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Different stability–indicating techniques for the determination of triclabendazole

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ABSTRACT

Three simple, accurate and sensitive methods were developed for the determination of triclabendazole (TCZ) in presence of its degradation product. Method (A) was depending on third derivative spectrophotometry ^{3}D, then measuring the peak amplitude at 266 nm. Method (B) was a TLC method, using silica gel 60 F_{254} plates; the optimized mobile phase was ethyl acetate/ methanol/ ammonium hydroxide (8:1:0.2 by volume). The spots were scanned densitometrically at 300 nm. Method (C) was an HPLC method, performed on C18 column using acetonitrile/ 0.05M potassium dihydrogen phosphate (60:40 v/v), the pH was adjusted to 3.5±0.2 with ortho-phosphoric acid as a mobile phase with a flow rate of 1.5 ml/min. Detection was performed at 300 nm. Linearity ranges were 5 – 40 µg/ml for method (A), 0.5 – 5 µg/band for method (B) and 0.5 – 5 µg/ml for method (C), the mean percentage recoveries were 100.0 ± 1.0%, 100.5 ±1.2% and 99.8 ±1.1% for methods (A), (B) and (C) respectively. The proposed methods were found to be specific for triclabendazole in presence of up to 70% of its degradation product for method (A) and 90% of its degradation product for methods (B) and (C). Statistical comparison between the results obtained by these methods and the manufacturer's method was done, and no significance difference was obtained.

KEYWORDS

Triclabendazole;
Degradation;
Derivative spectrophotometry;
HPLC;
TLC.

INTRODUCTION

Triclabendazole (TCZ), 5-Chloro-6-(2,3-dichlorophenoxy)- 2-(methylthio)benzimidazole, scheme 1. It is a benzimidazole anthelmintic drug used in veterinary medicine for the treatment of fascioliasis. It is also increasingly being used in the treatment of human fascioliasis, and is under investigation for the treatment of human paragonimiasis[1].

Literature survey reveals few analytical methods for quantification of the drug in pure form, in pharmaceutical dosage forms, in biological fluids and in the presence of its metabolites including continuous wavelet transform and derivative spectrophotometry[2] and HPLC methods[3-12].

However, there are no reported methods concerning the stability of TCZ or the determination of TCZ in presence of its degradation product. This work pre-

sents a study of oxidative degradation of TCZ, followed by the development of three stability-indicating methods, a derivative spectrophotometric method and two chromatographic methods for the determination of the drug in its pure powdered form, in the presence of its degradation product as well as in its dosage form.

Derivative spectrophotometric technique is of great utility for resolving some mixtures of compounds with overlapping spectra or determination of drugs in presence of their degradation products. TLC has become a routine analytical technique due to the possibility of application of several samples to be run simultaneously using a small amount of mobile phase, thus lowering the time and cost per analysis. HPLC has the advantages of its discriminating power to resolve the drug from its degradation product and its ability to operate at ambient temperature that would not contribute to the degradation of the drug.

EXPERIMENTAL

Instruments

An Agilent HPLC instrument was obtained from Aligant (Waldbronn, Germany), Densitometer-dual wave length flying spot was obtained from Shimadzu (Tokyo, Japan), UV lamp with short wave length 254 nm was obtained from Desaga (Waldbronn,Germany), UV-2400 PC Series Spectrophotometer with two matched 1cm quartz cell was obtained from Shimadzu (Tokyo, Japan), Thin layer chromatographic plates precoated with silica gel 60 F_{254} 10×20 cm were obtained from Fluka (Switzerland, Germany). The IR spectrophotometer was obtained from Bruker Optics (Ettlingen, Germany). Mass Spectrophotometer was obtained from Agilent Technologies, Wilmington, DE. (Hewlett Packard Model 5988A GC/MS, fragmentation patterns arise from electron impact ionization.

Reagents

Acetonitrile HPLC grade was obtained from s d fine-chem limited (Mumbai, India). Sodium hydroxide, sulphuric acid, ortho-phosphoric acid, disodium hydrogen phosphate, potassium permanganate, ethyl acetate, chloroform, methanol, ammonium hydroxide and potassium dihydrogen phosphate were obtained from ADWIC (Cairo, Egypt).

Samples

(a) Reference sample

TCZ-Pure sample was kindly supplied by Pharma Swede, Egypt. Its purity was found to be 99.2 ± 0.8% according to the manufacturer's method[13].

(b) Pharmaceutical formulation

Flukamid oral suspension-Batch number. IL005FM01 was supplied by Medco- Erp limited, Holland. Each 1 ml is claimed to contain 50 mg of triclabendazole.

(c) Degraded sample (equivalent to 1 mg/ml of intact TCZ)

TCZ (50 mg) was dissolved in 5ml methanol, a mixture of 1gm of potassium permanganate and 41.2 mg of disodium hydrogen phosphate dissolved in 10 ml water was added drop wise with continuous stirring. The solution was sonicated for 90 minutes. The whole mixture was extracted with 3×20 ml chloroform. The aqueous layer was reacidified with 20 ml dilute sulphuric acid, followed by 2 ml methanol and warmed gently to decompose excess potassium permanganate and to dissolve the formed manganese dioxide. The aqueous layer was further extracted with 2×20 ml chloroform and rendered just alkaline (pH aproximatly 7.5) using dilute aqueous solution of sodium hydroxide, then reextracted with 20 ml chloroform. The organic extracts were combined and evaporated till complete dryness. The residue was dissolved in 25 ml methanol and transferred quantitatively into a 50-ml volumetric flask. The volume was completed to the mark with the same solvent and filtered to prepare solution of concentration (equivalent to 1 mg/ml of intact TCZ) in methanol[14]. The degraded solution was tested for complete degradation by TLC using three different systems [toluene/ methanol/ chloroform/ ammonium hydroxide (5:4:6:0.1 by volume), methanol: 33% ammonium hydroxide (9:0.1 v/v) and ethyl acetate: methanol: 33% ammonium hydroxide (8:1:0.2 by volume)]. Only one spot was observed not corresponding to TCZ.

Standard solutions

1. TCZ stock standard solution: (1 mg/ml) in methanol.
2. Working standard solution: (0.1 mg/ml) in methanol.

3. Working standard solution: (0.1 mg/ml) in mobile phase for method (C).

Degraded solutions

1. Degradation product stock solution: (1 mg/ml) in methanol. Prepared as mentioned in section 2.3.3.
2. Working degradation product solution: (0.1 mg/ml) in methanol.
3. Working degradation product solution: (0.1 mg/ml) in mobile phase for method (C).

Laboratory prepared mixtures containing different ratios of TCZ and its degradation product

(a) Method (A)

Aliquots (1.8 - 0.6 ml) of TCZ were accurately transferred from its working standard solution (0.1 mg/ml) equivalent to (180 – 60 µg) into a series of 10-ml volumetric flasks. Aliquots (0.2 – 1.4 ml) of working degradation product solution (0.1 mg/ml) equivalent to (20 – 140 µg) were added, the volume was completed with methanol to prepare mixtures containing 10 – 70 % of the degradation product.

(b) Method (B)

Aliquots (4.5 – 0.5 ml) of stock standard solution (1 mg/ml) equivalent to (4.5 – 0.5 mg) were accurately transferred into a series of 10-ml volumetric flasks, then aliquots (0.5 – 4.5 ml) of degradation product stock solution (1 mg/ml) equivalent to (0.5 – 4.5 mg) were added. The volume was completed with methanol to prepare mixtures containing 10 – 90 % of the degradation product.

(c) Method (C)

Aliquots (0.45 - 0.05 ml) of TCZ were accurately transferred from its working standard solution (0.1 mg/ml) equivalent to (45 –5 µg) into a series of 10-ml volumetric flasks. Aliquots (0.05 – 0.45 ml) of working degradation product solution (0.1 mg/ml) equivalent to (5 – 45 µg) were added, the volume was completed with the mobile phase to prepare mixtures containing 10 – 90 % of the degradation product.

Procedures

(a) Method (A)

(A) Linearity

Aliquots (0.2, 0.4, 0.6, ……., 2 ml) of the drug working standard solution (0.1 mg/ml) were transferred into a series of 10-ml volumetric flasks. The volume was completed to the mark with methanol. The spectra of the prepared solutions were scanned and stored in the computer. The derivative spectra were recorded using $\Delta\lambda$=16 and scaling factor 500, the peak amplitudes of the third derivative spectra of TCZ were measured at 266 nm. A linear calibration curve was constructed relating the peak amplitude at 266 nm to the corresponding concentration of TCZ and the regression equation was computed.

(B) Accuracy

The accuracy of the results was checked by applying the previously mentioned procedure under linearity for different concentrations of pure TCZ within the linearity range. The concentrations of the drug were calculated from the regression equation. The mean recovery percentage and relative standard deviation were then calculated.

(C) Precision

(1) Repeatability

Three concentrations of TCZ stock standard solution (6, 10 and 14 µg/ml) were analyzed three times each, intra-day, using the previously mentioned procedure under section 2.7.1.1. The mean recovery percentage and relative standard deviation were then calculated.

(2) Intermediate precision

The above mentioned TCZ samples were analyzed on three successive days using the procedure stated under section 2.7.1.1. The mean recovery percentage and relative standard deviation were then calculated.

(D) Assessment of selectivity/specificity of the method

The third derivative spectra of the prepared mixtures (2.6.1.) were recorded. Then the procedure was completed as described in section 2.7.1.1. The concentration of TCZ was calculated by substitution in the corresponding regression equation.

(E) Application of the proposed method for the determination of TCZ in its pharmaceutical formulation

The contents of the Flukamid Oral-suspension bottle

were shacked well, then 1 ml equivalent to 50 mg TCZ was quantitatively transferred into a beaker, 25 ml methanol was added. The beaker was covered with watch glass and the solution was stirred for 30 minutes using a magnetic stirrer, filtered into a 50-ml volumetric flask, the residue was washed three times each with 5 ml methanol and filtered, the collected filtrates were accurately transferred to the volumetric flask, and the volume was completed to the mark with methanol to prepare solution of concentration (1 mg/ml) in methanol. Suitable dilutions were made with the mobile phase to prepare a solution of concentration (10 µg/ml) in methanol. Then the procedure was completed as described in section 2.7.1.1. The concentration of TCZ was calculated by substitution in the corresponding regression equation.

(b) Method (B)

(A) Linearity

Aliquots (0.5, 1, 2, 3, 4, 5 ml) of the stock standard solution (1 mg/ml) were transferred into a series of 10-ml volumetric flasks. The volume was completed to the mark with methanol. Ten µl of the prepared solutions, using 10 µl Hamilton syringe, was applied as separate compact bands 20 mm apart and 20 mm from the bottom of the plates. The chromatographic tank was saturated with the mobile phase [ethyl acetate/ methanol/ 33% ammonium hydroxide (8:1:0.2 by volume) for one hour in ascending manner to a distance of 7 cm from the spotting line at room temperature, air-dried, and the plates were scanned under the following conditions:

- Source of radiation: deuterium lamp.
- Photomode: Reflection.
- Scan mode: Zigzag.
- Result output: Chromatogram and area under the peak.
- Swing width: 10 mm.
- Wavelength: 300 nm.

The scanning profile for TCZ was obtained. The calibration curve relating the integrated peak area to the corresponding concentration was constructed and the regression equation was computed.

(B) Accuracy

The accuracy of the results was checked by applying the proposed method for the determination of different concentrations of pure TCZ within the linearity range. The concentrations were calculated from the regression equation. The mean recovery percentage and relative standard deviation were then calculated.

(C) Precision

(1) Repeatability

Three concentrations of TCZ stock standard solution (0.5, 1 and 2 µg /band) were analyzed three times each, intra-day, using the previously mentioned procedure under section 2.7.2.1. The mean recovery percentage and relative standard deviation were then calculated.

(2) Intermediate precision

The above mentioned TCZ samples were analyzed on three successive days using the procedure stated under section 2.7.2.1. The mean recovery percentage and relative standard deviation were then calculated.

(D) Assessment of selectivity/specificity of the method

Ten µl from the prepared mixtures (2.6.2.) was spotted on TLC plates and the procedure was completed as described in section 2.7.2.1. The concentration of TCZ was calculated by substitution in the corresponding regression equation.

(E) Application of the proposed method for the determination of TCZ in its pharmaceutical formulation

The dosage form was treated as under (2.7.1.5.) to prepare solution of concentration of (1 mg/ml). Suitable dilution was made to prepare solution (200 µg/ ml) in methanol. Then the procedure was completed as described in section 2.7.2.1. The concentration of TCZ was calculated by substitution in the corresponding regression equation.

(c) Method (C)

(A) Linearity

Aliquots (0.05, 0.1, 0.2, 0.3, 0.4, 0.5 ml) of the drug working standard solution (0.1 mg/ml) were transferred into a series of 10-ml volumetric flasks. The volume was completed to the mark with mobile phase. 20 µl of the previously prepared solutions was Injected in triplicate using the following chromatographic conditions:

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- Column: Nucleosil, size 125×4mm, C18 5µm. The column was equilibrated with the mobile phase until steady baseline obtained and column pressure was stabilized.
- Mobile phase: The mobile phase consisted of acetonitrile: 0.05M potassium dihydrogen phosphate in ratio (60:40 v/v). The pH was adjusted to 3.5±0.2 with ortho-phosphoric acid. The mobile phase was filtered using 0.45 µm membrane filters and degassed by ultrasonic vibrations for 30 min.
- Temperature: Ambient temperature.
- Flow rate: 1.5 ml/ min.
- Detector wavelength: 300 nm.
- Injection volume: 20 µl.

The chromatogram was obtained, the average peak area ratios obtained for each concentration of TCZ to that of external standard 5 µg/ml were plotted versus concentrations, and the regression equation was computed.

(B) Accuracy

The accuracy of the results was checked by applying the previously mentioned procedure under linearity for different concentrations of pure TCZ within the linearity range. The concentrations of the drug were calculated from the regression equation. The mean recovery percentage and relative standard deviation were then calculated.

(C) Precision

(1) Repeatability

Three concentrations of TCZ stock standard solution (2, 2.5 and 4 µg/ml) were analyzed three times each, intra-day, using the previously mentioned procedure under section 2.7.3.1. The mean recovery percentage and relative standard deviation were then calculated.

(2) Intermediate precision

The above mentioned TCZ samples were analyzed on three successive days using the procedure stated under section 2.7.3.1. The mean recovery percentage and relative standard deviation were then calculated.

(D) Assessment of selectivity/specificity of the method

Twenty µl from the prepared mixtures (2.6.3.) was injected into the liquid chromatograph. Then the procedure was completed as described in section 2.7.3.1. The concentration of TCZ was calculated by substitution in the corresponding regression equation.

(E) Application of the proposed method for the determination of TCZ in its pharmaceutical formulation

The dosage form was treated as under (2.7.1.5.) to prepare a solution of concentration of (1 mg/ml). Suitable dilutions were made in the mobile phase to prepare solution (2 µg/ml) of TCZ. Twenty µl from this solution was injected in triplicate into the liquid chromatograph under the previously mentioned chromatographic conditions in subsection (2.7.3.1.) of Linearity. The concentration of TCZ was calculated by substitution in the corresponding regression equation.

(F) System suitability

The tailing factors, the resolution factor, the selectivity factor, the theoretical plate count and the height equivalent to a theoretical plate (HETP) were calculated.

RESULTS AND DISCUSSION

The stability of TCZ was studied according to ICH guidelines[15] for:

(a) Acid and Alkaline Stress: 0.1M HCl/0.1M NaOH for 16 hrs, 0.2M HCl/0.2M NaOH for 16 hrs and 1MHCl/1M NaOH for 4 hrs and for 5 hrs.
(b) Oxidative Condition: 3% H_2O_2 for 2 hrs, 4 hrs, 6 hrs and for 10 hrs.
(c) Thermal Degradation: at 100°C in an oven for 2 hrs, 4 hrs and for 6 hrs.

TCZ shows highly stability to acidic, alkaline and thermal conditions, and it is insoluble in H_2O_2. The drug undergoes oxidative metabolism into its sulphoxide, sulphone and hydroxy derivatives by sheep liver microsomes[16]. So the interest was focused on the oxidation of the cited drug using $KMnO_4$.

Since this work was concerned with the development of stability-indicating methods for the determination of TCZ, the degradation product was prepared in laboratory as mentioned in 2.3.3. The structure of the isolated oxidative degradation product was confirmed using IR and MS spectroscopy, (Figures 1-4).

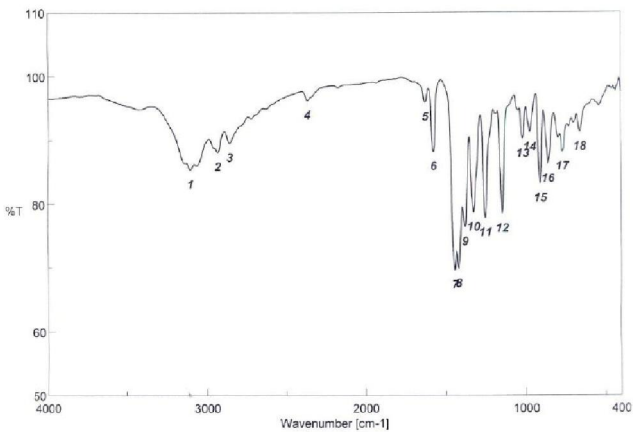


Figure 1 : IR spectrum of intact TCZ.

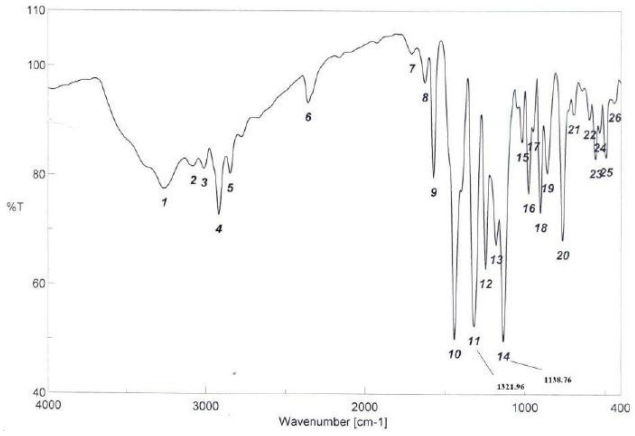


Figure 2 : IR spectrum of the degradation product of TCZ.

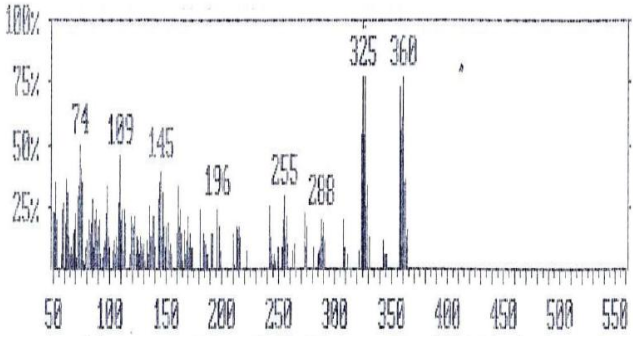


Figure 3 : Mass spectrum of intact TCZ.

The IR spectra of intact TCZ lacked the characteristic sulphone bands, (Figure 1). On the other hand the IR spectrum of the degradation product of TCZ is characterized by the absorption frequency of sulphone group at 1321.96 and 1138.76 cm^{-1}, (Figure 2). This result is proved by using MS spectroscopy; the MS spectrum of the intact TCZ is characterized by the molecular ion m/z at 360, (Figure 3). On the other hand the MS spectrum of the degradation product was characterized by the molecular ion m/z at 391, (Figure 4), which indicate the formation of sulphone derivative.

This finding suggested the degradation pathway and indicates that the degradation product of TCZ has the structure illustrated in scheme 1.

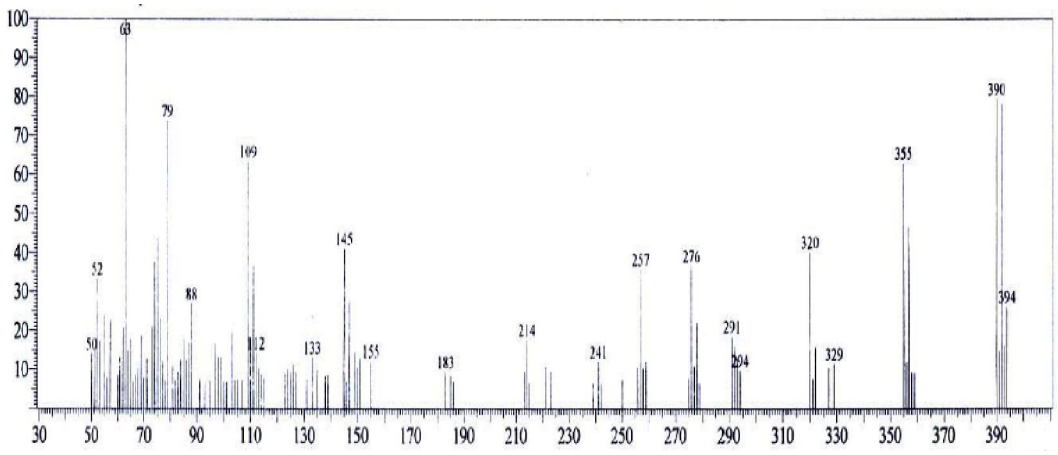
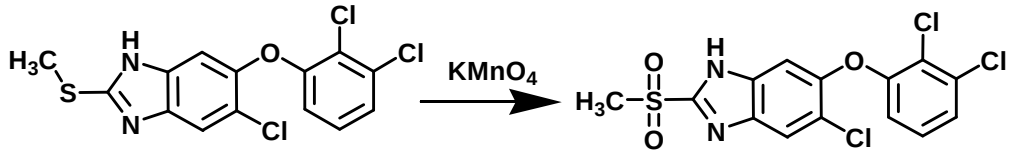


Figure 4 : Mass spectrum of the degradation product of TCZ.



Scheme 1 : The degradation pathway of TCZ.

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Method (A)

The present work is concerned with the use of derivative spectrophotometry for the quantitative determination of TCZ in the presence of its degradation product, in the pure powdered form as well as in pharmaceutical formulation.

Zero–order absorption spectra of TCZ and its degradation product in methanol show great overlap which can not permit direct measurement of the drug in the presence of its degradation product, (Figure 5).

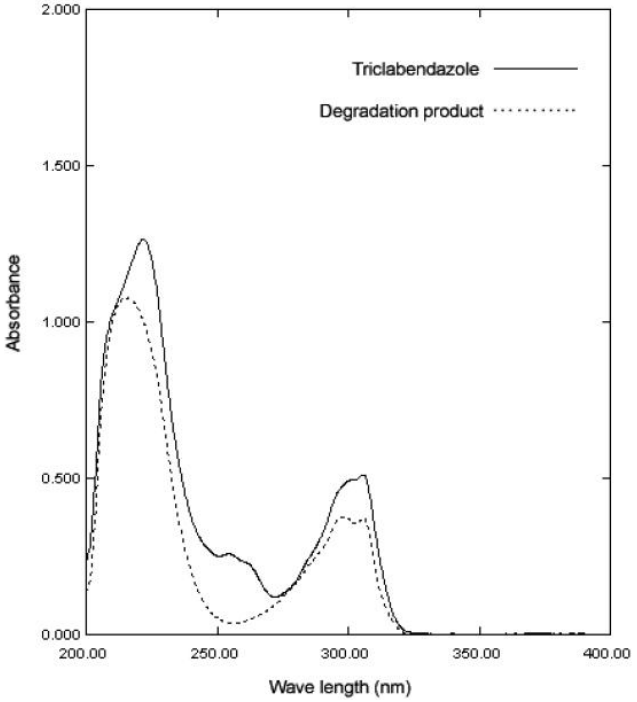

Figure 5 : Zero–order absorption spectra of TCZ (10μg/ml) and its oxidative degradation product (10 μg/ml) in methanol.

Derivative spectrophotometric method was applied. First and second derivative failed to solve the overlapping problem, (Figures 6, 7).

Upon examining the third derivative spectra of TCZ and its degradation product, (Figure 8), it is noticed that TCZ can be determined at 266 nm without interference from its degradation product.

The main parameters that affect the shape of the derivative spectra such as scanning speed, the wavelength increment over which the derivative is obtained ($\Delta\lambda$) and the scaling factor were studied and it was found that fast scanning speed, using $\Delta\lambda$=16 and scaling factor 500, gave best compromise in terms of signals to noise ratio, peak resolution and sensitivity throughout the determination.

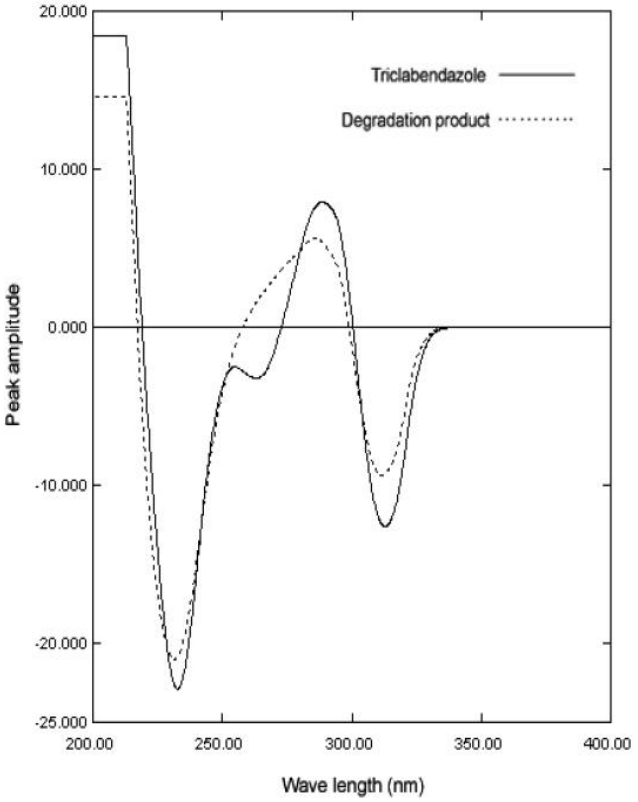

Figure 6 : First derivative spectra of TCZ (10 μg/ml) and its oxidative degradation product (10 μg/ml) in methanol.

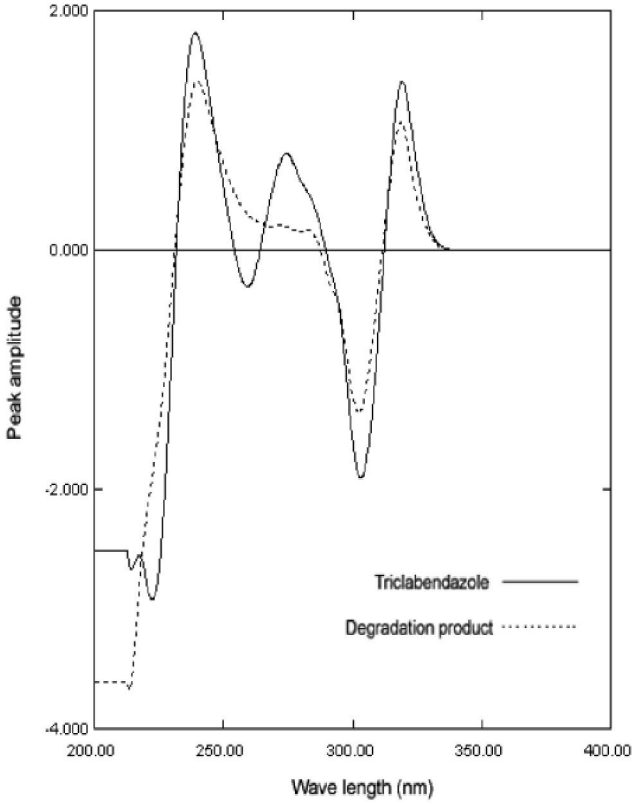

Figure 7 : Second derivative spectra of TCZ (10 μg/ml) and its oxidative degradation product (10 μg/ml) in methanol.

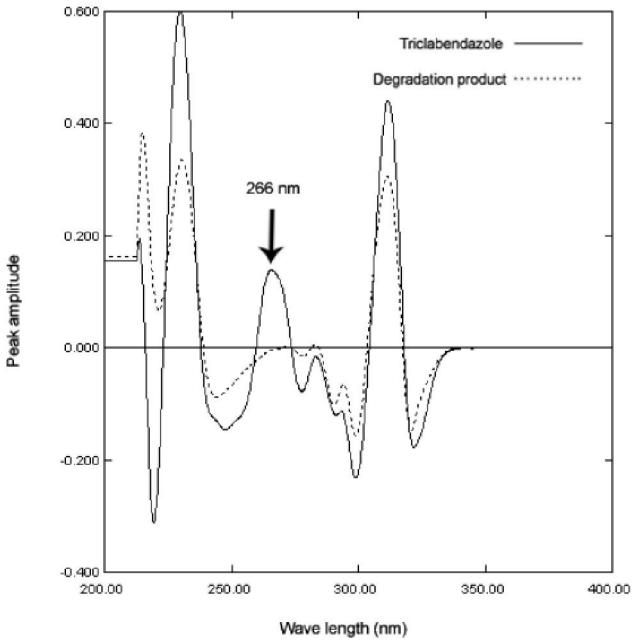

Figure 8 : Third derivative spectra of TCZ (10 µg/ml) and its oxidative degradation product (10 µg/ml) in methanol.

A linear relationship was obtained relating the peak amplitude at 266 nm to the corresponding concentration of triclabendazole over the range of (2-20 µg/ml), (Figure 9).

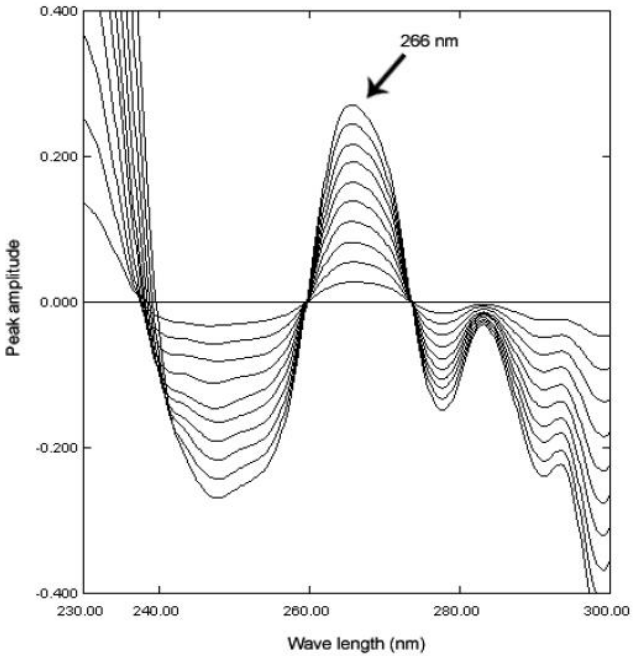

Figure 9 : Third derivative spectra of TCZ (2 -20 µg/ml) in methanol.

The regression equation was computed and found to be:

^3D = 0.0135 C + 0.0018 r = 0.9999

Where ^3D is the peak amplitude of third derivative spectra, C is the corresponding concentration µg/ml and r is the correlation coefficient.

The proposed method was successfully applied for the determination of the drug in pure powder form with mean percentage recovery of 100.0 ± 1.0%, (TABLE 1).

TABLE 1 : Results of validation parameters of the responses and the regression equations obtained by the proposed methods

Parameters	Method (A)	Method (B)	Method (C)
Slope [a]	0.0135	0.1068	0.1973
Intercept [a]	0.0018	0.0747	0.0186
Correlation coefficient	0.9999	0.9995	0.9997
Concentration range	2 – 20 µg/ml	0.5 – 5 µg/ band	0.5 – 5 µg/ ml
Average accuracy (%)	100.0	100.5	99.8
S.D.	1.0	1.2	1.1
R.S.D. %	1.0	1.2	1.1
Specificity± R.S.D.	99.4±1.0	99.8±0.6	99.9±1.2
Repeatability [b] %± R.S.D.	99.8±0.7	100.5±1.0	99.6±1.3
Intermediate precision [c] %± R.S.D.	98.9±0.4	99.4±0.6	99.8±0.3

[a] Results of five determinations; [b] n = 3×3; [c] n = 3×3

Method (B)

The proposed method is based on the difference in the R_f between the intact drug and its degradation product. The suitable mobile phase has been selected to achieve the best separation the drug from its degradation product; other necessary conditions have been established. Trials were carried out using different solvent systems to have a good separation of the drug from its degradation product. By using ethyl acetate: methanol: glacial acetic acid (8:1:0.2 by volume) separation of spots was occurred but it resulted in high R_f value of TCZ. Best separation of TCZ and its oxidative degradation product was obtained by using methanol: ethyl acetate: 33% ammonium hydroxide solution (8: 1: 0.2 by volume).

This selected mobile phase allows the determination of the drug without interference from the degradation product and without tailing of the separated spots provide better precision of the method. The instrumental conditions for densitometric measurement such as scan mode and wavelength detection were optimized. The scan mode chosen was zigzag mode, and the wave-

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length was 300 nm. TCZ was completely resolved from its degradation product and its R_f value was 0.84. On the other hand the R_f value of the degradation product was 0.56. This would permit quantitative determination of TCZ in presence of its oxidative degradation product, (Figure 10).

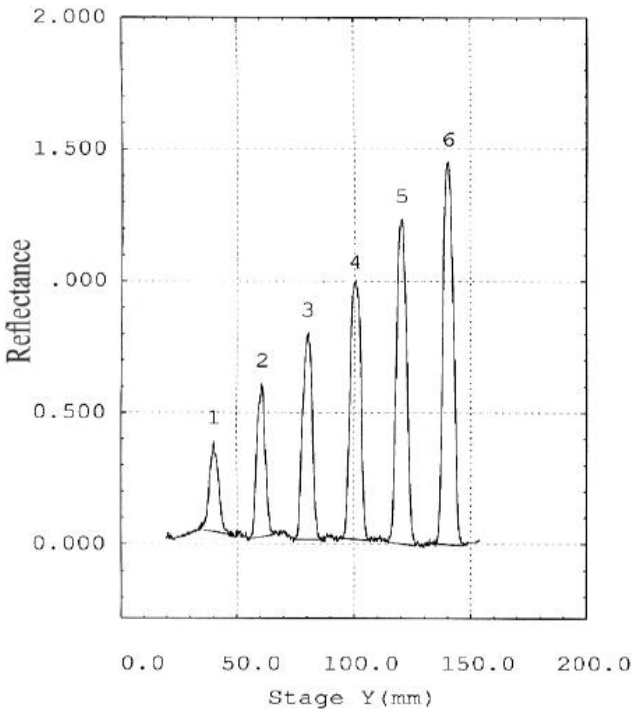

Figure 10 : Scanning profile of TLC chromatogram of TCZ at 300 nm.

A linear relationship between the concentration of TCZ and the integrated peak area was exist. The proposed method was found to be valid in the range of 0.5 – 5 µg/band and the regression equation was computed and found to be:

A = 0.1068 C + 0.0747 r = 0.9995

Where A is the integrated peak area $\times 10^{-4}$, C is the concentration of the drug in µg/band and r is the correlation coefficient.

The proposed method was successfully applied for the determination of the drug in pure powder form with mean percentage recovery of 100.5 ±1.2%, (TABLE 1).

Method (C)

The proposed method is based on the difference in the retention time between the intact drug and its degradation product. The suitable mobile phase has been selected to achieve the best separation the drug from its degradation product.

Different mobile phases with different ratios were investigated such as acetonitrile: water (10:90 v/v) and acetonitrile: methanol: 0.01M potassium dihydrogen phosphate (50:30:20 by volume), no good separation was obtained and also acetonitrile: 0.05M potassium dihydrogen phosphate in ratio (70:30 v/v), separation was occurred but tailed peaks were obtained. Satisfactory separation was performed with a mobile phase consisting of acetonitrile: 0.05M potassium dihydrogen phosphate in ratio (60:40 v/v) respectively, the pH was adjusted to 3.5±0.2 with ortho-phosphoric acid, with a retention time of 3.327 ± 0.03 min for TCZ and 2.293 ± 0.03 min for its oxidative degradation product. This would permit quantitative determination of TCZ in presence of its oxidative degradation product, (Figure 11).

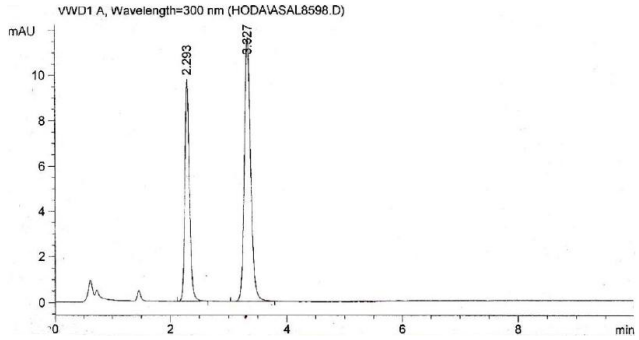

Figure 11 : HPLC chromatogram of the intact TCZ (2.5 µg/ml) and the degradation product (2.5 µg/ml) using the specified chromatographic conditions.

The average peak area ratios obtained for each concentration of TCZ to that of external standard 5 µg/ml were plotted versus the corresponding concentration of the drug. The proposed method was found to be valid in the range of 0.5 – 5 µg/ml, and the regression equation was computed and found to be:

A = 0.1973 C +0.0186 r = 0.9997

Where A is the peak area ratio, C is the concentration of the drug in µg/ml and r is the correlation coefficient.

The proposed method was successfully applied for the determination of the drug in pure powder form with mean percentage recovery of 99.8 ±1.1%, (TABLE 1).

System suitability test according to the United States Pharmacopoeia[17] was used to verify that the resolution and reproducibility of the chromatographic system were adequate for the analysis to be done. Accordingly, system suitability was checked by calculating the column efficiency (N), resolution (R), selectivity (α) and

tailing factor (T), where the system was found to be suitable, (TABLE 2).

TABLE 2 : Parameters of system suitability test of Method (C).

Parameter	Obtained value	
	TCZ	Degradation product
Relative retention time (α)	1.45	
Resolution (R)	6.38	
Capacity factor (K?)	4.40	2.72
Tailing factor (T)	0.87	0.86
Column efficiency (N)	4921	4681

Specificity of the methods was proven by the analysis of laboratory prepared mixtures containing different percentages of the degradation product. The proposed derivative spectrophotometric method was found to be specific for TCZ in presence of up to 70% of its degradation product while the proposed chromatographic methods were found to be specific for TCZ in presence of up to 90% of its degradation product (TABLE 3).

TABLE 3 : Results of analysis of TCZ in laboratory prepared mixtures containing different ratios of TCZ and its degradation product in pure powder form by the proposed methods.

Degradation %	Method (A) Concentration (µg/ml)			Method (B) Concentration (µg/ band)			Method (C) Concentration (µg/ml)		
	TCZ	Degradation product	Recovery %	TCZ	Degradation product	Recovery %	TCZ	Degradation product	Recovery %
10	18	2	99.6	4.5	0.5	99.6	4.5	0.5	101.7
20	16	4	101.4				4.0	1.0	98.2
30	14	6	99.5	3.5	1.5	99.8	3.5	1.5	100.8
40	12	8	99.4				3.0	2.0	99.8
50	10	10	98.6	2.5	2.5	100.7	2.5	2.5	99.4
60	8	12	98.2				2.0	3.0	99.6
70	6	14	98.8	1.5	3.5	100.1	1.5	3.5	98.4
80							1.0	4.0	101.1
90				0.5	4.5	99.0	0.5	4.5	99.7
Mean			99.4			99.8			99.9
S.D.			1.0			0.6			1.2
R.S.D. %			1.0			0.6			1.2

The usefulness of the proposed methods for the analysis of TCZ was studied by assaying Flukamid oral suspension, (TABLE 4). Samples were also spiked in order to asses the validity of the proposed method (TABLE 4).

TABLE 4 : Quantitative determination of TCZ in pharmaceutical formulation by the proposed methods and results of application of standard addition technique.

pharmaceutical formulation Flukamid oral suspension 5% B.N IL005FM01	Method (A) [a]	Method (B) [b]	Method (C) [c]
Found %	100.5±0.7%	99.6±0.3%	98.6±0.7%
Recovery % of standard added	98.9±0.8%	99.4±0.9%	101.1±0.7%

[a] **Average of four determinations;** [b] **Average of three determinations;** [c] **Average of six determinations**

Results obtained by the proposed methods for the determination of pure samples of the drug were statistically compared to those obtained by the manufacturer's method of the drug[13] and no significant differences were observed, (TABLE 5).

TABLE 5 : Statistical analysis between the results obtained for the determination of TCZ in pure samples by the proposed methods and those obtained by the manufacturer's method.

Item	Method (A)	Method (B)	Method (C)	Manufacturer's method*[13]
Mean	100.0	100.5	99.8	99.2
S.D.	1.0	1.2	1.1	0.7
R.S.D%	1.0	1.2	1.1	0.8
Variance	1.0	1.4	1.2	0.5
n	10	6	6	5
Student's t	1.6(2.160)	2.1(2.262)	1.1(2.262)	
F test	2.0(4.77)	2.8(5.05)	2.4(5.05)	

Figures in parentheses are the corresponding tabulated values at p = 0.05; *[13]Manufacturer's method HPLC, mobile phase water: acetonitrile (10:90 v/v) respectively, UV set at 258 nm and C18 column.

The accuracies were assessed by the determination of pure TCZ samples within the linearity ranges, the mean accuracies are given in TABLE 1.

The repeatability and interday precision were evaluated by assaying three freshly prepared solutions of the drug in triplicate on the same day and on three successive days respectively at concentrations within the linearity ranges for each method. RSD% shows the precision of the methods, (TABLE 1).

Validation of the proposed methods was made by measuring range, accuracy, precision, repeatability,

interday precision, linearity and specificity. Results obtained are depicted in TABLE 1. This data render the applicability of the proposed method for the quality control of the drug formulation.

The proposed derivative spectrophotometric, TLC and HPLC methods are precise, accurate, and sensitive. They can be used for the routine analysis of TCZ in pharmaceutical formulations. The ICH guidelines were followed throughout the study for method validation.

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