLC–Densitometry

In this work, a TLC–densitometric method was used for the determination of CT by separation from their degradation products, depending on the difference in R_F values. Complete separation of CT from its degradation products was achieved using ammonia–ethyl acetate–triethylamine (6:3.5:0.5, *v/v*) as the mobile phase. Densitometric scanning was performed at 272 nm with accepted results **(Figures 3 and 4).**

TLC–densitometry has the advantages, over the reported [35] and proposed RP-HPLC methods, of being simpler (simple developing systems with no pH adjustments); several samples can be run simultaneously using a small quantity of the mobile phase, thus lowering analysis time, cost per analysis, and has higher sensitivity. In addition, TLC–densitometry can successfully determine intact CT in the presence of up to 90% of the degradation products.

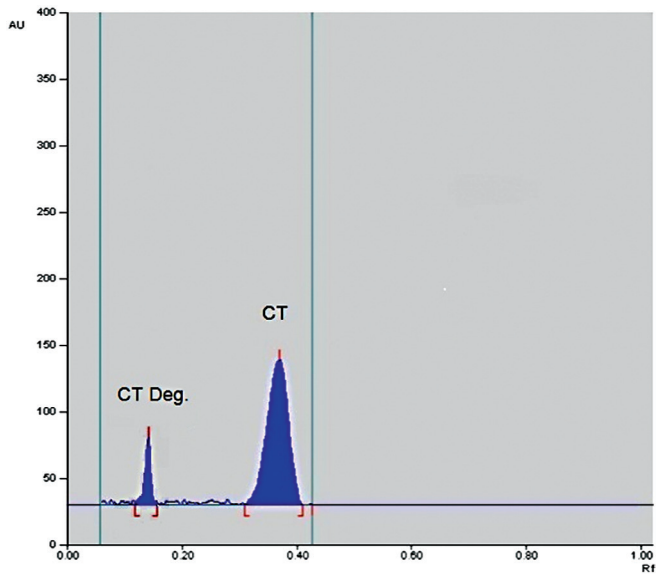

Figure 3

TLC chromatogram of the separated peaks of citicoline (CT; R_F = 0.35; 2 µg) and its alkaline-induced degradation products (CT Deg.; R_F = 0.1; 1 µg) using ammonia–ethyl acetate–triethylamine (6:3.5:0.5, *v/v*) as the mobile phase.

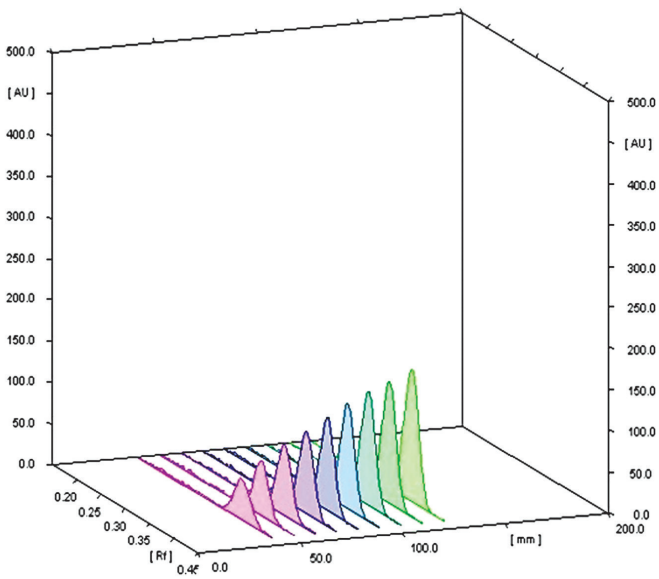

Figure 4

TLC chromatogram of the separated peaks of citicoline (CT; R_F = 0.35) over the concentration range of 0.60–2.20 µg band^{-1} using ammonia–ethyl acetate–triethylamine (6:3.5:0.5, *v/v*) as the mobile phase.

3.2 RP-HPLC

Good chromatographic separation of CT from its degradation products can be achieved by using a Zorbax SB-C18 (150 × 4.6 mm, 5-µm particle size i.d.) column, a mobile phase consisting of methanol–water–acetic acid (60:40:0.1, *v/v*, pH 4), the pH was adjusted to 4 using 0.1% orthophosphoric acid, UV detection at 272 nm, and a flow rate of 1 mL min^{-1} **(Figure 5).**

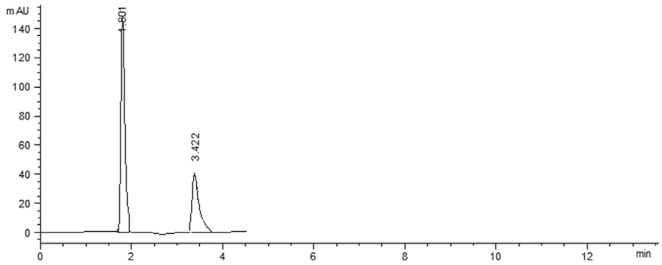

Figure 5

HPLC chromatogram of a resolved mixture of 100 µg mL^{-1} of citicoline (t_R = 1.801) and 50 µg mL^{-1} of its alkaline-induced degradation product (t_R = 3.422); the mobile phase is methanol–water–acetic acid (60:40:0.1, *v/v*, pH = 4).

Several trials were done to reach the optimum chromatographic separation, and the suggested chromatography system allows complete baseline separation in reasonable time.

RP-HPLC method has the advantages over the reported method [35] of using shorter column; this can reduce the volume of organic solvents used which is more economic in industrial scale. No buffers have been used which saves the column and increases its life time. It was capable of accurate determination of intact CT up to 90% of the degradation products and could quantitatively determine CT in wider concentration range (3–200 µg mL^{-1}) compared to TLC–densitometry.

Table 1

Results of regression and validation parameters of the proposed chromatographic methods for the determination of citicoline in the presence of its alkaline-induced degradation products.

Parameters	TLC	HPLC
Range	0.60–2.20 µg band^{-1}	3–100 µg mL^{-1}
Linearity		
Slope	0.7951	0.0508
Intercept	0.194	−0.0194
SE of slope	0.007136	0.0000701
SE of intercept	0.01143	0.006952
Correlation coefficient (*r*)	0.9998	1
Accuracy (mean ± SD)	100.77 ± 1.012	99.67 ± 0.862
LOD	0.0408 (µg band^{-1})	0.9479 (µg mL^{-1})
LOQ	0.1237 (µg band^{-1})	2.8726 (µg mL^{-1})
Precision (RSD %)		
Repeatability[a)]	0.192	0.266
Intermediate precision[b)]	1.224	0.311
Specificity (mean ± RSD)	101.06 ± 0.581	100.25 ± 0.768

LOD: limit of detection, LOQ: limit of quantitation
LOD = (SD of the response/slope) × 3.3, LOQ = (SD of the response/slope) × 10
[a)]The intra-day values of samples of CT for TLC (0.6, 1, and 1.4 µg band^{-1}) and for HPLC (20, 80, and 160 µg mL^{-1})
[b)]The inter-day values of samples of CT for TLC (0.6, 1, and 1.4 µg band^{-1}) and for HPLC (20, 80, and 160 µg mL^{-1})

Table 2

Determination of citicoline (CT) in the presence of its alkaline-induced degradation products (CT Deg.) in laboratory-prepared mixtures by the proposed methods.

Mixture No.	CT Deg. %	TLC–densitometry			RP-HPLC		
		Taken CT (μg band^{-1})	Found	Recovery %	Taken CT (μg mL^{-1})	Found	Recovery %
1	10	2.00	2.01	100.50	70.00	70.68	100.97
2	50	1.20	1.22	101.67	180.00	181.99	101.11
3	90	1.00	1.01	101.00	100.00	100.04	100.04
		Mean		101.06			100.25
		RSD%		0.581			0.768

3.3 Method Validation

The International Conference on Harmonisation (ICH) guidelines for method validation were followed for validation of the suggested methods [37].

3.3.1 Linearity and Ranges

The linearity of the proposed methods was evaluated by processing the different calibration curves on three different days. RP-HPLC showed higher correlation coefficients (r = 1) than TLC–densitometry (r = 0.9998).

Under the previously described experimental conditions, linear relationships were obtained by plotting drug concentrations against peak ratios for both chromatographic methods. For RP-HPLC methods, calibration curve was constructed in the range of 3–100 μg mL^{-1} and 0.6–2.2 μg band^{-1} for TLC–densitometry. The corresponding validation parameters are listed in **Table 1.**

3.3.2 Limits of Detection and Quantification

The limit of detection (LOD) and limit of quantification (LOQ) were calculated for CT using the proposed methods with a ratio of 3.3 and 10 of standard deviations of the blank and the slope of the calibration line, as shown in Table 1.

3.3.3 Accuracy

To study the accuracy of the proposed methods, procedures under linearity were repeated three times for the determination of different blind concentrations of pure CT. The accuracy expressed as % RSD is shown in Table 1.

Table 3

Determination of citicoline in Somazina® oral solution by the proposed chromatographic methods.

Parameters	TLC–densitometry	RP-HPLC
Somazina® oral solution 100 mg mL^{-1} B.N. A0260111 (mean ± SD)	100.83 ± 0.828	100.58 ± 0.294
Standard addition (mean ± SD)	100.78 ±1.530	100.09 ± 0.761

3.3.4 Precision

The precision of the proposed methods, expressed as RSD, was determined by the analysis of three different concentrations of pure drugs within the linearity range. Intra-day precision was assessed from the results of three replicate analyses of three pure CT samples (20, 80, and 160 μg mL^{-1} for RP-HPLC and 0.6, 1, and 1.4 μg band^{-1} for TLC–densitometric method) on a single day. Inter-day precision was determined from the same samples analyzed on three consecutive days. The total number used for each technique was nine. The results are presented in Table 1.

3.3.5 Specificity

Specificity was determined by analyzing different mixtures containing CT and its degradation products in different ratios (CT Deg. is 10%–90% of intact CT) **(Table 2).**

3.3.6 Application to Commercial Dosage Form

The suggested methods were successfully applied for the determination of citicoline sodium in Somazina® oral solution. The results shown in **Table 3** were satisfactory and with good agreement with the labeled amounts. Applying the standard addition technique, no interference due to excipients was observed and good results were obtained; compared with the reported one, no significant difference with respect to Student's t test and F test was obtained as shown from the results in Table 3.

Table 4

Statistical analysis of parameters required for system suitability testing of TLC.

Parameter	CT	CT Deg.	Limit [37]
Retention factor (R_F)	0.35 ± 0.01	0.1 ± 0.01	
Resolution (R_s)	3.38		$R_s > 2$
Tailing factor (T)	1	0.94	$T < 2$ $T = 1$ for symmetric peak
Selectivity factor (α)	3.78		$\alpha > 1$

In order to validate the suggested chromatographic methods, an overall system suitability testing was done to determine if the operating systems are performing properly. Good results were obtained as presend in **Tables 4 and 5.**

Table 5

Statistical analysis of parameters required for system suitability testing of HPLC.

Parameter	CT	CT Deg.	Limit [37]
Retention time (t_R)	1.801	3.244	$t_R > 1$
Resolution (R_s)	5.4		$R_s > 2$
Tailing factor (T)	1.16	1.33	$T < 2$ $T = 1$ for symmetric peak
Capacity factor (K')	2.602	5.844	$K' > 2$
Selectivity factor (α)	2.246		$\alpha > 1$
Column efficiency (N)	3524	4126	$N > 2000$ Increases with efficiency of the separation
Height equivalent to theoretical plate (HETP)	0.043	0.036	The smaller the value, the higher the column efficiency

When results obtained by applying the proposed methods for analysis of pure CT were compared to those obtained by applying the reported method [35], they showed no significant difference regarding accuracy and precision; results are given in **Table 6.**

Table 6

Statistical comparison of the results obtained by the proposed methods and the reported one for the determination of CT in the presence of its alkaline induced degradation products.

Parameter	TLC	HPLC	Reported HPLC method [35][b)]
Mean	99.91	100.08	100.12
SD	1.065	0.879	0.670
n	6	9	6
Variance	1.134	0.773	0.449
Student's t	0.407 (2.228)[a)]	0.149 (2.160)[a)]	
F	2.526 (5.0503)[a)]	1.722 (3.6875)[a)]	

[a)]The values in parentheses are the corresponding tabulated values at $p = 0.05$

[b)]HPLC method (C-18, using phosphate buffer and methanol [95:5, *v/v*] at pH = 4.5 with UV detection at 272 nm and flow rate 1 mL min^{-1})

Results obtained by the proposed methods for the determination of intact CT were statistically compared to those obtained by the reported method [35]. The values of the calculated t and F were less than the corresponding tabulated ones, which revealed that there was no significant difference with respect to accuracy and precision between the proposed methods and the reported one, as shown in Table 6.

In order to compare the ability of the proposed methods to determine pure CT, the obtained results were subjected to statistical analysis using one-way analysis of variance (ANOVA) test; there was no significant difference between all of the proposed methods **(Table 7).**

Table 7

Results of ANOVA (single factor) for comparison of the proposed methods and the reported one for the determination of CT in the presence of its alkaline induced degradation products.

Source of variation	Degree of freedom	Sum of squares	Mean square	F	F critical
Between columns	2	0.0938	0.0469	0.0707	3.5546
Within columns	18	11.9479	0.6638		
Total	20	12.0417			

4 Conclusion

A comparative study was carried out to compare the RP-HPLC and TLC–densitometry for the analysis of CT in the presence of its alkaline degradation products. It was found that the proposed chromatographic methods are simple, accurate, and reproducible stability-indicating methods for the quantitative analysis of CT in the presence of up to 90% of its alkaline degradation products. These methods have been successively applied in bulk powder, laboratory-prepared mixtures, and pharmaceutical formulation without interference from the excipients.

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