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Development and Validation of Spectrophotometric Methods for Quantitative Estimation of DiloxanideFuroate in Presence of Its Alkali-induced Degradation Product: A Comparative Study

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ABSTRACT

This study aimed to develop and validate two simple, accurate, selective, reproducible and sensitive spectrophotometric methods for the determination of diloxanidefuroate in the presence of its alkali-induced degradation product without preliminary separation. (A) ratio difference spectrophotometry method, where the peak amplitudes of ratio spectra were measured at270nm and 240 nm, (B)mean centering method, where the peak amplitudes were measured at270nm. All methods were applied in the range of (2- 30µg mL^{-1}). These methods were validated according toInternational Conference on Harmonization (ICH) guidelines and successfully applied for determination of diloxanidefuroate in Furamebe® tablets. The obtained results were statistically compared with those of the reported method by applying *t*-test and *F*-test at 95% confidence level and no significant difference was observed regarding accuracy and precision. The proposed methods are simple, rapid, economic, accurate and precise to determine diloxanidefuroate in the presence of its alkali-induced degradation product without previous separation steps.

INTRODUCTION

Diloxanidefuroate, (Figure 1), Chemically is 4-(N-methyl-2,2-dichloro-acetamido) phenyl-2-furoate having the molecular formula ofC14H11Cl2NO4 and the molecular weight of 328.1 g/mol[1]. It acts principally in the bowel lumen and it is used in the treatment of the intestinal amoebiasis. Diloxanidefuroate has been used in the treatment of the asymptotic carriers of Entamebahistolytica [2]. Several methods have been reported for the determination of diloxanidefuroate including titrimetric [3], electrochemical [4], spectrophotometric [5-14], and chromatographicmethods [15-23].

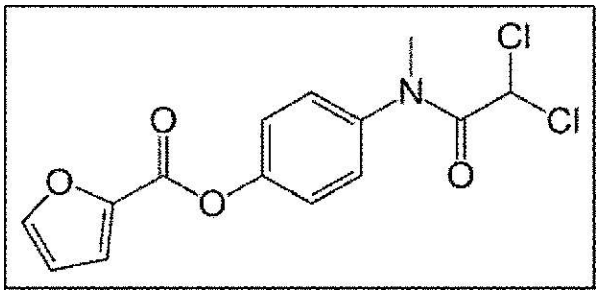

Figure 1: Structural formula of diloxanidefuroate .

The aim of this work is to develop and validate two simple, accurate, selective, reproducible and sensitive spectrophotometric methods for the determination of diloxanidefuroate in the presence of its alkali-induced degradation product without preliminary separation, which can be used foranalysis of diloxanidefuroatein raw material and pharmaceutical formulations. These methods include; ratio difference spectra and mean centering.

MATERIALS AND METHODS

Instruments

Shimadzu UV/visible double beam 1800 Spectrophotometer (Tokyo, Japan) with 1 cm matched quartz cells and UV Probe

software (2.35 ver.).

Soft wares

Mean centering was implemented in MATLAB 8.2.0.701 (R2013b) using PLS toolbox version2.1. TheStudent's *t*-test and *F*-test were performed using Microsoft Excel.

Samples

Pure standard

Pure materials of diloxanidefuroate were obtained as a gift sample from Sedico Pharmaceutical Company, 6 October City, Egypt.

Pharmaceutical preparation

Furamebe® tablets containing 500 mg of diloxanidefuroate per tablet (B.No.0014113), manufactured by Sedico Pharmaceutical Company,6 October City, Egypt.

Reagents and solvents

Methanol, analytical grade (El-Nasr Company, Egypt).

Hydrochloric acid, (El-Nasr Co., Egypt), prepared as 2N aqueous solution.

Sodium hydroxide, (El-Nasr Co., Egypt), prepared as 1N aqueous solution.

Preparation of standard solutions:

Standard solution (100 µg /mL) of diloxanidefuroate was prepared by transferring accurately weighed 10 mg of the powder into 100-mL volumetric flask, then dissolved in methanol and diluted up to the mark with the same solvent.

Preparation of the degradation products:

For 3 h, 0.3 g of pure diloxanidefuroatewere heated under reflux with 20 mL of 1N sodium hydroxide.The solution was allowed to cool and upon cooling, the first degradation product '4-hydroxy-N-methyl aniline' separates out. The precipitate was filtered, washed and recrystalized. The filtrate was acidified with 2N hydrochloric acid where the degradation product '2-furoic acid' separates out, which was filtered, washed and recrystallized.The second filtrate contains dichloro acetic acid which is liquid and miscible with water, so it has been difficult to separate it for further quantitation[9]. The obtained powder was used for the preparation of (100 µg /mL) stock solution of the degradation product.

Methods

Construction of calibration curves

Accurately measured aliquots equivalent to (20 □ 300 µg) ofdiloxanidefuroate were transferred from its standard solution (100 µg/mL) into a series of 10-mL volumetric flasks and the volume of each flask was diluted up to the mark with methanol, to reach the concentration range of (2 □ 30 µg/mL). The absorption spectra of these solutions were measured in the range of 200 to 400 nm against methanol as a blank.

Ratio difference spectroscopy method

The obtained absorption spectra weredivided by the 'the divisor' (the absorption spectrum of 15µg/ml of the degradation product), and theratio spectra thus obtained. The amplitudes difference of the ratio spectraat 270 and 240 nm (ΔP270240) versus the final concentrationsin µg/ml were plotted to get the calibrationgraph and the regression equation was derived.

Mean centering method

The obtained ratio spectra were mean centered.The measured mean centered values of the ratiospectra at 270nm versus the final concentrations in µg/ml were plotted to get the calibrationgraph and the regression equation was derived.

Assay of laboratory prepared mixtures

Aliquots of standard diloxanidefuroatesolution (100 µg/mL) and its degradation product solution (100 µg/mL), in the specified range, were introduced into a series of 10-mL volumetric flasks and diluted to volume with methanol. Procedure for each method was applied and the concentrations of diloxanidefuroatein the prepared mixtures were determined from the corresponding regression equation for each method.

Application to pharmaceutical formulation

Ten tablets of Furamebe®were accurately weighed, crushed and mixed well. An amount of the powder equivalent to 10 mg of diloxanidefuroate was weighed and dissolved in methanol by shaking for about 30 min. The solution was filtered and transferred quantitatively into 100 mL volumetric flask. The volume was then completed to the mark with methanol. Necessary dilutions were made to reach concentrations in the linearity range. The same procedures under the corresponding linearity were applied and the concentrations of diloxanidefuroatewere calculated from the corresponding regression equations.

RESULTS AND DISCUSSION

Degradation of diloxanidefuroate

It was reported that complete degradation of diloxanide furoatewas achieved upon heating under reflux with 1N sodium hydroxidefor 3 hours then acidified with 2N hydrochloric acid to give its degradation product as shown in Figure 2. The obtained powders were used for the preparation of the stock solutions of the degradation product. The isolated crystalline powder obtained from the acidic methanolic solution was confirmed by IR,^{1}HNMR and mass spectrometry.

Confirmation of degradation product

Confirmation of degradation product using IR techniques

IR spectrum of the intact diloxanidefuroate in Figure 3, showed peak of (C=O) of carboxyl group (-COOH) at 1726.32 cm^{-1}, while IR spectrum of degradation product showed disappearance of (C=O) stretch of carboxyl group which indicates the cleavage of ester linkage, and appearance of OH stretch at 3300 cm^{-1}in Figure 4.

Confirmation of degradation product using ^{1}H NMR techniques

The^{1} HNMR of the intact diloxanide furoate indimethyl sulfoxide (DMSO) in Figure 5. showed doublet signal of one proton (=CH) at 7.570-7.578 ppm, multiplet signals of one proton in furan ring at 6.795-6.807 ppm, doublet signal of one proton (=CH) at 7.492-7.513 ppm, two doublet signals of benzene ring at 7.403-7.425 ppm, singlet signal of three protons of methyl group (-CH3) at 3.240 ppm, singlet signal of one proton (-CH) at 6.260 ppm.

The^{1} HNMR of the degradation product in dimethyl sulfoxide

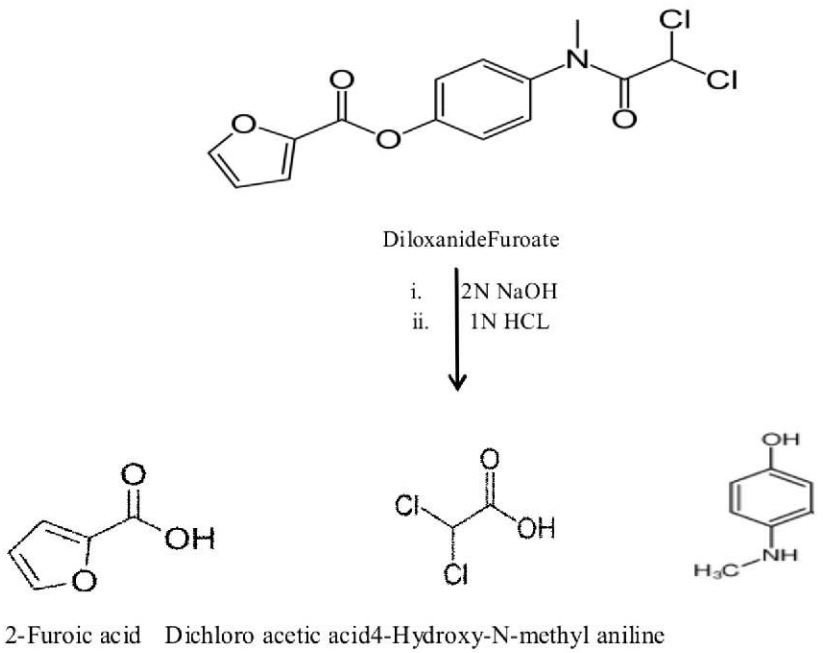

Figure 2: Degradation pathway of diloxanidefuroate.

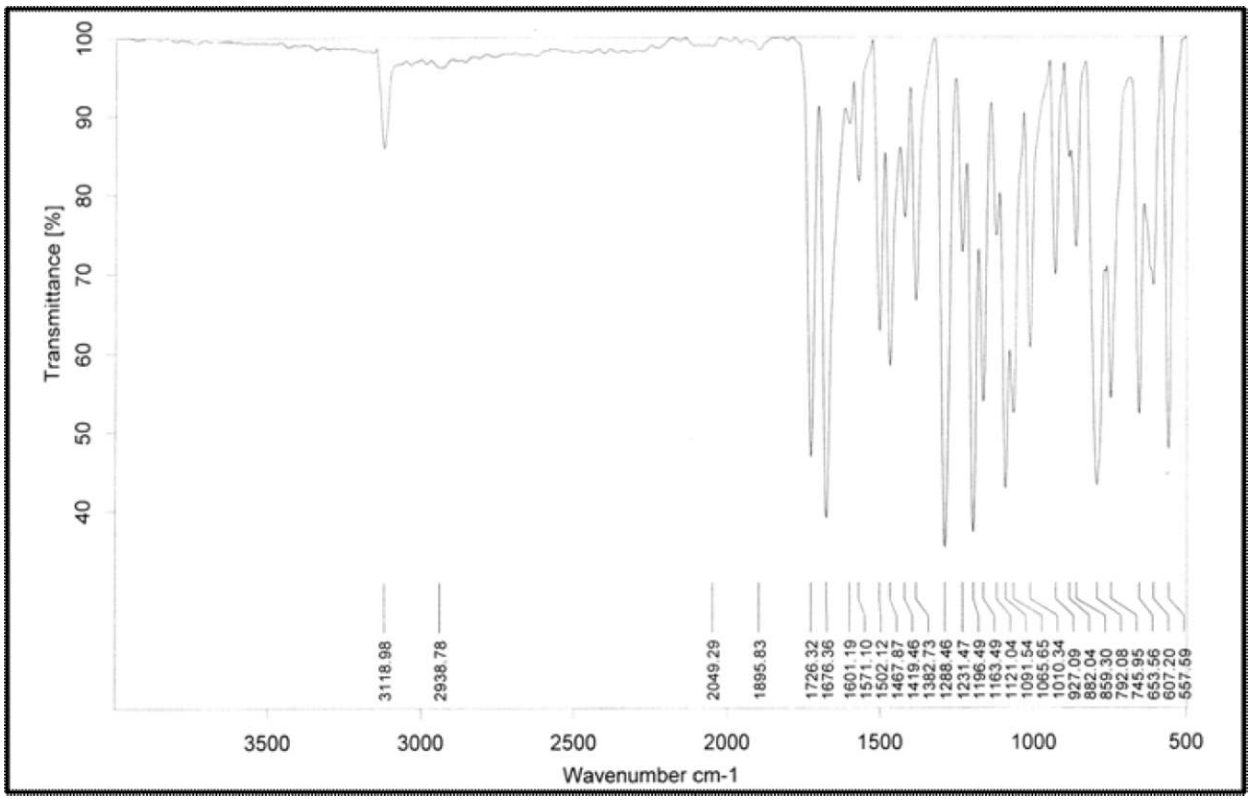

Figure 3: IRspectrum of intact diloxanidefuroate on K Br disc.

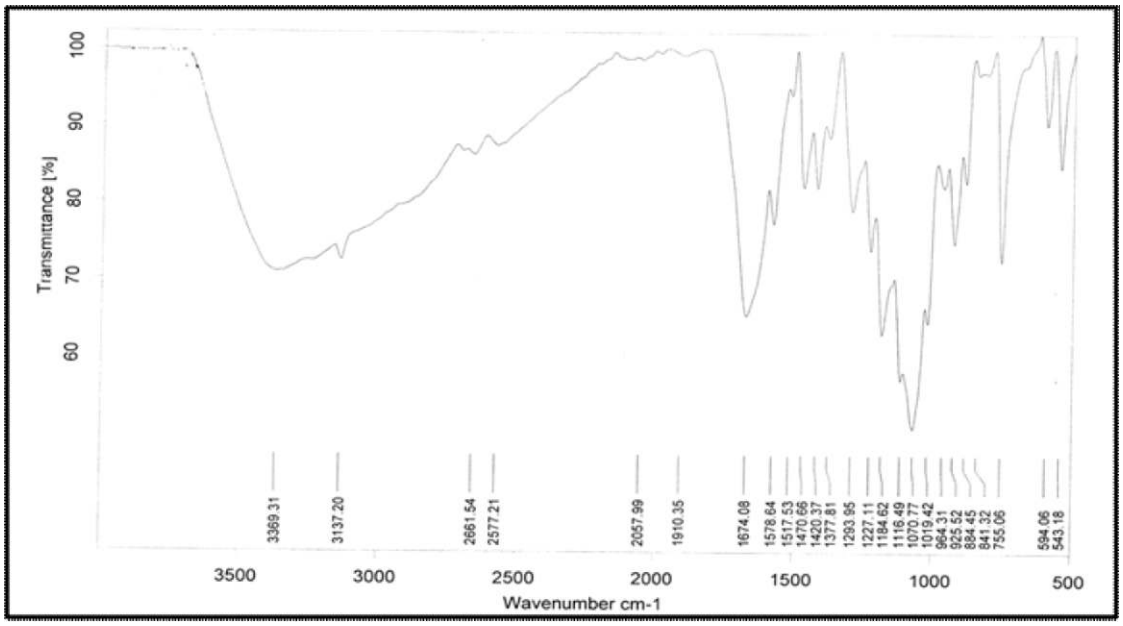

Figure 4: IR spectrum of diloxanidefuroate degradation production K Br disc.

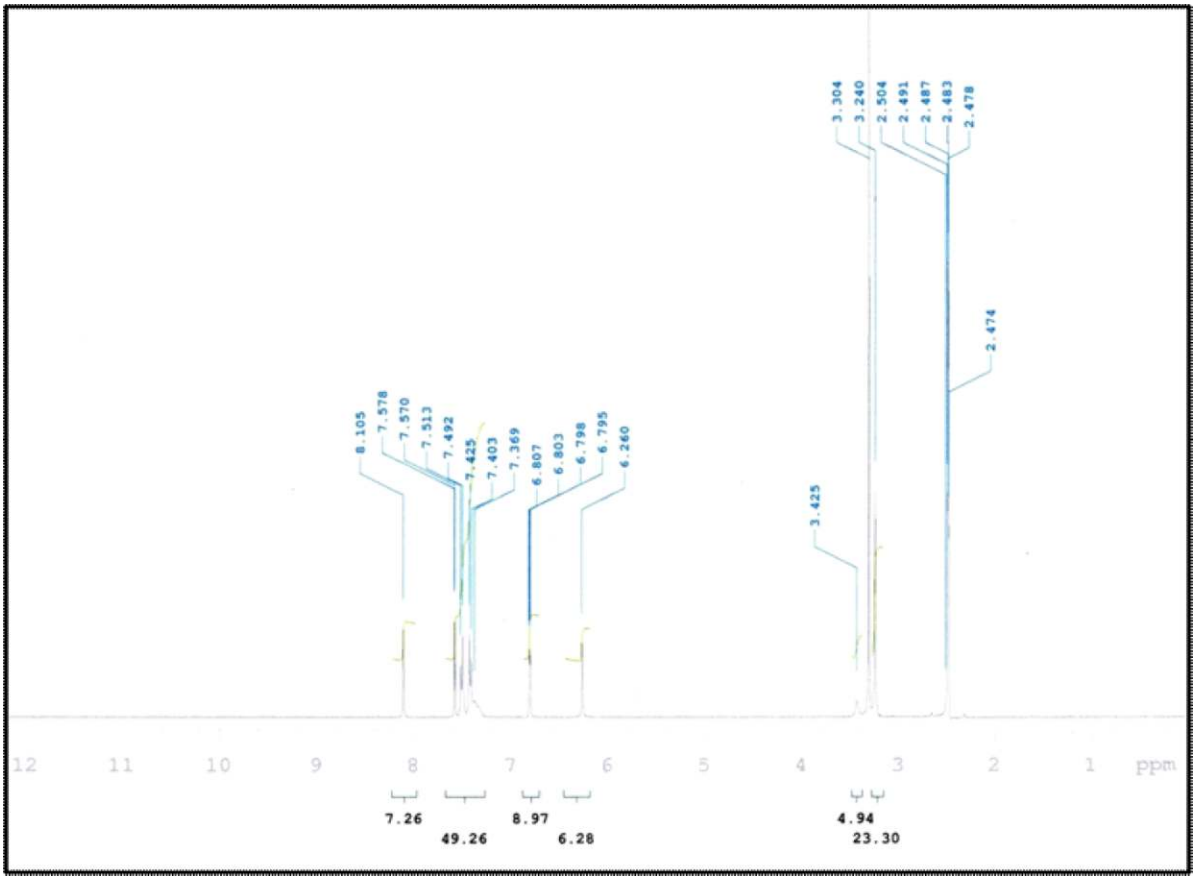

Figure 5: [1]HNMR spectrum of intactdiloxanide furoatein (DMSO).

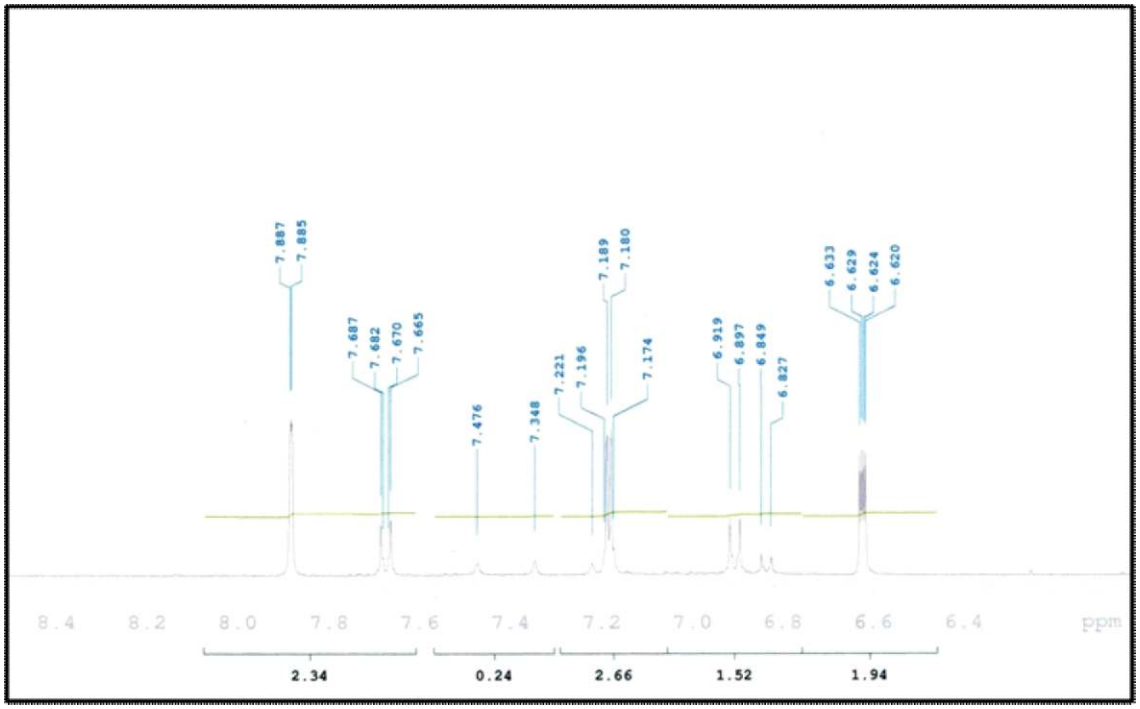

Figure 6: [1]HNMR spectrum of diloxanide furoate degradation productin (DMSO).

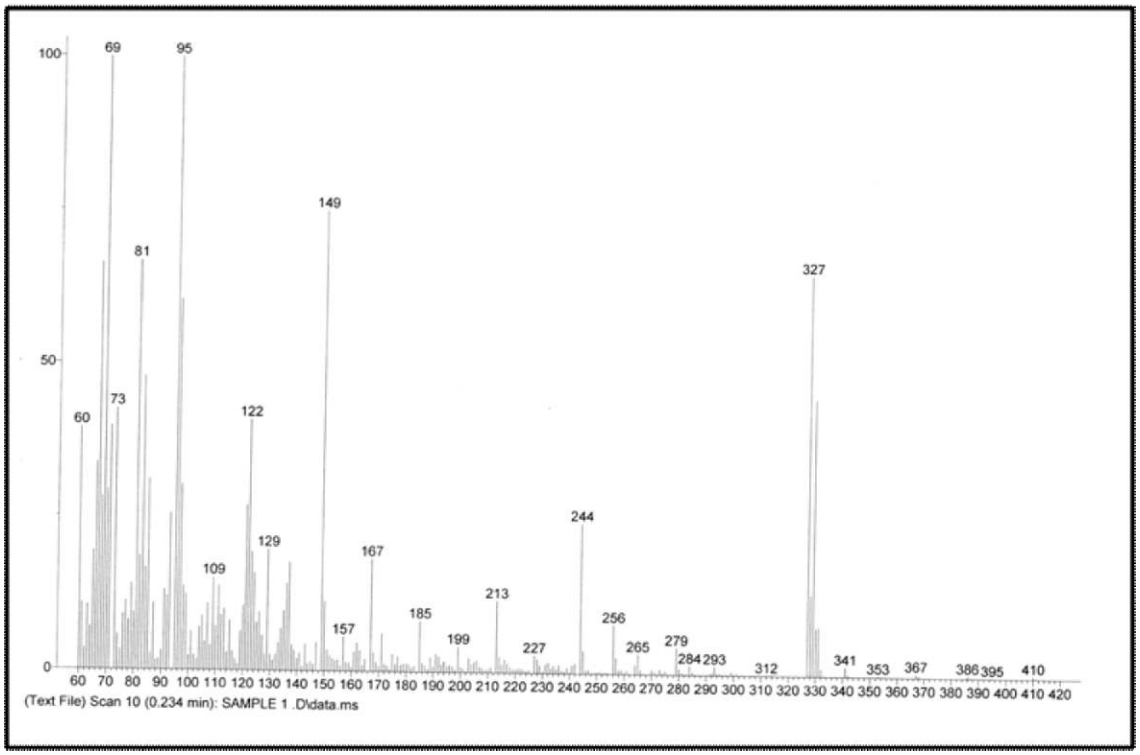

Figure 7: Mass spectrum of intact diloxanide furoate.

(DMSO) in Figure 6. showed singlet signal of one proton (-COOH) at 10.185 ppm indicating the cleavage of esterlinkage, doublet signal of one proton (-NH) at 7.180-7189 ppm indicating the cleavage of amide linkage.

Confirmation of degradation product using mass spectrometry

Mass spectrometry was performed for the intact drug and its degradation product and as shown in Figures 7-8. The molecular ion peak was obtained at m/z = 327.65 and m/z = 214.85,

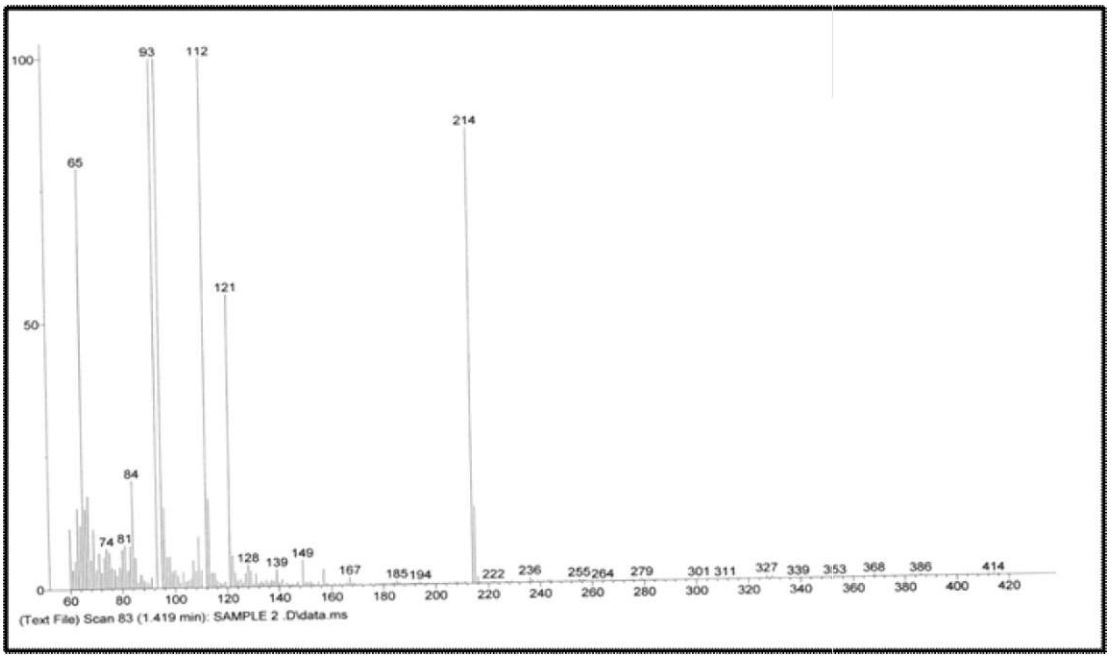

Figure 8: Mass spectrum of diloxanide furoate degradation product.

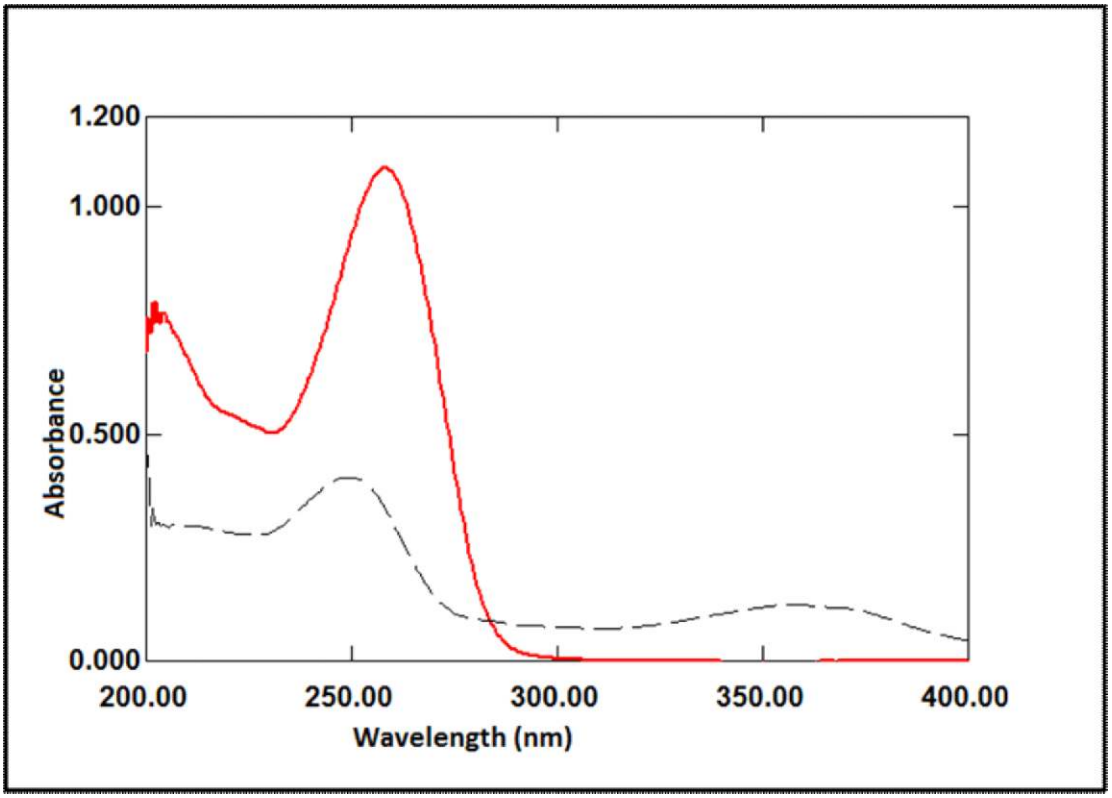

Figure 9: Zero order absorption spectra of diloxanidefuroate (15ug/mL) and diloxanide degradation product(15 ug/mL).

respectively indicating that the molecular weight of the degradation product is 214.85.

Spectral characteristics and methods development

The zero-order spectra of diloxanidefuroate and diloxanide degradation product shows severe overlapping, as shown in Figure 9. To be able to analyze diloxanidefuroate and overcome the interference from its degradation product, the zero order spectra of diloxanide furoate were divided by selective spectrum of diloxanide degradation product to get the ratio spectra, as shown in Figure 10. Careful choice of the divisor concentration was of great importance, so different concentrations of degradation product were tried asa divisor (2,5,10,15,20,25, and 30µg/ml); the best one was 15 µg/mL, as it produced minimal

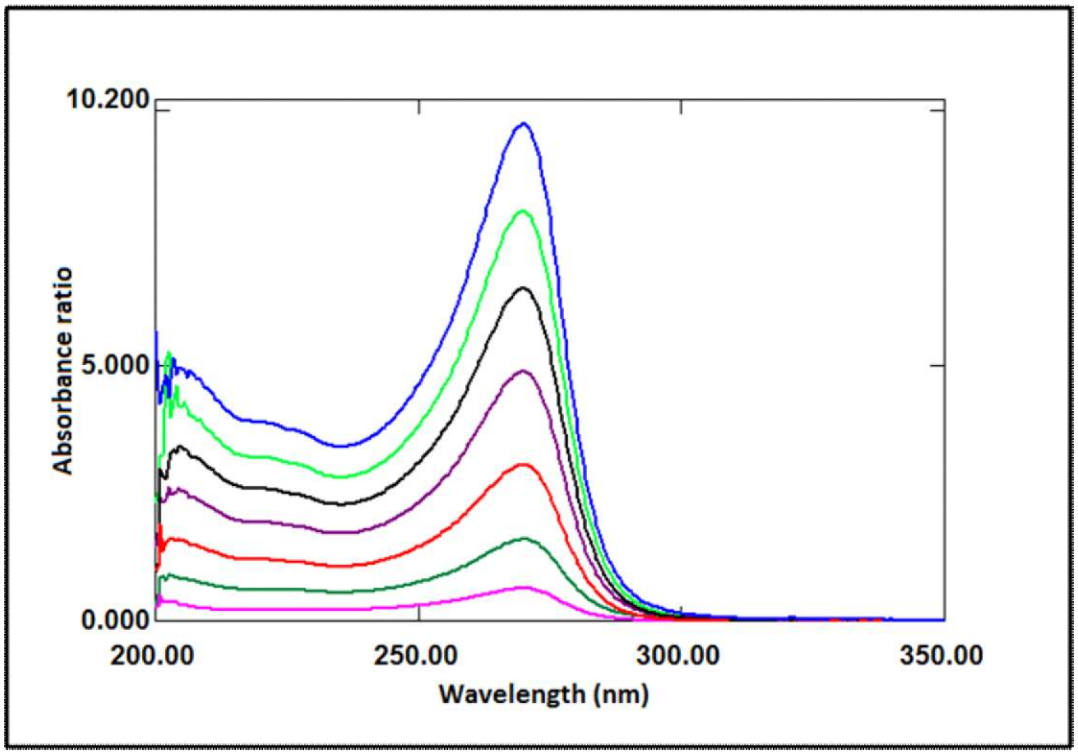

Figure 10: Ratio spectra of diloxanidefuroateat various concentrations (2-30 µg/ml) using 15 µg/ml of degradation product as a divisor.

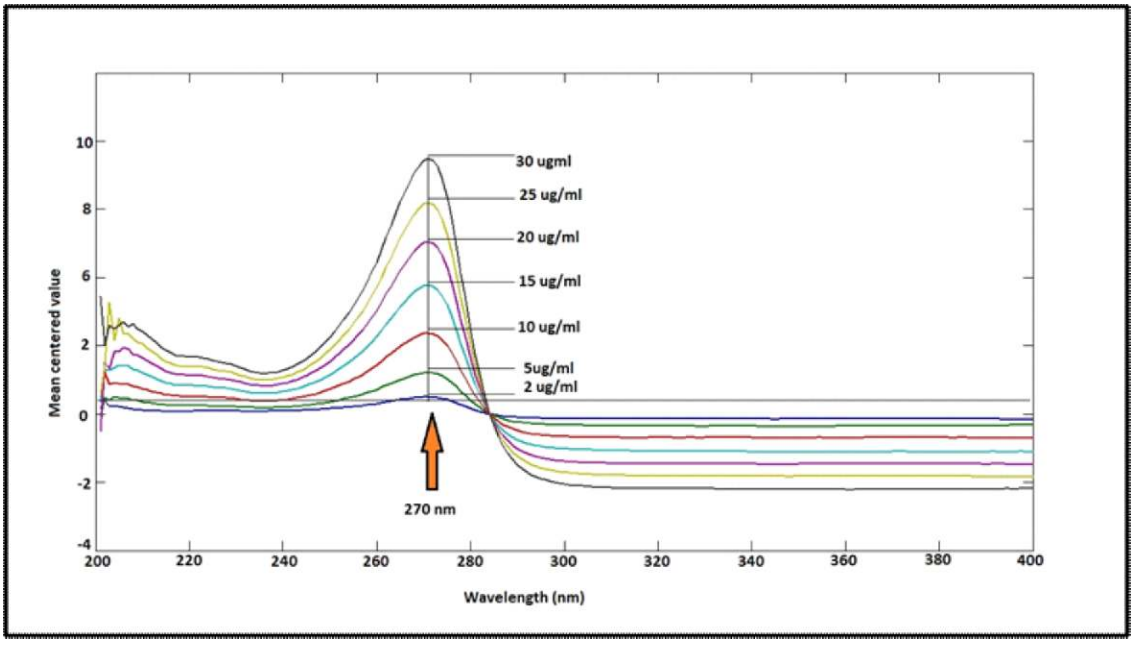

Figure 11: Mean centering of the ratio spectra of diloxanidefuroate at various concentrations (2-30 µg/ml) using 15 µg/ml of degradation product as a divisor.

noise and gave better results in accordance with selectivity. Then ratio spectra were manipulated by one of the following methods:

Ratio difference spectrophotometric method

In this method, the difference in peak amplitudes between 270 and 240 nm in the ratio spectra was proportional to the concentration of the drug without interference from its degradation product (divisor). The regression equation was computed from the linear relationship between the difference in peak amplitude at 270 & 240 nm and the corresponding

concentrations ofdiloxanidefuroate in the range 2-30 µg/mL.

Mean centering method

In this method, the obtained ratio spectra were mean centered. The mean centered values at 270nm were proportional to the concentrations of the drug without interference from its degradation product (divisor), as shown in Figure 11.The regression equation was computed from the linear relationship between the mean centered values of the ratio spectra at 270 nm and the corresponding concentrations of diloxanidefuroate in the range 2-30 µg/mL.

Methodvalidation:

The proposed methods was tested for linearity, range, limit of detection (LOD), limit of quantification (LOQ), accuracy, precision and selectivity according to International Conference on Harmonization (ICH) guidelines [24].

Linearity and range

Under the described experimental conditions, the calibration graphs for the methods were constructed by plotting the difference in peak amplitudes of the ratio spectra at 270 -240 nm versus drug concentrations in µg/mL for ratio difference and mean centering method respectively. The calibration graphs were linear over the concentration range of 2-30 µg/mL for both methods. The regression parameters are supplied in Table 1.

Limit of detection (LOD) and limit of quantification (LOQ)

LOD and LOQ were calculated according to ICH guidelines from the following equations:

$LOD = 3.3\sigma / S$

$LOQ = 10\sigma / S$

Where is the residual standard deviation of a regression line

Table 1 : Spectral and validation data for determination of diloxanidefuroate by the proposed methods:

Parameters	Ratio difference	Mean centering
Wavelength (nm)	240—270	270
Linearity range (µg/mL)	2—30	2—30
LOD (µg/mL)	0.266	0.375
LOQ (µg/mL)	0.807	1.082
Regression equation	$y^* = b\,x^{**} + a$	$y^* = b\,x^{**} + a$
Slope	0.2053	0.2492
Intercept	-0.0027	-0.0014
Correlation coefficient (r^2)	0.9999	0.9995
Accuracy (%R)***	99.59	100.16
Precision (%RSD)****		
- Repeatability	0.895	0.969
- Intermediate precision	1.167	1.378

* Peak area of diloxanidefuroate.

** Concentration of diloxanidefuroatein µg/ml.

***Average of three determinations of three concentration levels (5-15-25µg).

**** Relative standard deviation of nine determinations (tripled to determination of three concentrations).

Table 2 : Determination of diloxanidefuroatein laboratory prepared mixtures with its degradation product by the proposed methods:

method	Intact diloxanidefuroate (µg/ml)	Degradation product (µg/ml)	%Degradation product	Intact found (µg/ml)	%Recovery
Ratio difference	5	25	83.3	4.92	98.50
	10	20	66.7	10.02	100.24
	15	15	50.0	14.88	99.230
	20	10	33.3	20.11	100.58
	25	5	16.7	24.73	98.94
	Mean ± RSD				99.50 ±0.959
Mean centering	5	25	83.3	4.90	98.05
	10	20	66.7	9.85	98.51
	15	15	50.0	15.10	100.69
	20	10	33.3	20.14	100.71
	25	5	16.7	24.77	99.08
	Mean ± RSD				99.41±1.236

and S is the slope of the calibration curve.LOD and LOQ values ofdilxanidefuroate for each method were listed in Table 1.

Accuracy

Accuracy of the proposed methods, calculated as the mean percent recovery (%R), was assessed by applying the proposed procedures for triplicate determination of three concentration levels covering the specified range (5, 15 and 25µg/mL). The concentrations were obtained from the corresponding regression equations and the mean percent recoveries, shown in Table 1, indicate accuracy of the proposed methods. Accuracy of the methods was further assured by the use of the standard addition technique. It was performed by addition of known amounts of pure dilxanidefuroate to known concentrations of the pharmaceutical preparation and the resulting mixtures were assayed, and the results obtained were compared with the expected results in Table 3. The good recoveries of the pure added dilxanidefuroate suggested good accuracy of the proposed methods.

Precision

Precision of the proposed methods, calculated as percent relative standard deviation (%RSD) of the percent recoveries, was checked by applying the proposed procedures for triplicate determination of three concentration levels covering the specified range (5, 15 and 25µg/mL) in the same day (intra-day analysis) for repeatability and on three different days (inter day analysis) for intermediate precision. The results in Table 1 indicate precision of the method.

Selectivity

The selectivity of the methods was achieved by the analysis of different laboratory prepared mixtures of dilxanidefuroate and diloxanide degradation product within the linearity range. Satisfactory results listed in Table 2, and the results of the standard addition technique Table 3, prove that the proposed

Table 3 : Determination of diloxanidefuroate in Furamebe® tablets by the proposed methods and application of standard addition technique:

Proposed methods	Furamebe® tablets	Standard addition technique			
	% Recovery*± %RSD	Pharmaceutical taken (µg/ml.)	Pure added (µg/mL)	Pure found** (µg/mL)	% Recovery
Ratio difference	99.26 ± 1.082	10	10	9.87	100.20
			15	15.27	101.86
			20	20.60	101.81
	Mean ± %RSD				101.29 ± 0.932
Mean centering	99.44 ± 1.207	10	10	10.23	99.43
			15	15.41	100.11
			20	20.79	101.50
	Mean ± %RSD				100.35 ± 1.050

*Average of five determinations.
** Average of three determinations.

Table 4 : Statistical comparison between the results obtained by the proposed methods and the reported methods for the determination of diloxanidefuroate [9]in pharmaceutical form:

Parameters	Ratio difference	Mean centering	Reported method*
n	5	5	5
Mean %R	99.26	99.44	99.57
%RSD	1.082	1.207	1.227
Student's *t*-test (2.306)**	0.583	0.435	-----
*F*value (6.388)**	1.325	1.240	-

*UV spectrophotometric method utilizing first derivative spectra at 270 nm.
**The values in the parenthesis are tabulated values of *t* and *F* at (p= 0.05).

methods can selectively analyze the drug without any interference from its degradation product or the excipients.

Application to pharmaceutical formulation

The proposed methods were applied for the determination ofdiloxanidefuroate in its pharmaceutical formulation, Furamebe® tablets. Satisfactory results were obtained in good agreement with the label claim, and the results of the standard addition technique indicate no interference from excipients and additives Table 3.

Statistical analysis:

In order to compare the ability of the proposed methods for the determination of diloxanidefuroate in pharmaceutical preparation, Table 4 showed statistical comparison of the results obtained by the proposed methods and the reported method for diloxanidefuroate[9].Statistical analysis of the results obtained from both the methods revealed no significant difference between

the performance of the two methods regarding accuracy and precision as revealed by Student's *t*-test and *F*-test, respectively.

CONCLUSION

Two different methods manipulating the ratio spectra were applied for resolving the overlapping spectra of diloxanidefuroate and diloxanide degradation product namely; ratio difference, and mean centering. The results demonstrate the usefulness of the methods, which are simple, sensitive, precise, accurate and inexpensive so the proposed methods could be applied for routine analysis of pure drug or in its pharmaceutical formulation (either alone or in the presence of its degradation product).

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