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Simple stability indicating ultraviolet spectroscopic methods for the estimation of ezetimibe in presence of its alkaline degradation product

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ABSTRACT

Previously, Gajjar and Shah described the isolation and structure elucidation of major alkaline degradant of Ezetimibe (EZE) using preparative HPLC, [A. K. Gajjar, V.D. Shah. J. Pharm. Biomed. Anal. 55 (2011) 225-229] based on ^{1}H NMR, ^{13}C NMR and mass spectrometric studies. Then the last paper [Z. Santa, J. Koti, K. Szoke, K. Vukics, C. Szantay. J. Pharm. Biomed. Anal. 58 (2012) 125-129] illustrate that the correct structure of degradant to be (2R,3R,6S)-N,6-bis(4-fluorophenyl)-2-(4-hydroxyphenyl)-3,4,5,6-tetrahydro-2H-pyran-3-carboxamide. This work describe three simple, specific and accurate spectrophotometric which developed and validated for determination of ezetimibe (EZE) in presence of its alkaline degradation product without previous separation, namely (Ratio subtraction; amplitude modulation; simultaneous equation), calibration graphs were established in the range of 2-20ug/mL with good correlation coefficients. The developed methods have been successfully applied for the simultaneous analysis of ezetimbe (EZE) in its pharmaceutical dosage form. The methods were validated as per ICH guidelines; accuracy, precision and repeatability were found to be within the acceptable limit. © 2016 Trade Science Inc. - INDIA

KEYWORDS

Ratio subtraction;
Amplitude modulation;
Simultaneous equation.

INTRODUCTION

Ezetimibe (EZE), a selective inhibitor of intestinal cholesterol and related phytosterol absorption, is designated as 1-(4-fluorophenyl)-3(R)-[3-(4-fluorophenyl)-3(S)-hydroxypropyl]-4(S)-(4-hydroxyphenyl)-2-azetidinone (Figure 1), It blocks the intestinal absorption of dietary and biliary cholesterol, without affecting the uptake of triglycerides or fat soluble vitamins, this reduce the overall delivery of cholesterol to the liver, thereby promoting the synthesis of LDL receptors and a subsequent reductionin serum LDL-C[1-7]. The literature is enriched with several techniques for determination of (EZE) in pharmaceutical dosage forms and/or bio-

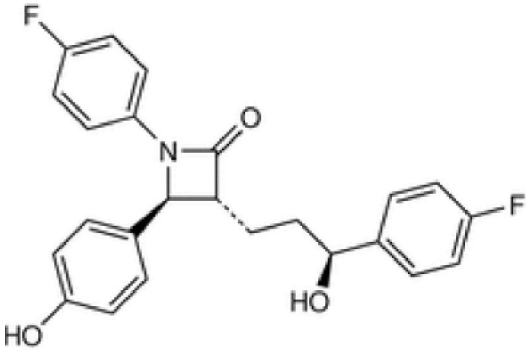

Figure 1 : Chemical structure of ezetimibe

logical fluids, including HPLC methods for the determination of ezetimibe are reported[8-32], TLC[15,18, 33-37], LC[38-45], chemometry[32,46], Spectrophotometric methods[13,30,31,47-68], UPLC[69, 70], densitometry[54,71], electrokinetic chromatography method[72], electrophoresis[73], voltammetry[74], spectrofluorometry[75], other related with degradation and elucidation of alkaline degradant of ezetimibe[76-78].

Reviewing the literature on the determination of (EZE) revealed the lack of any stability indicating spectrophotometric methods for the determination of (EZE) in presence of its alkaline degradation product, The aim of this work is to develop a simple, economic, rapid, sensitive, accurate and precise stability indicating methods for determination of (EZE) in presence of its oxidative degradate without sophisticated instruments or any separation steps.

THEORY

Theory of ratio subtraction[79]

A mixture of two components X and Y with overlapping spectra can be resolved by ratio subtraction if the spectrum of component Y is extended more than X. component X can be determined by dividing the spectrum of mixture by certain concentration of Y as a divisor (Y').

The will give a new curve that is represented by:

X / Y' + constant

If the constant determined directly from the spectra and subtracted, the the new spectrum multiplied by Y', the original spectrum of X is obtained.

This can be summarized in the following equation:

$$\therefore (X+Y) / Y' = (X / Y') + (Y / Y') \quad (1)$$

$$\therefore (X+Y) / Y' = (X / Y') + \text{Constant} \quad (2)$$

By subtraction of the constant from equation (2):

$$\therefore X / Y' + \text{Constant} - \text{Constant} = X / Y' \quad (3)$$

By multiplication of equation (3) by a divisor (Y'):

$$\therefore (X / Y') \times Y' = X \quad (4)$$

The constant can be determined directly from the spectrum (X+Y) / Y' by the straight line which is parallel to the wave length axis in the region where Y is extended.

Theory of amplitude modulation[53]

This method based on the same principle as the ratio subtraction method with simple modification step [use normalized spectrum as a divisor (1ug/mL)]. if you have a mixture of two components X and Y having overlapped spectra intersect at isoabsorptive point, and Y is extended over X and the spectra of X and Y shows isoabsorptive point at zero spectrum and consequently retained as an isosbestic point at the ratio spectrum.

The absorbance of the zero order absorption spectrum for mixture of X and Y at isoabsrptive point as follow:

$$A_{mix} = [a_x C_x] + [a_Y C_Y] \quad (5)$$

By dividing eq (5) with normalized spectrum of Y as a divisor to get ratio spectrum with isosbestic point (at the same wavelength of the zero order) so the following equation was obtained:

$$\frac{Amix}{[aY\,CY']} = \frac{[ax\,Cx]}{[aY\,CY']} + \frac{[aY\,CY]}{[aY\,CY']} \quad (6)$$

$$\frac{Amix}{[aY\,CY']} = \frac{[ax\,Cx]}{[aY\,CY']} + \text{Constant} \quad (7)$$

$$P_m = P_X + P_Y$$

Where, (Pm) is the amplitude of ratio spectrum of the mixture, (Px) is the amplitude of component X and (P_Y) is the amplitude of component Y. i.e. the recorded amplitude at isosbestic point of the ratio spectrum is equal to the sum of amplitude corresponding to X and that of Y.

The amplitude representing the component Y (P_Y) was the constant $\frac{[aY\,CY]}{[aY\,CY']}$, and it can be measured directly from the spectrum at the straight line that is parallel to the wavelength axis in the region where Y spectrum is extended.

Since, we use normalized divisor of Y (I ug/mL)

$$P_Y = \frac{[aY\,CY]}{[aY\,CY']}$$

$$P_Y = [CY] \quad (8)$$

∴ The recorded amplitude of the constant was modulated to concentration so it was representing the concentration of Y [CY], (CRecorded of Y).

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For determination of amplitude of X in the mixture, If we subtract the measured value of the constant from that of the mixture at isosbestic point of the ratio spectrum Eq.(10);

$P_X = Pm - PY$

$$P_x = \{ \frac{[ax\ Cx]}{[aY\ CY']} + \text{Constant} \} \text{ “ Constant} \quad (9)$$

$$P_X = \frac{[ax\ Cx]}{[aY\ CY']} \quad (10)$$

∴ At that isosbestic point $a_X = a_Y$ and normalized divisor of Y $C_{Y'}$ = 1µg/Ml

$$P_x = \frac{[ax\ Cx]}{[aY\ CY']} \quad (11)$$

$$P_x = [C_x] \quad (12)$$

∴ This obtained amplitude of ratio spectrum was modulated to concentration and it was representing concentration of X [C_X], ($C_{Recorded}$ of X)

The corresponding concentration of X or Y could be calculated by using the following regression equation:

$$C_{Recorded} = \text{Slope } C + \text{intercept}$$

Slope was found to be approximately one and intercept almost zero

Where; $C_{Recorded}$ represents the recorded amplitudes corresponding to the concentrations of either X or Y that obtained from the ratio spectrum using normalized spectrum of Y (1µg/mL) as a divisor and C represents the corresponding concentration of X or Y.

Theory of simultaneous equation

This method of analysis was based on the absorption of two components X and Y at the wavelength maximum of each other (λ_1 and λ_2). The absorptivity values were determined for both the components at the selected wavelengths, these values were mean of certain estimations. The concentration of both components in mixture can be calculated by using following equations:

$$Cx = (A_2 ay_1 - A_1 ay_2) / (ax_2 ay_1 - ax_1 ay_2)$$

$$Cy = (A_1 ax_2 - A_2 ax_1)/(ax_2 ay_1 - ax_1 ay_2)$$

Where, A1 and A2 are absorbances of mixture at the two selected wavelengths (λ_1 and λ_2).

ax_1 and ax_2 are absorptivities of component X at the two selected wavelengths(λ_1 and λ_2).

ay_1 and ay_2 are absorptivities of component X at the two selected wavelengths(λ_1 and λ_2).

Cx and Cy are concentrations of both components X and Y.

EXPERIMENTAL

Instruments

A double beam UV-Visible spectrophotometer (Shimadzu 1800, Japan) and it is connected to IBM compatible computer. The software UV-Probe Ver. 2.43. Hot plate (Torrey Pines Scientific, USA). Rota-Vapor SCI-Logics (RE-100-PRO) with Buchi pump.

Materials

A Ezetimibe was kindly provided by E.I.P.I.Co Company, Cairo, Egypt, with purity of 99.9%.

B Zetamibe® tablet: manufactured by Adwia; labeled to contain 10 mg of ezetimibe per tablet.

C Methanol (HPLC grade) obtained by Sigma-Aldrich company, Germany.

D 0.1 M methanolic sodium hydroxide solution.

E Whatman filter paper nº 41.

Standard solution

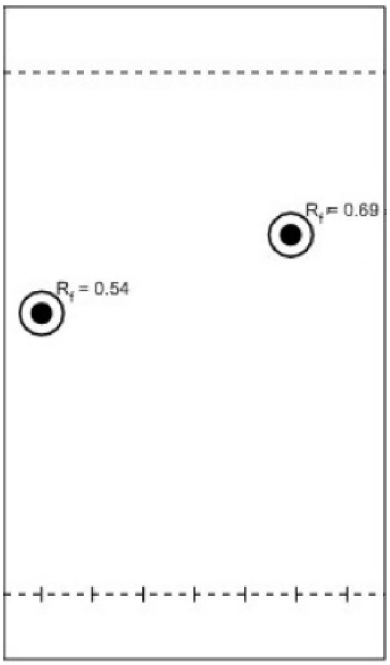

Figure 2 : TLC between starting material of EZE (R_f = 0.54), product (R_f = 0.69) after complete degradation

Stock solution of 1000 ug /mL for (EZE) was prepared by dissolving 100 mg of (EZE) in 100 ml pure. Different sets of working solution at various concentrations were prepared by appropriate dilution of the stock solution.

Alkaline degradation of EZE[77]

100 mg of EZE was dissolved in 50 mL (0.1 M methanolic sodium hydroxide), The solution was refluxed at 80º C for 30 minutes. The time required for complete degradation was followed by spotting on TLC plates at 10 minutes intervals for 30 minutes. The plates was developed using ethyl acetat: n-hexane (2:1 v/v), which indicate complete degradation with clear separation (Figure 2). After completion, the solution was cooled to room temperature then neutralize with 1M hydrochloric acid. The neutralized solution was filtered. Then evaporate the solution under vacuum till dryness, the residue was dissolved by pure methanol and filtered (several times), evaporate the filtrate using Rota-vapor under vaccum, the residue was dissolved in 100 pure methanol to give degradate stock solution of (1000 ug / mL).

Spectral characteristics

The absorption spectra of EZE and its alkaline degradation product were recorded over the range 200-400 with linearity range of 2 – 20 ug/mL.

Laboratory prepared mixtures

Accurate aliquots equivalent to (20-180 ug) of tizanidine HCL into series of 10 ml volumetric flasks from its working solution (100 ug/ml) and portion equivalent to (180 – 20 ug) of degradate from its working solution (100 ug/ml) were added to the same flasks and volumes were completed to mark with pure methanol and mixed well.

Pharmaceutical formulation

Ten zetamibe tablets were accurately weighed, crushed and mixed. An amount equivalent to 10 mg of EZE was weighed and transferred into 100 ml volumetric flask. To ensure complete extraction of drug, it was dissolved in pure methanol in 100ml volumetric flask, complete to the mark with methanol then filtered through Whatman filter paper nº 41 into 100 ml volumetric flask and the volume was completed to the mark with pure methanol. Filtration system was evaluated to ensure that filter does not adsorb any of drugs. Pharmaceutical solution was diluted to the working calibration ranges.

PROCEDURES

Construction of calibration curves (linearity)

Accurately measured aliquots equivalent to (0.2 6 2 ml) of EZE from working solution (100 ug/ml) were, separately transferred into a series of 10 ml volumetric flasks and the volume of each one was completed to the mark with pure methanol, to reach the concentration range of (2 6 20 ug/mL).

For determination of EZE and its alkaline degradant using ratio subtraction method

Zero absorption spectra of the resulting solution of EZE and its degradant which containing (2-20 ug/mL) were measured and stored in the computer. where, these absorption spectra of both EZE and its degradant show sever overlapping and degradant spectrum is extended more than EZE spectrum (Figure 3). Absorption spectra of the mixture of EZE and degradant containing different ratio as mentioned in [section 3.6.] were measured and recorded in the computer. Aliquot equivalent to 100 ug was transfer from the degradant working solution of (100 ug/mL) into 10 ml volumetric flask and completed to the volume with methanol to be used as a divisor (10 ug/mL).

The absorption spectra of the mixture were divided by the spectrum of 10 ug/mL (divisor) to give ratio spectra, then the constant was determined from the plateau region then subtracted from the ratio spectra, by multiplication of the obtained spectra by the spectrum of the divisor the orginal spectra of EZE were obtained. Absorbance at 232 nm (λmax of EZE) was determined against the corresponding concentration of EZE to give the corresponding regression equation.

For determination of EZE and its alkaline degradant using amplitude modulation method

Zero absorption spectra of the resulting solution

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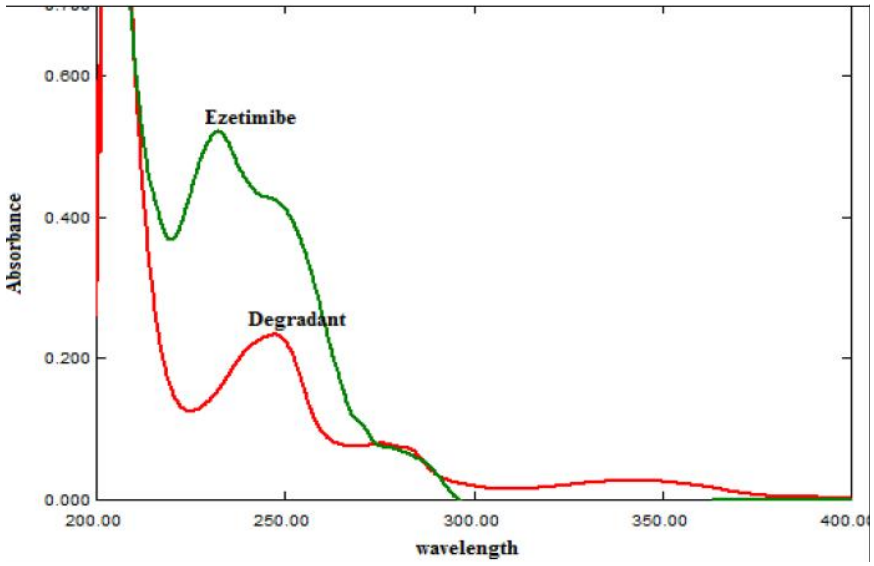

Figure 3 : Zero order absorption spectra of ezetimibe and its alkaline degradant

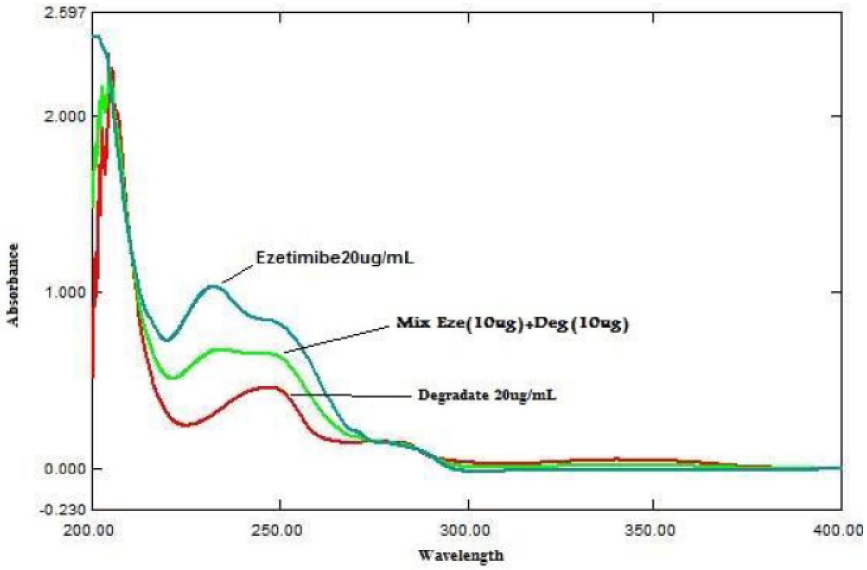

Figure 4 : Zero order absorption spectra of ezetimibe (20 ug/mL), degradant (20 ug/mL) and mixture of ezetimibe (10 ug/mL) with its degradant (10 ug/mL) with iso-absorptive point at 277 nm

of EZE and its degradant which containing (2-20 ug/mL) were measured and stored in the computer, where, these absorption spectra of both EZE and its degradant show sever overlapping with isoabsorptive point at 277 nm and degradant spectrum is extended more than EZE spectrum (Figure 4). Absorption spectra of the mixture of EZE and degradant containing different ratio as mentioned in [section 3.6.] were measured and recorded in the computer. Aliquot equivalent to 10 ug was transfer from the degradant working solution of (100 ug/mL) into 10 ml volumetric flask and completed to the volume with methanol to be used as a divisor (1 ug/mL).

The absorption spectra of the mixture were divided by the normalized spectrum of 1 ug/mL (divisor) to give ratio spectra, then the constant was determined from the plateau region then subtracted from the ratio spectra. We obtain the spectra which have the absorbance at isoabsorptive point equal to the corresponding concentration of EZE.

For determination of EZE and its alkaline degradant using simultaneous equation method

Prepared solution of EZE in the range of (2 6 20 ug/mL) and of its degradant in the range of (2 6 20

ug/mL), were scanned in the spectrum mode from 400 to 200 nm. The absorption spectra which obtained is selected for analysis. From the overlain spectra of both EZE intact and its alkaline degradant (Figure 3), the wavelengths which selected for quantitation for each concentration of EZE and degradant in the linearity range are 232 and 247 nm for EZE and its degradant which are λmax for both respectively.

Absorptivity for each concentration of both EZE and its degradant at 232 and 247 nm was determined. The concentration of both EZE and its degradant present in the mixture as mentioned in (section 3.6.) was determined by using the simultaneous equation calculation as mentioned in (section 2.3.).

Accuracy

Accuracy was assured by carrying out the previously mentioned procedures under linearity for the determination different concentration of pure tizanidine HCL. The concentration was calculated from the corresponding regression equations.

Precision

Intra-day precision (Repeatability)

Three concentrations of EZE were analyzed three times intraday using the previously mentioned procedures. The percentage of recoveries of each concentration of EZE and its relative standard deviation were calculated using the suggested methods (TABLE 2).

Intermediate precision(Reproducibility)

Three concentrations of EZE were analyzed on three successive days using the procedure stated under linearity. The percentage of recoveries of each concentration of EZE and its relative standard deviation were calculated using the suggested methods (TABLE 2).

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

The LOD and LOQ parameters were determined from regression equation,

LOD = 3.3 Sy /a

LOQ = 10 Sy / a

Where, (Sy) is a standard error of the calibration curve and (a) is the slope of the corresponding calibration curve (TABLE 1).

Application to laboratory prepared mixtures

Laboratory prepared mixtures containing different ratios of tizanidine HCL and its oxidative degradate within their calibration ranges were prepared. The spectra of these mixtures were recorded and the procedures under construction of calibration curves were then followed but using the recorded spectra of the prepared mixtures. Recoveries were calculated as previously mentioned in accuracy, and percentages of degradate in mixtures were calculated (TABLE 3).

Application to pharmaceutical formulation

Different concentrations within calibration range of each method (ratio subtraction method, amplitude modulation and simultaneous equation) were prepared from the solution of the pharmaceutical preparation, the spectra of these prepared concentrations were recorded and procedures under construction of calibration curves were followed using the re-

TABLE 1 : Spectral data for determination of EZE by proposed methods

Parameters	Ratio subtraction	Amplitude modulation	Simultaneous equation	
Wavelength (nm)	232	277	232 λmax of EZE intact 247 λmax of degradant	
Linearity range (μg ml^{-1})	2 – 20	2 – 20	2 ? 20	
LOD (μg ml^{-1})	0.115	0.084	0.036	0.044
LOQ (μg ml^{-1})	0.347	0.255	0.108	0.133
Regression equation*				
Slope (*b*) Intercept (*a*)	0.0534-0.004	1.0082-0.0467	0.0526 -0.0037	0.0429 - 0.0034
Regression coefficient (r^2)	0.9999	0.9999	0.9999	0.9999

*** *y= a + bx* where *y* is the response and *x* is the concentration.**

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TABLE 2 : Intraday and interday accuracy and precision for the determination of EZE by the proposed methods

Method	Conc µg.ml^{-1}	Intraday			Interday		
		Found Conc. ± SD	Accuracy (R%)	Precision (RSD%)	Found Conc. ± SD	Accuracy (R%)	Precision (RSD%)
Rato subtraction	4	4.007 ± 0.019	100.19	0.467	4.001 ± 0.029	100.03	0.715
	10	9.956 ± 0.029	99.56	0.287	9.938 ± 0.029	99.38	0.288
	14	13.908 ±0.057	99.34	0.411	13.920 ±0.029	99.43	0.205
Amplitude modulation	4	4.016 ± 0.010	100.41	0.249	4.015 ± 0.011	100.37	0.285
	10	9.969 ± 0.016	99.69	0.162	9.971 ± 0.025	99.71	0.246
	14	13.951 ± 0.030	99.65	0.215	13.937 ± 0.024	99.55	0.172
Simultaneuos equation	4	4.037 ± 0.029	100.93	0.719	4.044 ± 0.019	101.09	0.470
	12	12.029 ± 0.038	100.24	0.316	12.060 ± 0.048	100.50	0.397
	20	19.950 ± 0.067	99.75	0.335	19.956 ± 0.057	99.78	0.286

TABLE 3 : Determination of EZE and its alkaline degradation product in their laboratory prepared mixtures by the proposed methods

	Intact in (µg ml^{-1})	Degradate in (µg ml^{-1})	Percent of degradate	Intact found in (µ ml^{-1})	Recovery % of intact
Ratio subtraction	18	2	10.00	18.02	100.12
	16	4	20.00	15.97	99.81
	14	6	30.00	13.94	99.55
	12	8	40.00	12.05	100.44
	10	10	50.00	10.06	100.56
	8	12	60.00	8.02	100.28
	6	14	70.00	6.06	101.06
	4	16	80.00	4.05	101.21
	2	18	90.00	2.00	99.81
	Mean ± SD%				100.32 ± 0.566
Amplitude modulation	18	2	10.00	18.00	100.01
	16	4	20.00	16.08	100.53
	14	6	30.00	13.92	99.43
	12	8	40.00	12.02	100.19
	10	10	50.00	9.96	99.62
	8	12	60.00	7.97	99.59
	6	14	70.00	6.01	100.17
	4	16	80.00	4.01	100.15
	2	18	90.00	2.03	101.40
	Mean ± SD%				100.12 ± 0.60
Simultaneous equation	18	2	10	18.12	100.67
	16	4	20	15.99	99.97
	12	8	40	11.99	99.91
	8	12	60	7.93	99.08
	4	16	80	3.99	99.78
	2	18	90	2.00	100.11
	Mean ± SD%				99.92 ± 0.52

corded spectra of the pharmaceutical formulation prepared solution.

The validity of the methods was assessed by applying the standard addition technique (TABLE 4).

TABLE 4 : Application of standard addition technique to the analysis of Sirdalud® tablets by applying the proposed methods

		Sirdalud® tablets		
Method	Taken µg ml^{-1}	Pure added µg ml^{-1}	Pure found µg ml^{-1}	Recovery %
Ratio subtraction	2	2	1.97	98.50
		6	6.00	99.94
		12	11.99	99.91
		14	14.10	100.75
	Mean ± RSD%			99.45 ± 0.825
Amplitude modulation	2	2	2.01	100.29
		6	6.00	99.94
		12	11.87	98.95
		14	13.94	99.56
	Mean ± RSD%			99.73 ± 0.698
Simultaneous equation	2	2	1.98	98.84
		6	6.01	100.16
		14	13.87	99.06
		16	15.94	99.62
	Mean ± RSD%			99.42 ± 0.597

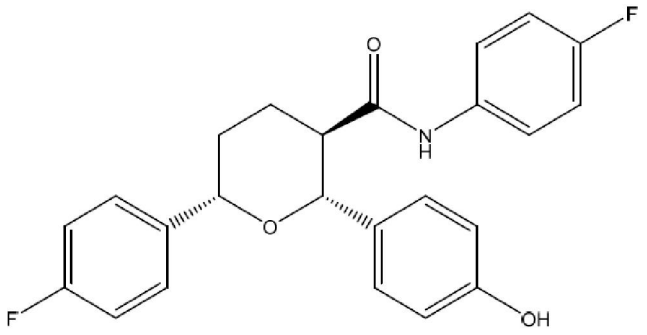
RESULTS AND DISCUSSION

Alkaline degradation process of EZE was previously achieved by Gajjar and Shah and use preparative HPLC for isolation of the degradant, confirming the degradation using ^{1}H NMR, ^{13}C NMR and mass spectrometric studies[76]. After that, the work in paper of [Z. Santa, J. Koti, K. Szoke, K. Vukics, C. Szantay. J. Pharm. Biomed. Anal. 58 (2012) 125-129] illustrate that the correct structure of degradant is (2R,3R,6S)-N,6-bis(4-fluorophenyl)-2-(4-hydroxyphenyl)-3,4,5,6-tetrahydro-2H-pyran-3-carboxamide (Figure 5).

By reviewing the literature, revealed the lack of any spectrophotometric methods for the determination of (EZE) in presence of its alkaline degradation product, the aim of this work is to develop three simple, accurate, precise methods for developing EZE in presence of its alkaline degradation product without need of any preseparation step or sophisticated instrument.

Ratio subtraction method

As mentioned previously in (section 2.1.), extended zero absorption spectrum of degradate than zero absorption spectrum of intact EZE and presence of isosbestic point are necessary for application of this method as shown in (Figure 3), (Figure 4). the benefit of the extension is to give constant value when we use a certain concentration of degradant as a divisor (10 ug/mL), this constant can simply be determined directly from the obtained ratio spectrum at the region of degradant absorption spectrum extension, and by subtraction of this constant the new ratio spectrum (EZE spectrum / a divisor spectrum) was obtained. By multiplication of new ratio spectrum with a divisor spectrum (10 ug/mL), the spectrum which represent EZE intact without interference of degradant will be obtained. Good linearity is obtained in the concentration range of (2



(2R,3R,6S)-N,6-bis(4-fluorophenyl)-2-(4-hydroxyphenyl)-3,4,5,6-tetrahydro-2H-pyran-3-carboxamide

Figure 5 : Structure of alkaline degradation product of ezetimibe

20 ug/mL). The corresponding regression equation was computed.

$Y_{232} = 0.0534\ X - 0.004\ r^2 = 0.9999$

Where Y_{232} is the absorbance at λmax of EZE (232 nm), X is the concentration in ug/ mL and r^2 is the regression coefficient as shown in (TABLE 1).

Amplitude modulation method

This method needs the same condition of ratio subtraction method (presence of isoabsorptive point (277nm) and extension of degradant spectrum than of intact EZE) as shown in (Figure 3), (Figure 4), but with simple modification step, which is using normalized spectrum of degradant as a divisor (i.e. 1 ug/mL). As mentioned in (section 2.2.) the division of mixture spectrum of EZE and degradant by normalized spectrum of degradant (1 ug/mL) as a divisor, give ratio spectrum with constant value, this constant can simply be determined directly from the obtained ratio spectrum at the region of degradant absorption spectrum extension, and by subtraction of this constant the new ratio spectrum (EZE spectrum / a divisor spectrum) was obtained.

$\therefore a_{EZE} . C_{EZE} / a_{deg} . C_{deg}$

where, a_{EZE} and a_{deg} are the absorptivity of both intact EZE and degradant.

C_{EZE} is the concentration of intact EZE.

C_{deg} is the normalized spectrum of a divisor (1 ug/mL)

Since, The absorptivity of each EZE (a_{EZE}) and degradant (a_{deg}) at isoabsorptive point is equal to each other, and C_{deg} is equal to 1.

So, the absorbance at isoabsorptive point is equal to corresponding concentration of EZE

$\therefore A = C_{EZE}$

Good linearity is obtained in the concentration range of (2 20 ug/mL). The corresponding regression equation was computed.

$Y_{277} = 1.0082\ X - 0.0467\ r^2 = 0.9999$

Where Y_{277} is the absorbance at wavelength of isoabsorptive point (277 nm), X is the concentration in ug/ mL and r^2 is the regression coefficient as shown in (TABLE 1).

Simultaneous equation method

In this method the absorption of both EZE and its alkaline degradation product was determined at maximum wavelength of each other (232 and 247 nm) for EZE and degradant respectively, the absorptivity values were determined for both EZE and degradant at the selected wavelengths, these values were mean of certain estimations. The concentration of both components in mixture can be calculated by using following equations:

$C_{EZE} = (A_1 ax_2 - A_2 ax_1) / (ax_2 ay_1 - ax_1 ay_2)$

Where, A1 and A2 are the absorbances of the mixture at the two selected wavelengths (232 and 247 nm) respectively.

ax_1 and ax_2 are the absorptivities of the degradant at the two selected wavelengths (232 and 247 nm) respectively.

ay_1 and ay_2 are the absorptivities of EZE intact at the two selected wavelengths(232 and 247 nm) respectively.

C_{EZE} is the corresponding concentrations of EZE.

Good linearity is obtained in the concentration range of (2 20 ug/mL). The corresponding regression equation was computed at 232 nm.

$Y_{232} = 0.0526\ X - 0.0037\ r^2 = 0.9999$

Where, Y_{232} is the absorbance at λmax of EZE (232 nm), X is the concentration in ug/ mL and r^2 is the regression coefficient as shown in (TABLE 1).

The other corresponding regression equation was computed at 247 nm.

$Y_{247} = 0.0429\ X - 0.0034\ r^2 = 0.9999$

Where, Y_{247} is the absorbance at λmax of degradant (247 nm), X is the concentration in ug/ mL and r^2 is the regression coefficient as shown in (TABLE 1).

Accuracy and precision

According to the ICH guideline, three replicate determination of three different concentration of the studied drug in pure form within their linearity ranges were performed in the same day (intra-day) in three successive days (inter-day) for each method. concentrations of (4, 10 and 14 ug mL^{-1}) were used in both of ratio subtraction and amplitude modulation methods, and concentrations of (4, 12 and 20 ug mL^{-1}) were used in simultaneous equation method, and Accuracy as recovery percent (R%), and precision as percentage relative standard deviation (RSD%) were calculated and results were listed in (TABLE 2).

TABLE 5 : Statistical comparison between the results obtained by applying the proposed spectrophotometric and reported methods for determination of EZE in sirdalud® tablets

	Ratio subtraction	Amplitude modulation	Simultaneous equation	Reported method[65]
*N**	5	5	5	5
$X^?$	100.07	99.63	99.79	100.17
SD	0.564	0.656	1.006	0.460
Variance	0.564	0.659	1.008	0.460
*t***	0.293 (2.306)	1.510 (2.306)	0.776 (2.306)	——
F**	1.502 (6.388)	2.033 (6.388)	4.776 (6.388)	——

*** No. of experimental, ** The values in the parenthesis are tabulated values of t and F at (p= 0.05)**

Specificity

The specificity of the proposed methods were assured by applying the laboratory prepared mixtures of EZE and its degradate. The results were listed in (TABLE 3).

Pharmaceutical applications

The proposed methods were applied to the determination of the studied drug in Zetamibe ® tablets. The statistical comparison between the results obtained by applying the proposed methods and those obtained by applying the reported method[65] showed less calculated t and F values revealing no significant difference in accuracy and precision (TABLE 5).

Statistical comparative discussion of proposed methods

All data mentioned above related to previous tables and figures introduce a comparative discussion for three techniques which applied for manipulating of EZE and its alkaline degradation product, we observe that all of these proposed methods (ratio subtraction, amplitude modulation and simultaneous equation) have the same correlation coefficient (r2=0.9999), but we know that "more small values of LOD and LOQ, more sensitive the methods". So according to LOD and LOQ, simultaneous equation method seems to be more sensitive than the others, since it has LOD and LOQ smaller than that of the others (Figure 1). Amplitude modulation method is the second one in sensitivity after simultaneous equation method, since its LOD and LOQ is smaller than that of ratio subtraction method.

On the other hand, simultaneous equation method seems to be simplest one, since simultaneous equation not need division step using a divisor and not need subtraction of constant step which involved in the other methods.

In (TABLE 3) we can note that percent of degradate concentration related to intact concentration for all three methods is high which reach to 90%, these three techniques have great benefit in cases where high degree of interference could be found among spectra.

Statistical comparison of the results obtained by the proposed methods and official method was shown in (TABLE 5). The calculated [t and F] values were less than the theoretical ones indicating that there was no significant difference between the proposed and the official method with respect to accuracy and precision.

Finally, the proposed methods are simple without requirement for sophisticated technique or instruments, they also sensitive, selective and can be used for manipulation of EZE in their available dosage forms.

CONCLUSION

By reviewing the literatures, we observe that, this manuscript is the first one which deals with manipulating of ezetimibe in presence of its alkaline degradation product using simple, accurate, precise sensitive, economic spectrophotometric methods without any separation steps or sophisticated instruments.

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