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Densitometric Determination of Ezetimibe in the Presence of its Alkaline Degradation Product

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ABSTRACT

Objectives: A simple, accurate, selective and sensitive densitometric method was developed for the determination of ezetimibe in the presence of its alkaline degradation product. **Methods:** TLC-densitometric separation of ezetimibe from its degradation products was carried out on silica gel plates using ethyl acetate: n-hexane (2:1 v/v) as a developing system. This method depends on quantitative densitometric evaluation of ezetimibe at 230 nm over a concentration range of 1–8 µg/spot. Ezetimibe and its alkaline degradation product were resolved with R_f values of 0.49 and 0.68 respectively. **Results:** The proposed method has been successfully applied to the analysis of ezetimibe in pharmaceutical dosage form without interference from additives and the results were statistically compared with the reported method. **Conclusion:** TLC- densitometric technique has provided a simple, straightforward method for separating ezetimibe and its alkaline degradation product simultaneously.

Keywords: *Alkaline degradation product; Ezetimibe; TLC-densitometry*

INTRODUCTION

Ezetimibe (EZE), a selective inhibitor of intestinal cholesterol and related phytosterol absorption, is designated as 1-(4-fluorophenyl)-3(R)-[3-(4-fluoro-phenyl)-3(S)-hydroxypropyl]-4(S)-(4-hydroxy-phenyl)-2-azetidinone, 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 reduction in serum LDL-C [1-2]. The literature is enriched with several techniques for determination of (EZE) in pharmaceutical dosage forms and/or biological fluids, including spectrophotometric methods[3-11], chemometry[12], spectrofluorometry[13], electrochemical[14-16], electrokinetic chromatographic method[17], TLC[18-20], LC [21-23], HPLC[3, 24-30], other related with degradation and elucidation of alkaline degradant of ezetimibe[31, 32].

Reviewing the literature on the determination of (EZE) in the presence of its alkaline degradation product, revealed the lack of any quantitative determination method based on separating technique.

The aim of this work is to develop a simple, economic, rapid, sensitive, accurate and precise densitometric method for determination of (EZE) in the presence of its alkaline degradation product without sophisticated instruments or any pretreatment.

Theory of TLC- densitometric chromatography method

This technique offers a simple way to quantify separated drugs directly on the TLC plate by measuring the optical density of the separated bands. The amounts of compounds are determined by comparing to a standard curve from reference materials chromatographed simultaneously under the same condition[33-34].

MATERIAL AND METHODS

Instruments

- TLC-densitometer: Precoated TLC-plates, silica gel 60 F_{254} (20 cm × 20 cm, 0.25 mm), E. Merck (Darmstadt-Germany). Camag TLC scanner 3 S/N 130319 with winCATS software. Camag linomat 5 autosampler (Switzerland). Camag microsyring (100 ul).
- UV lamp with short wavelength (246 nm.) (Desega-Germany).
- Chromatographic tank (25 × 25 × 9 cm).
- Hot plate (Torrey Pines Scientific, USA).
- Rota-Vapor SCI-Logics (RE-100-PRO) with Buchi pump.

Material

Pure standard

- Ezetimibe was kindly provided by E.I.P.I.Co Company, Cairo, Egypt, with purity of 99.9%, the recovery of certainconcentrations (4,8,10,20 µg/mL)were calculated against the 1ry standard using the reported method (ratiosubtraction)[5].

Pharmaceutical dosage form

- Zetamibe® tablet: manufactured by Adwia; labeled to contain 10 mg of ezetimibe per tablet, (Batch No. 133822)

Chemical and reagent

- Methanol (HPLC grade) obtained from Sigma-Aldrich company, Germany.
- 0.1 M methanolic sodium hydroxide solution.
- Whatman filter paper nº41.
- Ethyl acetate, n-hexane, chloroform and acetonitrile (All of them are HPLC grade).

Standard and sample solutions

Standard solution:

A stock solution of (1000 µg/mL) for (EZE) was prepared by dissolving 100 mg of (EZE) in 100 ml methanol.10 mL of this stock solution was transferred into 100-ml volumetric flask and diluted to the mark to give a working solution of (100 µg/mL).

Alkaline degradation of EZE[5,32]

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 minute intervals for 30 minutes. The plates were developed using ethylacetate: n-hexane (2:1 v/v), which indicate complete degradation with complete separation. The cooled solution was neutralized with 1M hydrochloric acid, then filtered. The filtered solution was evaporated under vacuum till dryness, the residue was dissolved by pure methanol and filtered (several times), evaporate the filtrate using Rota-vapor under vacuum, the residue was dissolved in 100 pure methanol to give a degradate stock solution of (1mg/mL). 10 mL of this stock solution was transferred into 100-mL volumetric flask and then, diluted to the mark with methanol to give a working solution of (100 µg/mL) (**Figure 1)**.

Pharmaceutical dosage form

Ten zetamibe® tablets were accurately weighed, crushed and finely powdered in a mortar. A quantity of the powder equivalent to one tablet was accurately weighed and transferred to a 100-mL volumetric flask then 50 mL of methanol was added. The flask was sonicated for 15 min. Then filtered through Whatman filter paper no 41 into 100-mL volumetric flask, the residue was washed with methanol. All the filtrate and washings were collected and the volume was adjusted to 100 ml with methanol to obtain the solution claimed to contain 100 µg/mL of EZE. Filtration system was evaluated to ensure that the filter does not adsorb any of the drug.

Procedure

Construction of calibration graph

The aliquots of stock solution of **EZE** (1000µg/mL) which equal to (1, 2, 3, 4, 5 and 6 µg/mL) were spotted as bands of 5mm width on TLC plates (20 ×10 cm). Bands were applied at 15 mm intervals and 15mm from the bottom and side edges of the plate. Linear ascending development was performed in a chromatographic tank which previously saturated with ethyl acetate: n-hexane (2:1 v/v) for 30 minutes at room temperature. **EZE** was scanned at 230 nm. A calibration curve relating the optical density of each spot to the corresponding concentration of **EZE** was constructed. The regression equation was computed for the studied drug and used for determination of unknown samples of ezetimbe (**Figure 2a** and **b).**

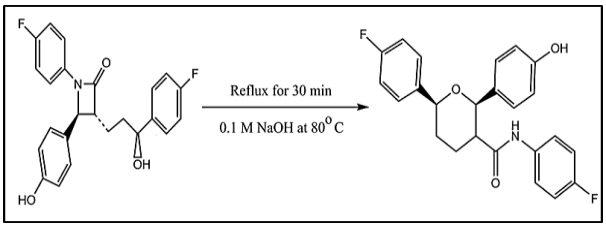

Figure 1. Degradation pathway of ezetimibe under the alkaline condition.

Laboratory prepared mixtures

Accurate aliquots equivalent to (20 -70 µg) of EZE are transferred into a series of 10-mLvolumetric flasks from its working solution (100 µg/mL) and

portions equivalent to (6.0 – 10 µg) of its alkaline degradate from its working solution (100 µg/mL) were added to the same flasks and then, diluted to the mark with methanol and mixed well. The procedure under 2.7.1. section was repeated with laboratory prepared mixtures which, give a high degree of resolution, (**Figure 3 a** and **b**).

Analysis of pharmaceutical dosage form

The procedure mentioned above. was repeated and the proposed method should be applied in different concentrations of EZE which, covering the concentration range. Tablet content of EZE was calculated using the corresponding regression equation.

RESULTS AND DISCUSSION

To the best of our knowledge, the literature survey revealed that there is no separating technique was developed for the determination and quantification of EZE in the presence of its alkaline degradation product. It was found that the complete degradation of EZE proceeds optimally in alkaline media and at elevated temperature.

The structure of the alkaline degradate was elucidated and confirmed by using ^{1}H NMR technique where, the ^{1}H NMR of intact ezetimibe (**Figure 4),** showed a characteristic proton of aliphatic (OH) at 4.86 ppm, aromatic (OH) at 9.40 ppm and aliphatic proton (CH_2) at 1.75 ppm. While, ^{1}H NMR of degradate (**Figure 5**), showed disappearance of aliphatic (OH) at 4.86 ppm and appearance of characteristic proton of (CO-NH) at 9.98 ppm and aromatic OH still present at 9.06 ppm [4].

So, the above findings are in agreement with the reported mechanism of recyclization of ezetimibe under alkaline conitions [32]as shown in **Figure 1**.

The role of this method is to develop a simple, accurate and selective densitometric method for the determination of EZE in the presence of its alkaline degradation product.

Spectral characteristics

The absorption spectra of EZE and its alkaline degradation product were recorded over the range 200-400 (**Figure 6**).

TLC-densitometric[34,35]

To improve separation of bands, it was necessary to investigate the effect of different variables. The study of the optimum parameters for maximum separation was carried out as follows:

Mobile phase[5]

Different developing systems of different composition and ratios were tried for separation, e.g. hexane-ethyl acetate (1:1 and 2:1, v/v), hexane-ethyl acetate-acetic acid (8:2;0.1, v/v/v), chloroform-hexane-acetonitrile (5:3:2, v/v/v) and hexane-methanol (9:1, v/v). The best mobile phase was methanol-chloroform (6:4, v/v). This selected mobile phase allows the determination of EZE in the presence of its alkaline degradate without tailing of the separated bands.

Band dimension

Different band dimension was tested in order to obtain sharp and symmetrical separated peaks. The optimum bandwidth chosen was 5 mm and the inter-space between bands was 15 mm.

Scanning wavelength

Different scanning wavelengths (251, 246, 240, 230 and 235 nm) were tried. Peaks at 230 nm were more sharp, symmetrical and minimum noise was obtained.

Slit dimension of scanning light beam

The slit dimension of the scanning light beam should ensure complete coverage of band dimensions on the scanned track without interference of adjacent bands. Different slit dimensions were tried, where 5 mm × 0.2 mm proved to be the slit dimension of choice which provides highest sensitivity.

This method is based on the difference in the R_f values of EZE (R_f = 0.49 ± 0.02) and its alkaline degradate (R_f = 0.68 ± 0.01) (**Figure 5 a)**.

Method validation

The linearity range, limit of detection (LOD), limit of quantification (LOQ), selectivity, accuracy and precision of the proposed method was tested according to International Conference on Harmonization (ICH) guidelines[36].

Linearity and range

The calibration graph for the proposed method was constructed by plotting the area under peak versus drug concentrations of EZE. The regression plot was found to be linear over the range of (1-8 µg/band).

The linear regression equations for the graphs were:

$$Y = 3688.4\,X - 17408 \qquad r = 0.9996$$

Where, ***y*** is the area under peak values, ***x*** is the drug concentration and ***r*** is the correlation coefficient. Linearity range, regression equation, intercept, slope and correlation coefficient for the calibration data are supplied at **Table 1**.

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

LOD and LOQ were calculated according to ICH guidelines[49] from the following equations:

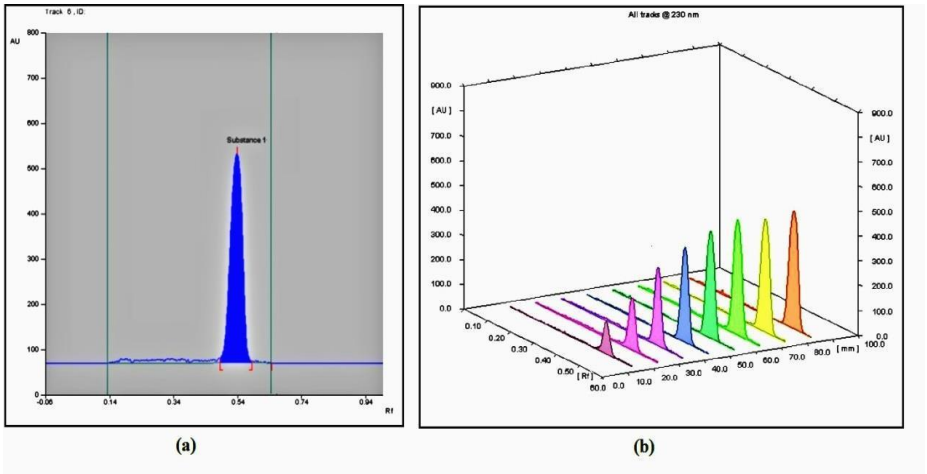

Figure 2. Thin layer chromatogram of separated peaks of EZE at various concentrations (1-8 µg/spot) with R_f value (0.49 ± 0.01) at 230 nm.

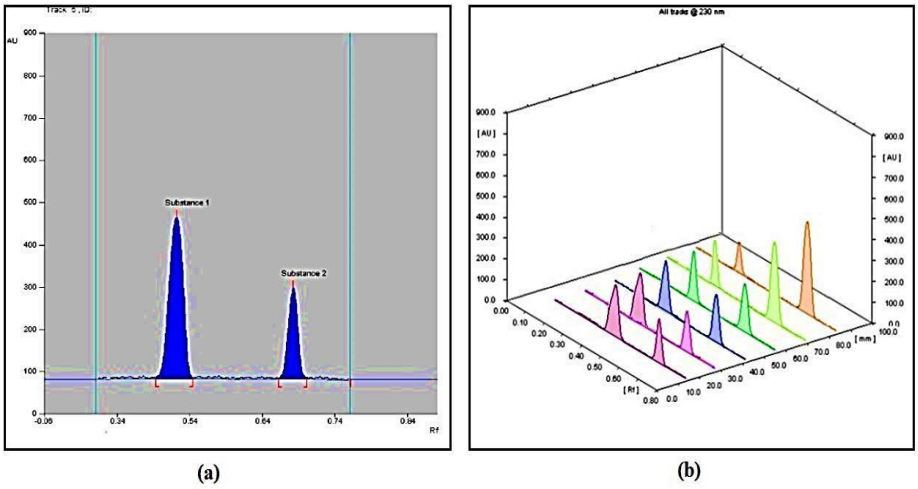

Figure 3. Thin layer chromatogram of separated peaks of mixture of EZE and its alkaline degradation product in range of (7 - 2 µg/spot) and (1−6 µg/spot), respectively.

LOD = 3.3 Sa / slope
LOQ = 10 Sa / slope

Where Sa is the residual standard deviation of a regression line.

LOD and LOQ values of EZE for the proposed method were listed in the **Table 1**.

Selectivity

The selectivity of the methods was achieved by the analysis of different laboratory prepared mixtures of EZE and its alkaline degradation product within the linearity range. Satisfactory results listed in the **Table 2**, and the results of the standard addition technique **Table 3**, prove that the proposed method can selectively analyze EZE without any interference from its alkaline degradate or the excipients.

Accuracy

The accuracy of the proposed method calculated as the mean percent recovery (% R), by applying the proposed procedure for triplicate determination of three concentration levels covering the specified range for EZE (3, 4 and 5 µg/band). The concentrations were obtained from the corresponding regression equations and the mean percent recoveries, shown in **Table 1**. The accuracy of the methods was further assured by the use of the standard addition technique. It was performed by the addition of known amounts of pure EZE to known concentrations of the pharmaceutical preparation and the resulting mixtures were assayed, and the results obtained were compared with the expected results **Table 3**. The good recoveries of the pure added of EZE suggested good accuracy of the proposed methods.

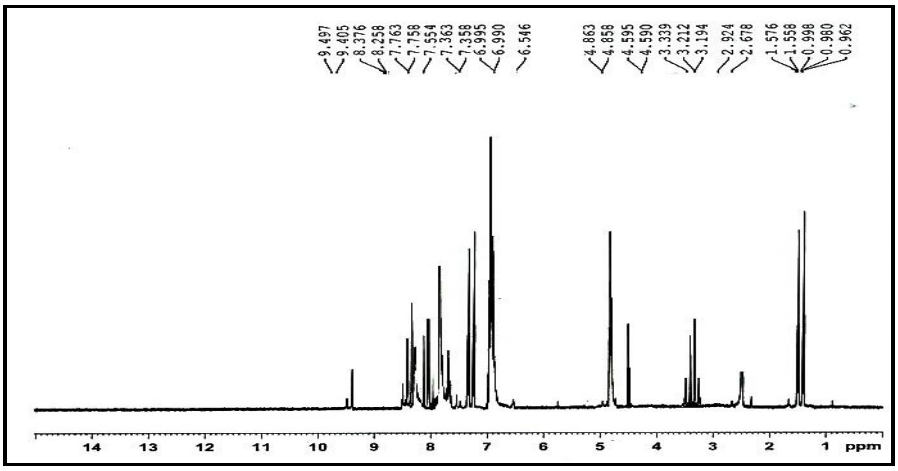

Figure 4. ^{1}H NMR of intact ezetimibe in DMSO.

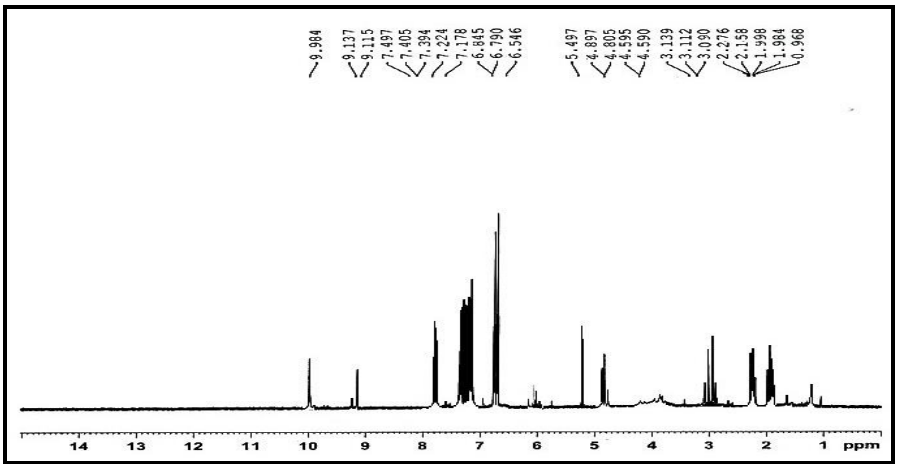

Figure 5. ^{1}H NMR of alkaline degradation product of ezetimibe in DMSO.

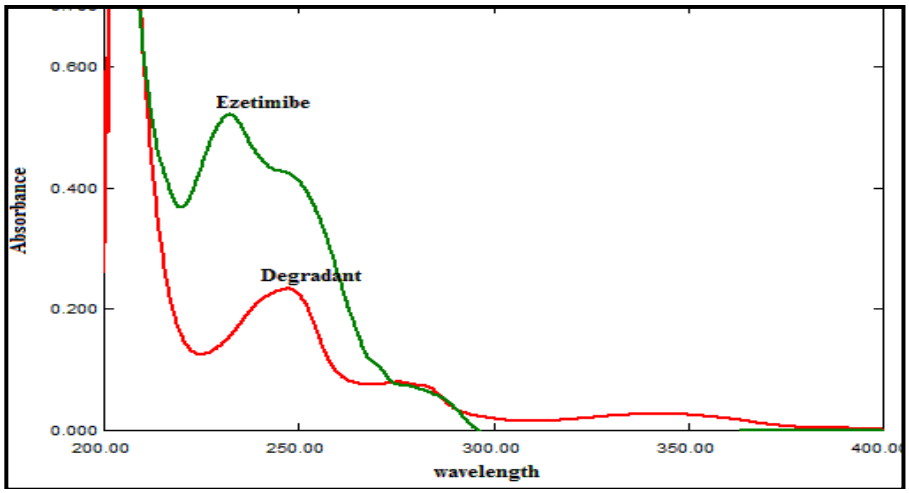

Figure 6. Zero order absorption spectra of ezetimibe (10 µg/mL) and its alkaline degradate (10 µg/mL).

Table 1. Regression and validation data for the determination of EZE by the densitometric method

Parameters	EZE
Wavelength (nm)	230
Linearity range (µg/band)	1-8
LOD (µg/mL)	0.189
LOQ (µg/mL)	0.574
Regression equation	
Slope Intercept	3688.4 -17408
Correlation coefficient (*r*)	0.9996
Accuracy (% R)*	100.26
Precision (% RSD)* - Repeatability (intra-day) - Intermediate precision (inter day)	 0.911 1.318
Robustness (% RSD): - Mobile phase content ± 2%: - Detection wavelength	 0.972 1.031

**Average of three determinations of three concentration levels.*

Table 2. Determination of EZE in laboratory prepared mixtures by the densitometric method

Concentration taken (µg/spot)		Concentration found (µg/spot)	% Recovery
EZE	**Deg.**	**EZE**	**EZE**
2	6	1.97	98.50
3	5	3.04	101.33
4	4	3.97	99.25
5	3	5.03	100.60
6	2	5.98	99.67
7	1	6.96	99.43
Mean ± % RSD			99.80 ± 1.012

Deg. Is the ezetimibe alkaline degradation product.

Table 3. Determination of EZE in Zetamibe® tablets by the densitometric methodand application of standard addition technique

Zetamibe® tablet	Standard addition technique			
% Found[b] ± %RSD	**Claimed concentration (µg/band)**	**Pure added (µg/band)**	**Pure found[a] (µg/band)**	**% Recovery**
EZE	**EZE**	**EZE**	**EZE**	**EZE**
99.85 ± 0.993	2	2	2.03	101.50
		4	3.95	98.75
		5	5.03	100.60
Mean ± %RSD				100.28 ± 1.398

[a]*Average of three determinations.*
[b] *Average of the recoveries for five different concentrations.*

Table 4. System suitability parameters for determination of EZE by the densitometric method

Parameters	Densitometric method	Reference values
Resolution (R)	2.28	**R > 2**
Tailing factor (T)	0.96	**T < 2**
Capacity factor (K)	1.04	**1–10 acceptable**
Theoretical plates (N)	2916	**N > 2000**

Table 5. Statistical comparison between the results obtained by densitometric method and the reported method for the determination of EZE[5] in pharmaceutical form

Parameters	Densitometric method	Reported method (absorbance ratio)[5]
n	5	5
Mean % R	99.85	100.01
SD	0.991	1.091
% RSD	0.993	1.091
Student's *t*-test (2.306)*	1.026	-
***F* value** (6.388)*	1.370	-

**The value in the parenthesis are the corresponding theoretical values of t and F at (P=0.05).*

Precision

The Precision of the proposed method 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 for each drug (3, 4 and 5µg/band) in the same day (intra-day analysis) for repeatability and on three different days (inter day analysis) for intermediate precision. The results were listed in the **Table 1**.

Robustness

The robustness of the proposed method tested by applying deliberated changes in experimental parameters such as the working wavelengths (± 2 nm) and a mobile phase content (± 2%). These minor changes did not have any significant effect on the peak area or separation of EZE from its alkaline degradation products and % RSD of the responses were < 2 %, confirming the robustness of the procedures, as shown in **Table 1**.

System suitability

System suitability was checked by calculating different parameters including resolution, tailing factor, capacity factor and number of theoretical plates. The obtained values were found to be in the acceptable ranges when compared to USP reference values as shown in **Table 4**.

Application to the pharmaceutical preparation

The proposed method was applied for the determination of EZE in zetamibe® tablets. The obtained results **Table 3** assure that the excipients did not interfere with the proposed method.

Statistical analysis

Statistical analysis of the results obtained by the proposed method and the reported method (ratio subtraction) of EZE in pharmaceutical form using student's t-test and F-value revealed that there is no significant difference between the proposed and the reported method [5] regarding the accuracy and precision **Table 5**.

CONCLUSION

TLC- densitometric technique has provided a simple, straightforward method for separating ezetimibe and its alkaline degradation product simultaneously. Its major advantage that it can be run using a small quantity of the mobile phase, thus lowering the analysis time and the cost with higher sensitivity rather than other described methods.

Conflict of Interest

The authors declare that they don't have any conflict of interest.

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