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THREE STABILITY INDICATING SPECTROPHOTOMETRIC AND SPECTRODENSITOMETRIC METHODS FOR THE DETERMINATION OF THIOCOLCHICOSIDE IN PRESENCE OF ITS DEGRADANT

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ABSTRACT

Three simple, selective, accurate and green methods were developed for the determination of thiocolchicoside in the presence of its degradation product without preliminary separation or need to an advanced HPLC system. The first derivative spectrophotometry method D^1 was developed with good selectivity without interference of the degradation product. The peak amplitudes of D^1 spectra of TCC at 412.8 nm (corresponding to the zero-crossing point of degradation product) TCC could be determined over a concentration range of 5-30 µg/ml with mean percentage recovery of 100.28±0.138%. The second method is Dual wave length spectrophotometry DW, Linear correlations were obtained between the differences in absorbance values at 256.8-290.8 nm and the corresponding concentrations of TCC while the degradant shows the same absorbance value. TCC could be determined over a concentration range of 5-40 µg/ml with mean percentage recovery 100.44±0.136%. The third method is TLC densitometric method,it was used for the quantitative determination of thiocolchicoside in presence of its degradation product using silica gel plates and a mixture of ethyl acetate: methanol (85:15 v/v) as a developing system at 254 nm in the range of 4-40 µg/spot with recovery percentage 100.00±1.181%. These methods were checked by the analysis of laboratory-prepared mixtures of TCC and its degradation product in different ratios and its pharmaceutical dosage form without any

interference. There was no statistically significant difference in results compared with the official method.

Highlights:

- Three simple, inexpensive, accurate and precise stability indicating methods of analysis.
- First comparative study for analysis of TCC by spectrophotometric and spectrodensitometric methods.
- No need for preliminary separation steps or sophisticated chromatographic systems.
- Very applicable methods for routine analysis in quality control laboratories.

Keywords: Thiocolchicoside (TCC), Stability indicating method (SIM), Derivative spectrophotometry (D^1), Dual wavelength spectrophotometry (DW), TLC Densitometry

INTRODUCTION

Thiocolchicosde is an amide drug which its amide group is unstable under normal temperature. It is more susceptible to degrade during storage or shipping process to inactive or even toxic byproducts **[1]**. So, the need to find a technique for eliminating the interference of degradation products and developing stability indicating methods is in a growing importance **[2]**. Derivative spectrophotometry is a well-established technique that is able to enhance the resolution of overlapping spectral bands. This operation allows the removal of spectral interferences and as a consequence leads to increase in the selectivity of assay **[3]**. The first part deals with the application of first derivative spectrophotometry (D^1) **[4]** for the determination of TCC in the presence of its degradation product. In the proposed D^1 method the wavelength corresponding to zero crossing of degradation product was used for the determination of the intact drugs **[5]**. The second part deals with the application of Dual wavelength spectrophotometry (DW).It is a direct spectrophotometric technique which is a simple, reproducible and rapid technique commonly used for separation of drug mixtures with high accuracy and precision using the zero-order absorption spectra without prior separation or chemical derivatization **[6]**. The principle of this method is that the absorbance difference at two points on the spectra is directly proportional to the component of interest, independent of the interfering component **[7]**. The prerequisite for this method is the selection of two wavelength values where the interfering component shows the same absorbance value, while the component of

interest shows a significant difference in absorbance which is proportional to its concentration **[8]**. DW is used to solve the problem of the overlapping zero order absorption spectra of TCC and its degradation product. Careful choice of the wavelength pair is the most important factor that affects the sensitivity and the selectivity of this method **[9]**.

Thin-layer chromatography (TLC) is a powerful method equally appropriate for both qualitative and quantitative analytical tasks. Many applications of TLC-densitometric methods for identification and quantitation of impurities and impurity level targets, constituents, active substances and degradation products, and also for kinetics studies, process development and optimization, process monitoring have been demonstrated **[10]**. It is superior to other analytical techniques in terms of total cost and time for analysis as it does not require tedious cleanup, with the use of appropriate mobile phases and reagents, all interfering agents will be omitted. It is a very rapid, accurate and precise chromatographic technique for different components assays **[11]** and can be used for routine quality control of pharmaceutical products **[12]**.

Experimental:

Apparatus:

Spectrophotometer: SHIMADZU dual beam UV-visible spectrophotometer (Kyoto, Japan), model UV-1650 PC. Thin layer chromatography (HPTLC) plates precoated with silica gel 60 F254 0.25 mm thickness (Merck, Germany). CAMAG Linomat 5, autosampler (Muttens, Switzerland) CAMAG TLC densitometric Scanner 3S/N 130319 in the reflectance absorbance mode (Muttens, Switzerland). WINCATS software was used for densitometric evaluation (Muttens, Switzerland).

Reference Samples:

TCC reference standard was purchased from Memphis pharmaceutical Co., and its purity was certified to be 99.99%.

Pharmaceutical Formulation:

Relaxin ® tab. manufactured by Memphis Company (Cairo, Egypt) batch number 1311600 was labeled to contain 4 mg TCC per tablet.

Degraded samples:

Preparation of the degradation product of TCC:

An accurately weighed portion of 100 mg of pure TCC was dissolved in 25 ml of 1M NaOH, and then the solution was refluxed in a 250-mL round-bottom flask for 2 hours till complete degradation, **Figure 1**. The solution was then neutralized using 1M HCl and evaporated in a water bath till complete dryness; the

residue was dissolved by adding 30 mL methanol portion wise with stirring, filtered and transferred quantitatively into 100-mL measuring flask. The volume was completed to the mark to form stock solution of alkaline degradation product equivalent to 0.1 mg/ mL of TCC. A volume of the prepared degraded solution was evaporated again to obtain a powder that was used for the elucidation of the structure of the degradation product by IR and mass spectroscopy.

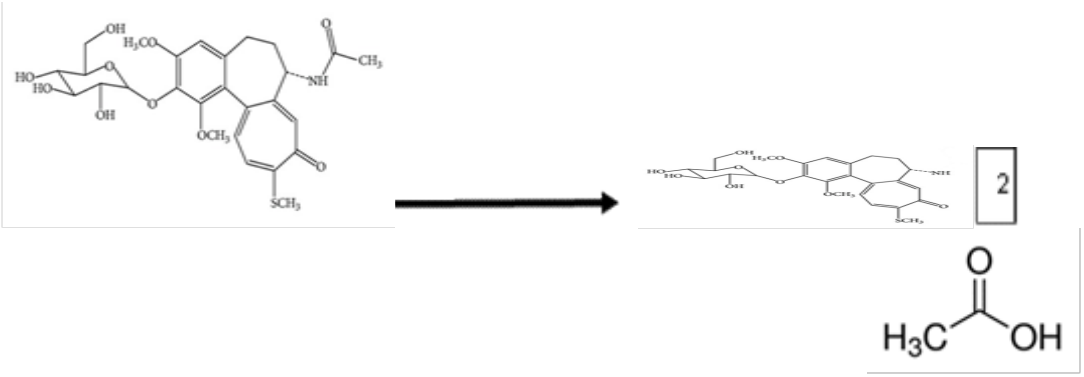

Figure 1: Alkaline degradation pathway of thiocolchicoside

MATERIALS AND REAGENTS

All chemicals used throughout this work were of analytical grade, and distilled water was used as a solvent (Adwic, Cairo, Egypt).

Standard solutions:

TCC standard solutions with a concentration of 0.1 mg/mL in distilled water were prepared in a 100-mL volumetric flask by dissolving 10 mg of pure TCC in 100 ml of distilled water.

TCC degradation product stock solutions with concentrations of 0.1 mg/mL equivalent were prepared by dissolving weight of degradation product equivalent to 10 mg of TCC in 100 ml of distilled water.

Procedures:

Construction of calibration graphs:

First derivative spectrophotometry:

The zero-order (D°) absorption spectrum of 15.0 µg/mL of TCC and its degradation product solutions were recorded against distilled water as

a blank over the range of 350 – 450 nm. Then the first derivative (D^1) spectra were recorded **[13]**. Aliquots (0.5–3.0 mL) of TCC stock solution which concentration is (100µg/ml) were separately transferred into a series of 10-mL volumetric flasks, and the volumes were completed with distilled water in order to prepare concentration range (5µg/ml -30 µg/ml). The zero-order spectra were recorded using distilled water as a blank. The first derivative of the obtained spectra was recorded using $\Delta\lambda$=4 nm and the scaling factor 10 for TCC. The peak amplitudes of the obtained first derivative spectra were measured at

412.8 nm. Calibration graph relating the peak amplitude to the corresponding concentrations of TCC was constructed. The corresponding regression equation was computed **[14]**.

Dual wavelength spectrophotometry (DW):

The zero-order absorption spectrum of 15.0 µg/mL intact TCC and its degradation product equivalent to 15.0 µg/mL intact TCC were recorded against distilled water as a blank over spectral range between 200 – 300 nm. Aliquots (0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0 mL) of TCC stock solution which concentration is (100 µg/ml) were transferred into a series of 10-mL volumetric flasks, and the volumes were completed with distilled water to obtain concentrations (5µg/ml-40µg/ml). The zero-order spectra were recorded using distilled water as a blank. Linear correlations were obtained between the differences in absorbance values at 256.8-290.8 nm, and the corresponding concentrations of TCC. The corresponding regression equation was computed for TCC.

TLC Densitometry

Aliquots of TCC standard solution were spotted in triplicates on TLC plates to have a range (4 – 40 µL/spot), using an autosampler with micro syringe (100 µL), Spots width (3mm), spacing (14.2 mm) and 15 mm from bottom edge of the plate using an applicator. Linear ascending development was performed in a chromatographic tank previously saturated with the developing system consisting of methanol: ethyl acetate (15:85) v/v, for 45 min at room temperature (about 25C°). The developed plates were air dried and scanned at 254 nm. The scanning profile for TCC was obtained, and the calibration curve relating the optical density of each spot to the corresponding concentrations of TCC was constructed. The corresponding regression equation was then computed.

Analysis of laboratory mixtures:

Laboratory prepared methods with different concentrations of TCC and its degradation product were prepared and analyzed by the proposed methods.

Analysis of pharmaceutical preparations:

First derivative spectrophotometry:

Ten tablets were individually weighed and the average weight was calculated then the 10 tablets were grinded. An amount of powder equivalent to 10 mg (10000 µg) TCC was accurately weighed and transferred into a 100-mL volumetric flask. About 50 mL distilled water were added then the solution was sonicated for 30 minutes to enhance the extraction of the drug. The volumes were filtered into

100-ml volumetric flask and residues were washed, finally the volume filtered was completed to the mark with distilled water. (0.5, 1.0 and 2.0 ml) of aliquot were transferred into a 10-mL volumetric flask; the volume was then completed with the water to get final concentration of (5.0µg/ml, 10.0µg/ml and 20.0µg/ml) respectively. The same procedures under construction of calibration curve were applied. The concentrations of TCC were obtained from the computed regression equation and the accuracy of the method was further assessed by applying the standard addition technique.

Dual wavelength spectrophotometry (DW):

Relaxin® solution with a concentration equal to 10µg/mL was prepared by weighing 10 tablets individually, calculating the average weight then grinding 10 tablets. An amount of powder equivalent to 10000 µg TCC was accurately weighed and transferred into a 100-mL volumetric flask. About 50 mL distilled water were added then the solution was sonicated for 30 minutes to enhance the extraction of the drug. The volumes were filtered into 100-ml volumetric flask and residues were washed, finally the volume filtered was completed to the mark with distilled water. Aliquots (1.5,2.0 and 2.5 ml) of TCC stock solutions were transferred into a series of 10-mL volumetric flasks, and the volumes were completed with distilled water to obtain (15µg/ml,20 µg/ml and 25 µg/ml) respectively. The same procedures under construction of calibration curve were applied. And the concentrations of TCC were calculated from the corresponding regression equation.

TLC Densitometry:

Weigh 10 tablets individually of Relaxin® 4mg which is labeled to contain 4 mg TCC per tablet, take the average weight. Prepare a stock solution with a concentration equal to 10 µg/mL TCC by grinding 10 tablets and take a weigh equivalent to 10 mg (10000 µg) TCC in a 100-mL volumetric flask, dissolve, filter and fill till mark with distilled water (stock solution 1). From this stock solution transfer 1.0 mL into a 10-mL volumetric flask and complete the volume with distilled water to get a concentration equal to 10 µg/mL (stock solution 2). Transfer aliquots (1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0 mL) of TCC stock solution into a series of 10-mL volumetric flasks, and complete to mark with distilled water. Into a 100ml volumetric flask, dissolve 40mg of intact TCC in 100ml distilled water. Transfer quantitively 1ml to 10 ml volumetric flask, complete till mark with distilled water (40µg/mL). Add (5, 6 and

7 ml) of this solution to 2.0 ml of stock solution 2 in three 10ml volumetric flasks and complete the volume of each one with distilled water. The same procedures under construction of calibration curve were applied. Calculate the concentration of the added TCC from the corresponding regression equation.

RESULTS AND DISCUSSION

The zero-order absorption spectra of TCC and its corresponding degradation product showed sever overlap that hindered its direct spectrophotometric measurements **[15]**, **Figure 2**.

First derivative spectrophotometry (D^1):

It is an analytical technique of great utility for extracting both qualitative and quantitative information from spectra composed of unresolved bands **[16]** and for eliminating the effect of baseline shifts and baseline tilts by using the first or higher derivatives of absorbance with respect to wavelength **Figure 3 [17]**. A rapid, simple, and low-cost spectrophotometric method based on measuring the peak amplitudes of D^1 spectra of TCC at 412.8 nm (corresponding to the zero-crossing point of degradation product) was developed with good selectivity without interference of the degradation product **[18]** as shown in **Figure 4**. In order to optimize D^1 method, different scaling factors were tested, where a scaling factor = 50 showed good resolution, and suitable signal to noise ratio was achieved using $\Delta\lambda$= 4 for TCC.

The linearity of the peak amplitudes of the D^1 curves at the selected wavelength values was studied. Linear relationships were obtained in the concentration ranges of 5-30 µg/mL for TCC, then the regression equation was computed and found to be:

A=0.015 C+0.023 r=0.9995

Where C is the concentration of the drug in µg/mL, A is the peak amplitude of the first derivative curve at 412.8 nm, and r is the correlation coefficient. This method was checked by the analysis of laboratory-prepared mixtures of TCC with its degradation product in different ratios. TCC could be determined in the presence of up to 75% of its degradation product with mean percentage recovery of 100.28±0.138%.

The proposed method was successfully applied to the analysis of TCC in Relaxin® 4mg tab. and. The method was also assessed by the standard addition technique. The validity of the proposed method was assessed according to the ICH guidelines **[19]**. The results obtained from the proposed method were statistically compared to the results of the USP official assay method for drug which is a HPLC

method **[20]**. The obtained t and F values indicated that there was no significant difference between the two methods regarding accuracy and precision.

Dual Wavelength Spectrophotometry (DW):

Dual Wavelength Spectrophotometry (DW) is a very effective and simple method for the analysis of TCC in the presence of its degradation product with no need for further derivatization of raw data **[21]**. In order to optimize DW, different pairs of wavelengths were tried for TCC. The absorbance values at 256.8 and 290.8 nm showed the best selectivity for the analysis of TCC **(Figure 5) [22]**.

Linear correlation was obtained between the differences in absorbance values at the selected wavelengths, and the corresponding concentrations of TCC in the range of 10–40 µg/mL **Figure 6**, and the regression equation was computed and found to be:

▲A=0.015 C+0.025 r=0.9995

Where C is the concentration of TCC in µg/mL, ▲A is the difference in absorbance values and r is the correlation coefficient. This method was checked by the analysis of laboratory-prepared mixtures of TCC and its degradation product in different ratios. TCC could be determined in presence of up to 75% of its degradation product with mean percentage recovery of 100.44±0.136%. The proposed method was successfully applied to the analysis of TCC in its pharmaceutical formulations. The method was also assessed by the standard addition technique. The validity of the proposed method was assessed according to the ICH guidelines **[19]**. The results obtained from the proposed method were statistically compared to the results of USP official assay method for drug which is a HPLC method **[20]**. The obtained t and F values indicated that there is no significant difference between the two methods regarding accuracy and precision.

TLC Densitometry:

A simple, time saving and selective stability indicating TLC densitometric method was developed and validated for determination of TCC in bulk powder, in presence of its degradation product and in its pharmaceutical formulations.

In order to obtain a suitable developing system for optimum separation, several mixtures of solvents were tried, such as methanol: chloroform, methanol: toluene and methanol: dimethyl formamide. But there was no successful system where poor separation was obtained. A mixture of methanol: ethyl acetate was tried in different ratios to obtain optimum separation **Figure 6**. The obtained TLC chromatograms were promising, and after fine adjustment of the ratios, the optimum

developing system was found to be methanol: ethyl acetate (1.5: 8.5) v/v, where symmetric peaks with good resolution were obtained as shown in **Figure 7**. The Rf values (± 0.02) were 0.60 and 0.32 for TCC and its degradation product respectively. Well defined spots were obtained when the chromatographic tank was previously saturated with the mobile phase for 45 minutes at room temperature. The instrumental conditions such as slit dimension and wavelength detection were optimized. Detection at 254 nm was suitable with minimum noise.

TLC densitometric scanning chromatograms of different concentrations of TCC was performed. Calibration curve was constructed representing the relationship between the relative peak area ratios and the corresponding concentrations in the range of 4.0 – 40.0 µg/spot. The regression equation was computed and found to be:

TCC: A=0.049C+0.024 r=0.9990

Where A is the peak area ratio (to that of a concentration of 20 µg/spot in of TCC as an external standard), C is the concentration in µg/spot and r is the correlation coefficient. The validity of the proposed method was assessed according to the ICH guidelines, **Table 1**.

The selectivity of the proposed methods was proved by the analysis of laboratory prepared mixtures containing different ratios of TCC with its degradation product. The results proved that the method is highly selective for the determination of the TCC in the presence of up to 90 % of its degradation product as shown in **Table 2** with recovery percentage 100.00±1.181 for TCC.

The suggested method was successfully applied for the determination of TCC in its pharmaceutical formulation (Relaxin® tablets). The results were satisfactory and with good agreement with the labeled amounts. Validity of the method was assessed by applying the standard addition technique, which showed no interference due to excipients as shown from the results in **Table 3**. The suggested procedure was subjected to validation scheme according to the ICH guidelines **[19]**. Finally, statistical comparison of the results obtained by the suggested method and those obtained by applying the official HPLC method **[20]**. The obtained calculated t and F-values were less than the corresponding theoretical ones indicating no significant difference with respect to accuracy and precision, **Table 4**.

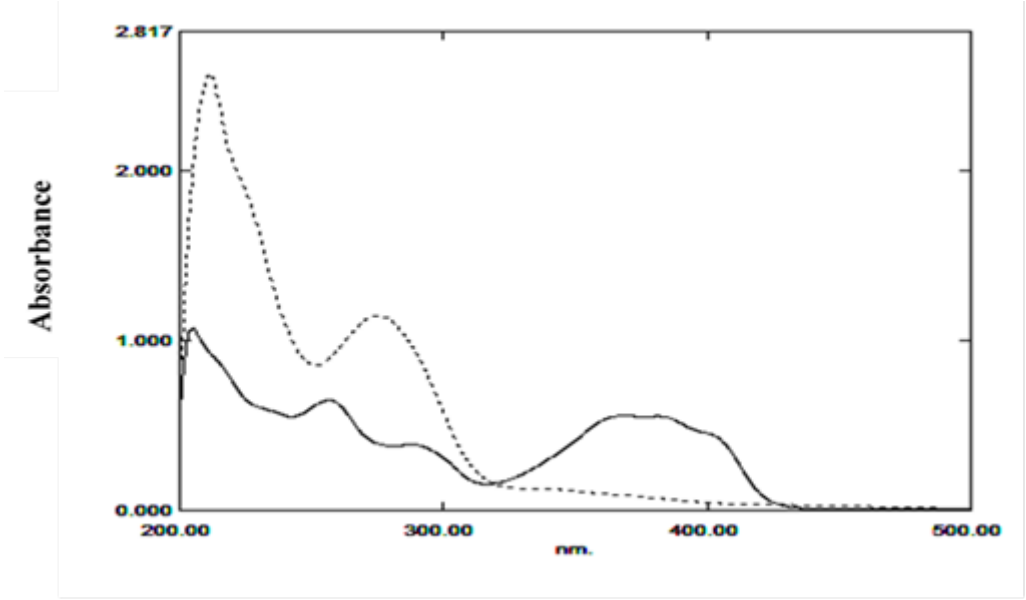

Figure 2: Zero order absorption spectra of 15 µg/ml TCC (__) and 15 µg/mL of degradation product equivalent (---) using distilled water as a blank

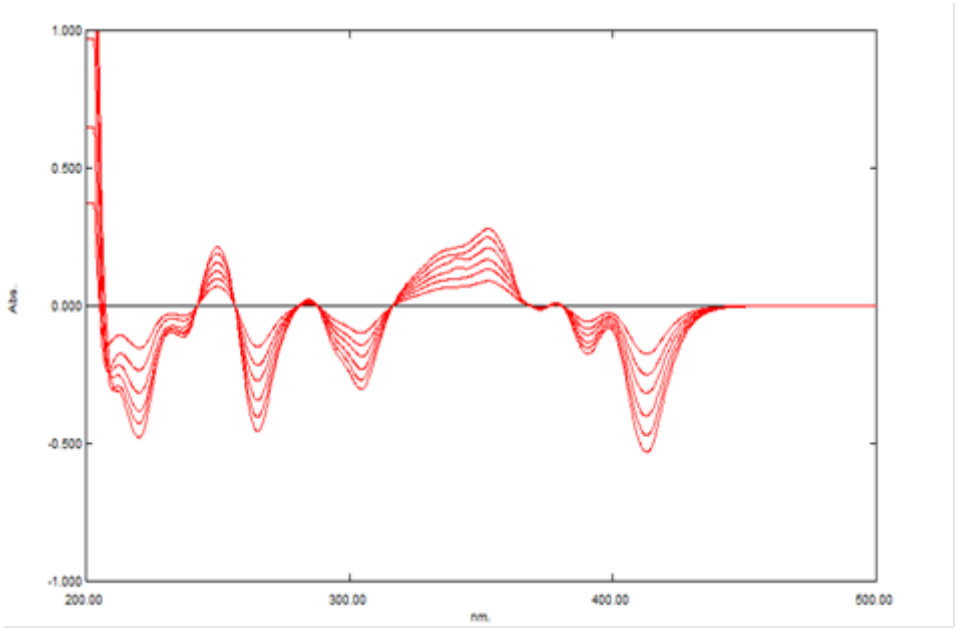

Figure 3: First derivative spectra D1 of TCC (5.0- 30.0 µg/mL) using distilled water as a blank

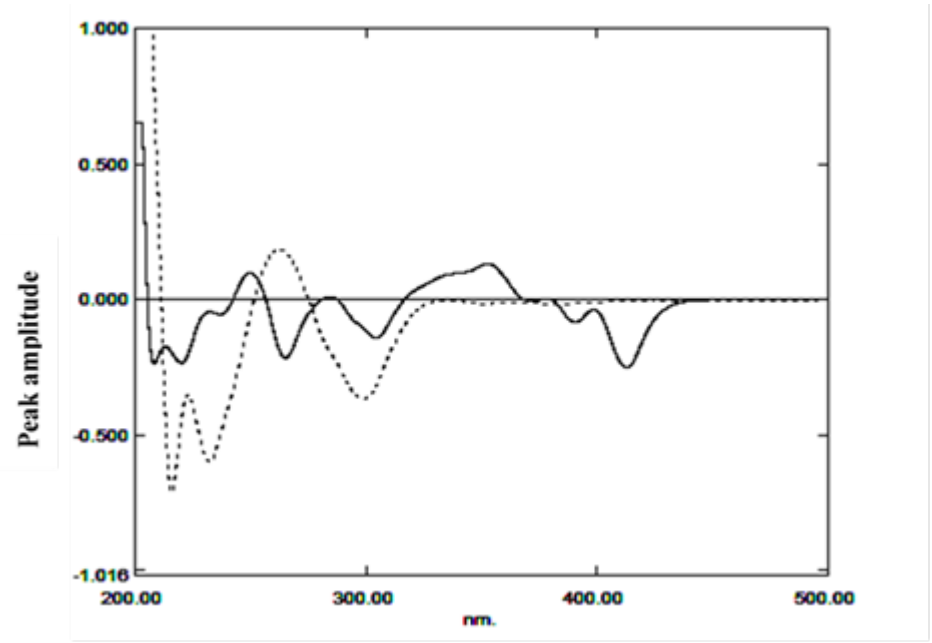

Figure 4: First derivative absorption spectra of TCC 15 µg/mL (___) and the degradation product equivalent to 15 µg/mL (-----)

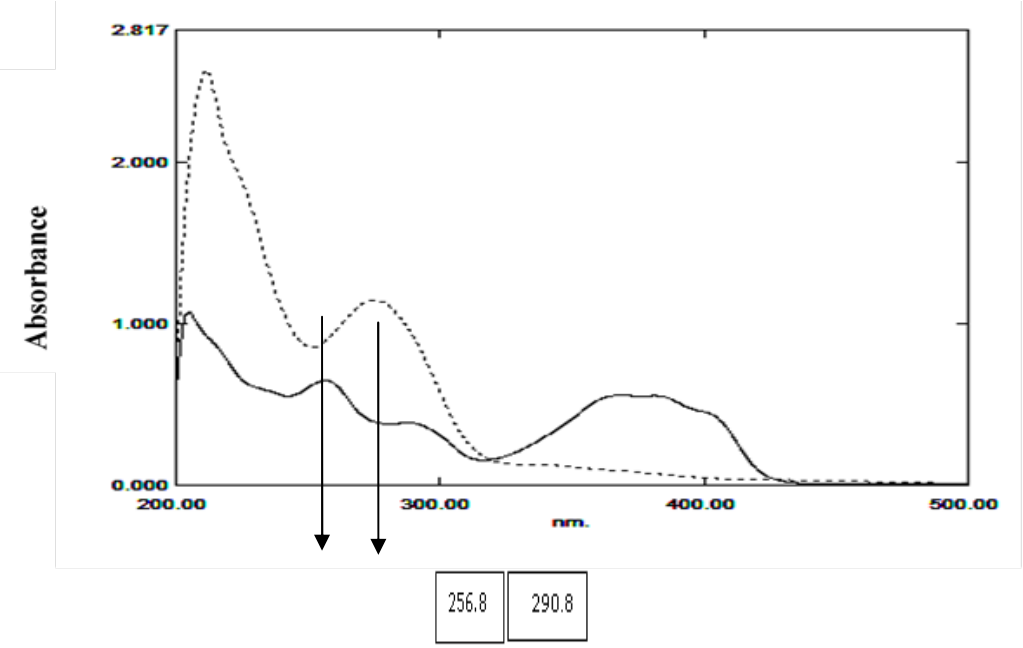

Figure 5: Zero order absorption spectra of 15 µg/mL TCC (___) and 15 µg/mL degradation product equivalent (-----) using distilled water as a blank

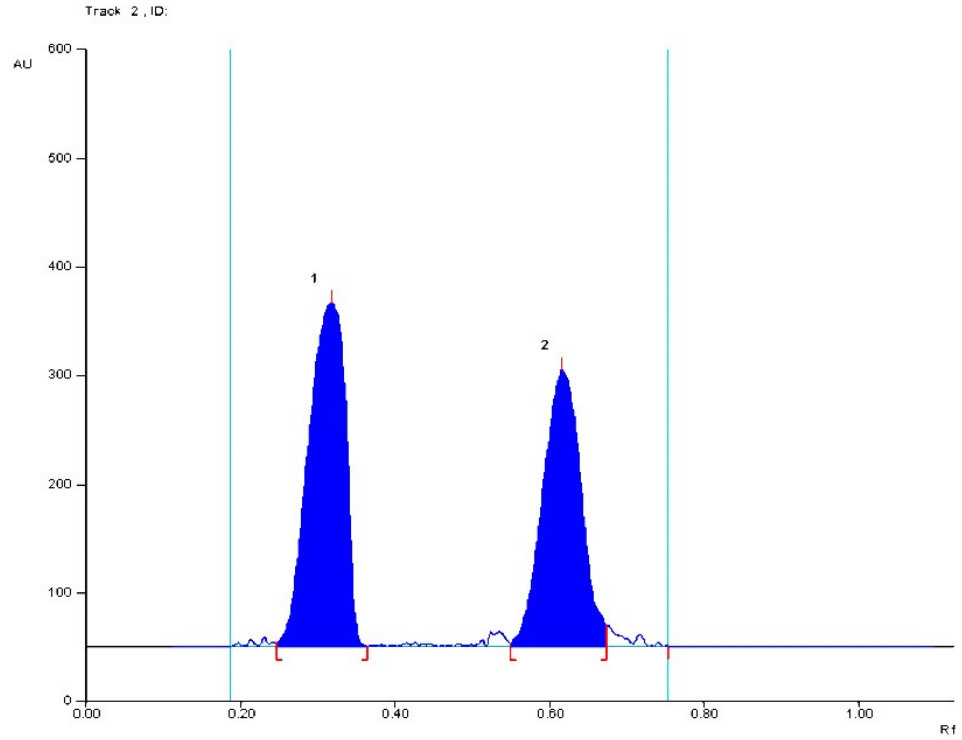

Figure 6 :2D chromatogram of a resolved mixture of alkaline degradation product of TCC (1) (Rf=0.32±0.02) and intact drug (Rf=0.60±0.02) (2) by TLC separation using ethyl acetate: methanol: (8.5:1.5, v/v)

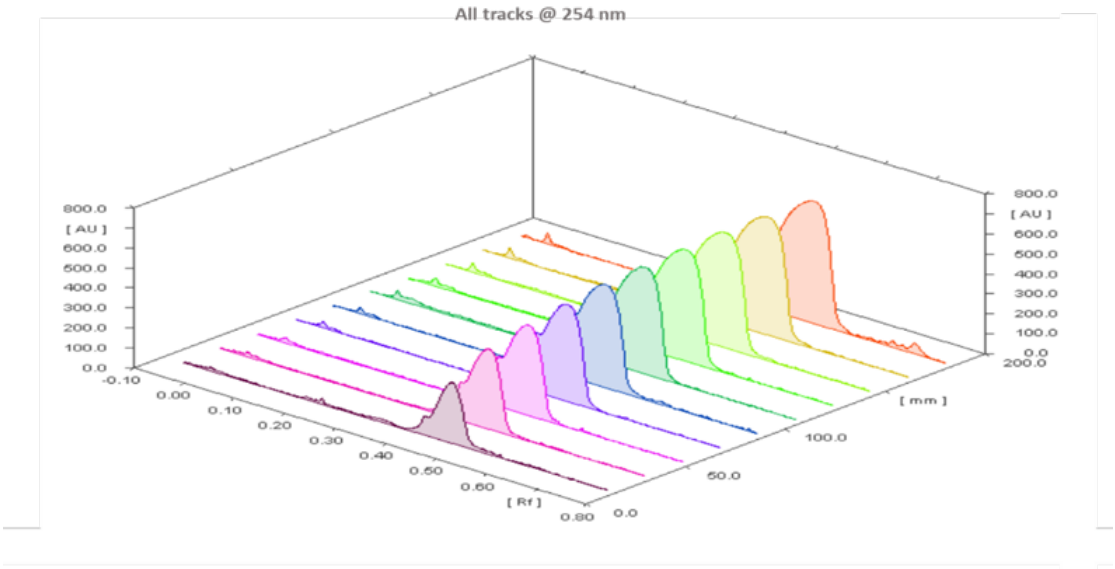

Figure 7: Scanning profile of the TLC chromatogram (3D) of TCC (4 – 40 µg/spot), Rf 0.48± 0.02, at 254 nm using methanol: ethyl acetate (1.5:8.5, v/v) as a developing system

Table 1: Assay validation sheet of the proposed methods for the determination of pure TCC

Parameter	D^1 method	DW method	TLC densitometry
Range	5-30 (µg/mL)	5-40 (µg/mL)	(4-40) µg / spot
Slope	0.015	0.015	0.049
Intercept	0.023	0.007	0.024
SE of slope	0.0002	0.0002	0.0024
SE of intercept	0.0005	0.0001	0.0006
Correlation coefficient (r)	0.9995	0.9995	0.9990
Specificity	100.28±0.138	100.44±0.136	100.00±1.181
Accuracy (mean±SD)	100.26 ± 1.349	100.23±1.253	100.60±0.205
LOD	1.05	0.31	0.26
LOQ	3.18	0.94	0.79
Precision (RSD)*			
Repeatability	100.27	100.42	100.20
Intermediate precision	100.28	100.44	98.90

*The intraday and the inter-day precision RSD values of samples of concentration of 15, 20 and 25 µg/mL of TCC for D1 and DW and 15, 20 and 25 µg/spot for TLC

Table 2: Determination of TCC in laboratory prepared mixtures by the proposed methods

% Degradation	Conc. taken of intact in (µg/mL) for D^1 and DW and in (µg/spot) for TLC	Conc taken of degradant equivalent to TCC in (µg/mL) for D^1 and DW and in (µg/spot) for TLC	%R		
			D^1	DW	TLC
10	36	4			100.83
12.5	35	5		100.63	
20	32	8			99.67
25	30	10	100.33	100.33	
30	28	12			99.25
37.5	25	15	100.24	100.16	
40	24	16			98.68
50	20	20	100.15	100.45	101.58
62.5	15	25	100.20	100.67	
75	10	30	100.50	100.40	
Mean			100.28	100.44	100.00
SD			0.138	0.136	1.181
%RSD			0.137	0.135	1.181

* Average of three determinations

Table 3: Quantitative determination of TCC in Relaxin (4mg/ tablet) and the results of application of standard addition technique by the proposed methods

Relaxin (4 mg/tablet)	D^1	DW	TLC
Batch no.1311600	100.27±0.049	100.21±0.05	100.22±0.311
Recovery % of standard added	100.97±0.049	100.42±0.249	100.60±0.205

* Average of three determinations

Table 4: Statistical analysis of the results obtained by the proposed methods and the official method for the determination of TCC in pure powder form

Item	D^1 method	DW	TLC	Official method [a]
Mean	100.26	100.23	100.25	100.92
SD	1.349	1.250	1.769	0.812
Variance	1.82	1.56	3.13	0.66
n	6	8	10	5
Student's t-test [b]	0.984 (2.262)	1.076(2.228)	1.027(1.771)	
F-value [b]	2.758 (6.26)	2.364(6.16)	4.742(6.01)	

a= HPLC method-RP chromatography- C18 column-UV detector at wave length 370 nm (USP40) [21].
B= Figures between parentheses represent the corresponding tabulated values of t and F at P = 0.05.

CONCLUSION

The most striking features of these developed methods are selectivity, simplicity, sensitivity, precision, accuracy and inexpensively. So, the proposed methods could be used in routine and quality control analysis of TCC in their pure powder form or in their combined preparations. The results demonstrate the usefulness of these methods for the selective determination of TCC without any interference even with its corresponding degradation products with no need to prior separation or to use other sophisticated methods like HPLC chromatography.

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Graphical abstract