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Validated chromatographic methods for determination of some anti-hyperlipidemic used drugs

Maha Farouk¹, Omar Abdel-Aziz^{1*}, Reham Nagi¹, Laila Abdel-Fattah²

¹Analytical Pharmaceutical Chemistry Department, Faculty of Pharmacy, Ain Shams University, African Union Authority St. Abbassia, Cairo, (EGYPT)

²Analytical Chemistry Department, Faculty of Pharmacy, Cairo University, El-Kasr El Einy St. El Tahrir Square, Cairo, (EGYPT)

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ABSTRACT

The present study describes the development and subsequent validation of accurate, precise and reproducible HPLC and HPTLC methods for the analysis of Pravastatin (**I**), Simvastatin (**II**) and Ezetimibe (**III**) at ambient temperature. (**I**) was determined in presence of its acid, alkaline and oxidative-degradates, as stability-indicating study by the mentioned chromatographic techniques, utilizing acetonitrile: 0.1% acetic acid pH 3 ± 0.1 (50:50 v/v) as a mobile phase and chloroform: ethanol: glacial acetic acid (9:1:0.2 v/v) as a developing system. While, acetonitrile: acetic acid pH 3 (60:40 v/v) and diethyl ether: chloroform (9:1 v/v) was used as a mobile phase and as a developing system for determination of (**II**) and (**III**) in presence of each other and in presence of their acid, alkaline and oxidative-degradates, respectively, by the proposed chromatographic techniques. All the proposed methods were validated according to the International Conference on Harmonization (ICH) guidelines and successfully applied to determine the mentioned drugs in pure form, in laboratory prepared mixtures and in pharmaceutical preparations. The obtained results were statistically compared to the official and manufacturer's methods of analysis (for (**I**) and (**II**) and (**III**), respectively) and no significant differences were found.

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KEYWORDS

Pravastatin;
Simvastatin;
Ezetimibe;
HPLC;
HPTLC;
Stability indicating methods.

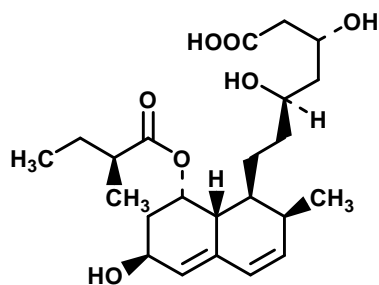
INTRODUCTION

Pravastatin sodium (**I**) and Simvastatin (**II**) are examples of statins that act by competitively inhibiting HMG-COA reductase enzyme that catalyzes the rate-limiting step in cholesterol biosynthesis^[1]. The ICH-guidelines^[2] requires performing stress-testing of the drug substance that can help in identifying the likely degradation-products, also can be useful in establish-

ing the degradation-pathways and validating the stability-indicating power of the analytical procedures used. Moreover, validated stability-indicating method should be applied in the stability study^[3]. Stability-indicating methods can be specific one that evaluates the drug in the presence of its-degradation products, excipients and additives^[4].

Most methods for (**I**) analysis utilized high performance liquid chromatographic techniques in biological

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Pravastatin M.W.=446.5

fluids^[5-13], thin layer chromatography^[14], gas chromatography^[15,16], capillary electrophoresis^[17] and polarography^[18] were reported. Different methods have been reported for determination of **(II)** including, spectrophotometric methods^[19,20], high performance liquid chromatographic techniques^[21-36] and gas chromatographic methods^[37-39]. Ezetimibe **(III)** is the first in a new class of anti-hyperlipidemic drugs known as cholesterol absorption inhibitors. It blocks effectively intestinal absorption of dietary and biliary cholesterol^[40]. Different methods used for **(III)** analysis using high performance liquid chromatographic techniques^[41-43], high performance thin layer chromatographic technique^[44] and spectrophotometric methods^[45,46] were reported.

The main goal of this work is to establish accurate, precise, rapid and reproducible chromatographic methods for the determination **(I)**, **(II)** and **(III)** in presence of their-degradates, as stability indicating study, and simultaneous determination of **(II)** and **(III)** in binary mixtures, which can be used for the routine quality control analysis of these drugs in raw material and pharmaceutical preparations and for stability studies.

EXPERIMENTAL

Chemicals and reagents

Pravastatin sodium was kindly provided by Bristol-Mayers Squibb and certified to contain 99.99%. Lipostat[®] tablets: batch number: J42992, manufactured by Bristol-Mayers Squibb Company. Each tablet was labeled to contain 20 mg of Pravastatin sodium. Simvastatin was kindly supplied by Amriya Pharmaceutical industries (Egypt) and certified to contain 99.95%. Ezetimibe was kindly supplied by Global Napi Pharmaceuticals (Egypt) and certified to contain 99.99%. Inegy[®] tablets: batch number: NE16760,

manufactured by Global Napi Pharmaceuticals. Each tablet was labeled to contain 20 mg of Simvastatin and 10 mg Ezetimibe.

Acetonitrile, diethyl ether, ethanol, methanol and bi-distilled water (Riedel-dehaen, Sigma-Aldrich, Germany), glacial acetic acid and hydrogen peroxide 30% (E.Merck, Germany), hydrochloric acid and sodium hydroxide (BDH), each 'aqueous 0.1M' and chloroform (Adwia).

All chemical and reagents used through this work are of chromatographic analytical grade. Bi-distilled water is used throughout the whole work and is indicated by the word 'water'.

Instruments

The HPLC system-Bio-Tek Kontron instrument 'SRL Via G Fantoli 16/15 – 20138 Milan, Italy' comprised an isocratic pump model series 422, connected to PC and software package 1.8 (Kromasystem 2000 version 1.8.1a), Knauer injector with Hamelton fixed-needle syringe 'P.No.A50-0024 and Unit 50.0 µl', 50-µl loop and a 540+ photodiode array detector. The chromatographic separation was performed using supelcosil C18 column (5µm, 250 x4.6 mm i.d.) at ambient temperature. Ultrasonic vibrator, Crest ultrasonic-Tru/Sweep, Model 575 TAE, N.Y., USA. Disposable membrane filters, 0.45µm, Phenomenex, Nylon, Millipore, USA.

For HPTLC, a Desaga densitometer model CD60 (Germany), AS 30 Desaga applicator, Desaga UV lamp with short wavelength (254nm), HPTLC aluminium plates pre-coated with silica gel 60 F₂₅₄ (E.Merck).

A (Jenway 3510, UK) pH-meter, equipped with combined glass electrode for pH adjustment.

Standard solutions

Standard solutions of the investigated drugs

Stock standard solutions of **(I)** having concentration of (1.0 mg.ml⁻¹), were prepared in water and methanol for HPLC and HPTLC analysis, respectively, where the first one was further diluted with the mobile phase to have a concentration of 100 µg.ml⁻¹ used as a working standard HPLC-solution, and the last one was used as a working standard HPTLC-solution.

While, stock standard solutions of **(II)** and **(III)**, each having concentration of (1.0 mg.ml⁻¹ and 2.0 mg.ml⁻¹)

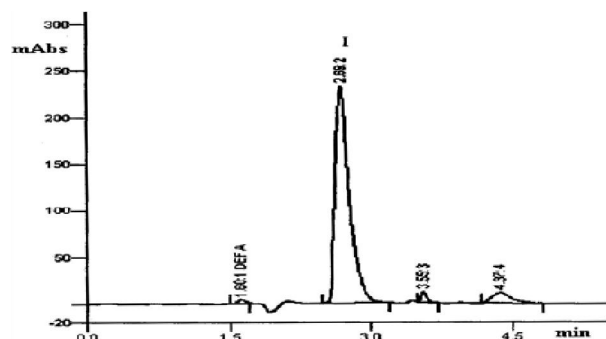


Figure 1a : HPLC chromatogram of mixture solution containing Pravastatin sodium (I) $20.0\mu\text{g}\cdot\text{ml}^{-1}$ with its acid-degradates $10.0\mu\text{g}\cdot\text{ml}^{-1}$

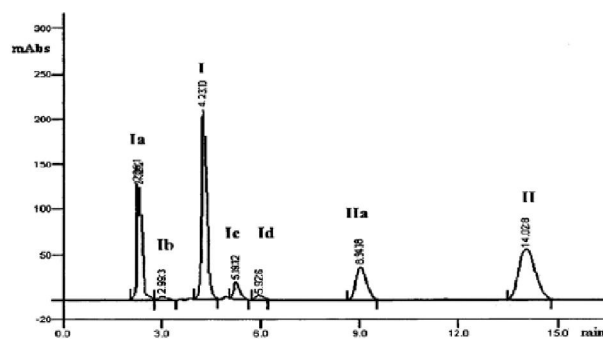


Figure 2a : HPLC chromatogram of mixture solution containing Ezetimibe (I) and Simvastatin (II) ($20.0\mu\text{g}\cdot\text{ml}^{-1}$ each) with Ezetimibe acid-degradates (Ia, Ib, Ic & Id) $20.0\mu\text{g}\cdot\text{ml}^{-1}$ and Simvastatin acid-degradaate (IIa) $20.0\mu\text{g}\cdot\text{ml}^{-1}$

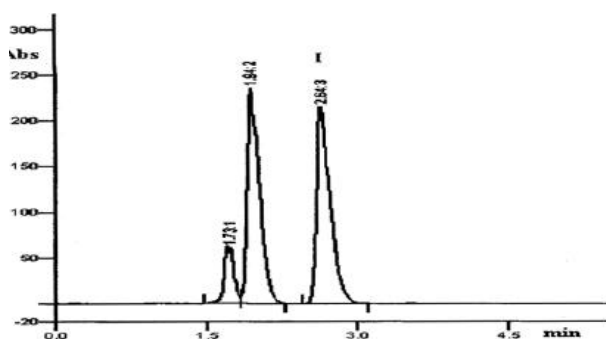


Figure 1b : HPLC chromatogram of mixture solution containing Pravastatin sodium (I) $20.0\mu\text{g}\cdot\text{ml}^{-1}$ with its alkaline-degradates $20.0\mu\text{g}\cdot\text{ml}^{-1}$

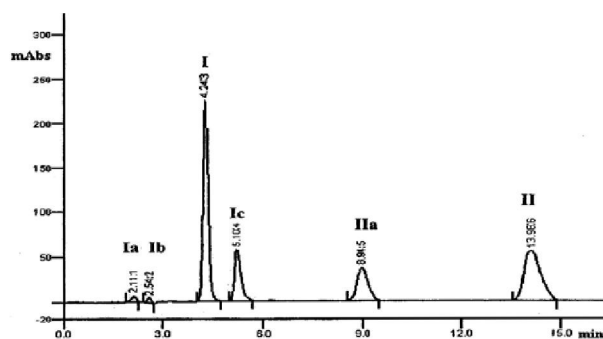


Figure 2b : HPLC chromatogram of mixture solution containing Ezetimibe (I) and Simvastatin (II) ($20.0\mu\text{g}\cdot\text{ml}^{-1}$ each) with Ezetimibe alkaline-degradates (Ia, Ib & Ic) $20.0\mu\text{g}\cdot\text{ml}^{-1}$ and Simvastatin alkaline-degradaate (IIa) $20.0\mu\text{g}\cdot\text{ml}^{-1}$

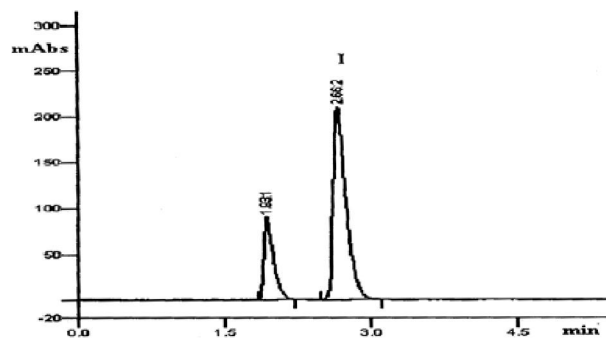


Figure 1c : HPLC chromatogram of mixture solution containing Pravastatin sodium (I) $20.0\mu\text{g}\cdot\text{ml}^{-1}$ with its oxidative-degradates $5.0\mu\text{g}\cdot\text{ml}^{-1}$

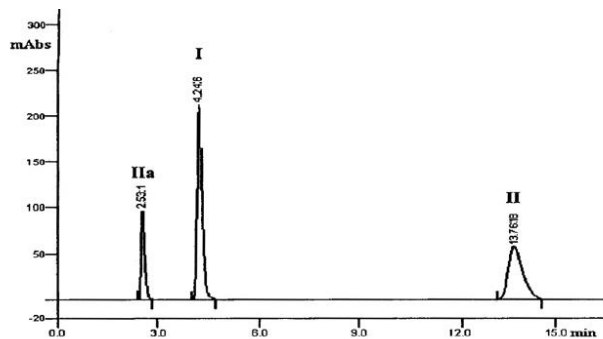


Figure 2c : HPLC chromatogram of mixture solution containing Ezetimibe (I) and Simvastatin (II) ($20.0\mu\text{g}\cdot\text{ml}^{-1}$ each) with Simvastatin oxidative-degradates (IIa) $10.0\mu\text{g}\cdot\text{ml}^{-1}$

for HPLC and HPTLC, respectively, were prepared in acetonitrile, where working standard HPLC-solutions of (II) and (III), having concentrations of $250\mu\text{g}\cdot\text{ml}^{-1}$ were prepared by further dilution with the mobile phase, while HPTLC working standard solutions of (II) and (III) were prepared by further dilution with acetonitrile to have a concentration of $500\mu\text{g}\cdot\text{ml}^{-1}$.

Standard solution of pravastatin degradates

Pravastatin sodium

Stock standard solutions of 'acid and alkaline-degradates', were prepared, by mixing 10 ml of the stock standard solution of (I), separately, with 20 ml of '0.1M HCl and 1.0 M NaOH', heating in water-bath at 70°C for '2 and 3.5' hours, respectively, cooling,

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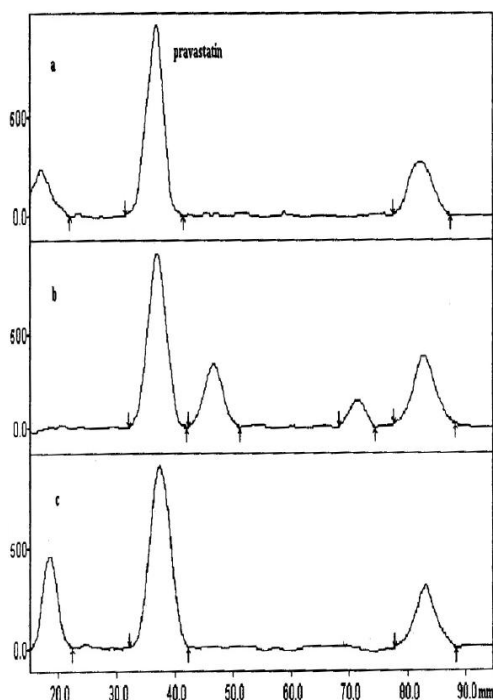


Figure 3 : HPTLC chromatograms of mixture solutions containing Pravastatin sodium $3.0\mu\text{g.spot}^{-1}$ with: (a) Its oxidative-degradates $1.0\mu\text{g.spot}^{-1}$, (b) Its acid-degradates $2.0\mu\text{g.spot}^{-1}$ and (c) Its alkaline-degradates $2.0\mu\text{g.spot}^{-1}$

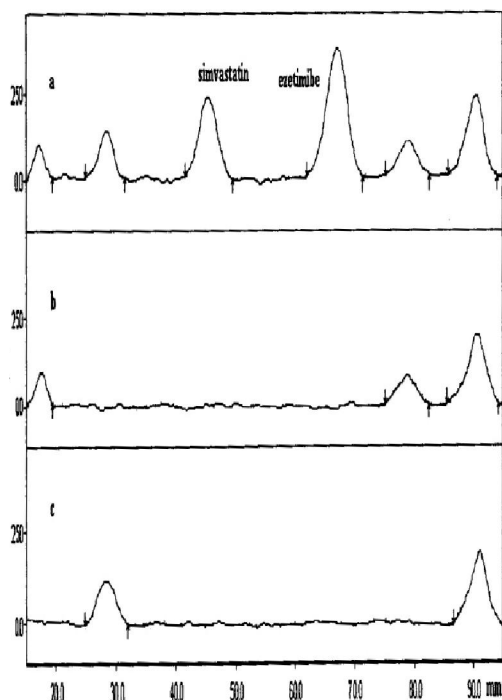


Figure 4a : HPTLC chromatograms (a) mixture solutions containing Ezetimibe and Simvastatin ($2.0\mu\text{g.spot}^{-1}$ each) with their acid-degradates $2.0\mu\text{g.spot}^{-1}$, (b) Ezetimibe acid-degradates $2.0\mu\text{g.spot}^{-1}$ and (c) Simvastatin acid-degradates $2.0\mu\text{g.spot}^{-1}$

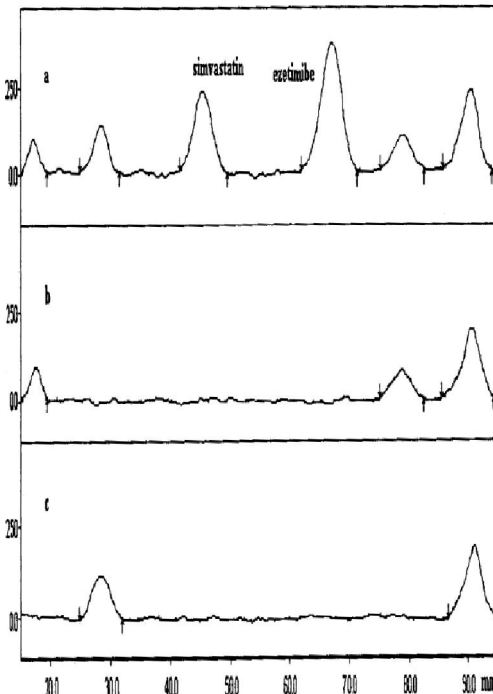


Figure 4b : HPTLC chromatograms (a) mixture solutions containing Ezetimibe and Simvastatin ($2.0\mu\text{g.spot}^{-1}$ each) with their alkaline-degradates $2.0\mu\text{g.spot}^{-1}$, (b) Ezetimibe alkaline-degradates $2.0\mu\text{g.spot}^{-1}$ and Simvastatin alkaline-degradates $2.0\mu\text{g.spot}^{-1}$

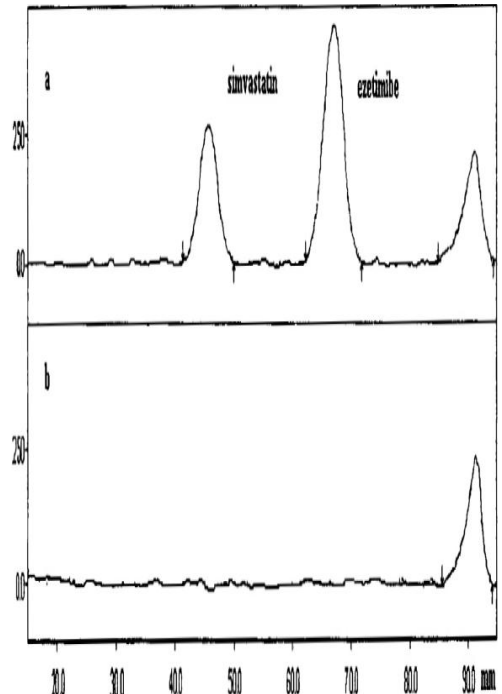


Figure 4c : HPTLC chromatograms (a) mixture solutions containing Ezetimibe and Simvastatin ($2.0\mu\text{g.spot}^{-1}$ each) with Simvastatin oxidative-degradates $2.0\mu\text{g.spot}^{-1}$ and (b) Simvastatin oxidative-degradates $3.0\mu\text{g.spot}^{-1}$

then neutralizing the media with '0.1M NaOH and 0.1M HCl' and making the volumes to 50 ml with water and methanol for HPLC and HPTLC analysis, respectively, to obtain a concentration of 200 $\mu\text{g}\cdot\text{ml}^{-1}$.

Standard stock solution of oxidative-degradates, were prepared by mixing 10 ml of the stock solution of (I) with 10 ml 30% H_2O_2 , leaving at room temperature for 24 hrs and then making volume to 50 ml with water and methanol for HPLC and HPTLC analysis, respectively, to obtain a concentration of 200 $\mu\text{g}\cdot\text{ml}^{-1}$.

Complete degradation was checked by using HPTLC system; silica gel 60 F_{254} plates and chloroform: ethanol: galacial acetic acid (9: 1: 0.2, v/v/v) as a developing system or HPLC system; Supelcosil C18 5 μm column and acetonitrile: acetic acid (pH 3.0) (50: 50, v/v) as a mobile phase.

Then, all the prepared standard stock degraded solutions, used for HPLC technique were further diluted with the mobile phase to prepare the working stock solutions having a concentration of 40 $\mu\text{g}\cdot\text{ml}^{-1}$.

Simvastatin and ezetimibe

Stock standard solutions of (II) and (III) each having concentration of 2.0 $\text{mg}\cdot\text{ml}^{-1}$ in acetonitrile were used in the forced degradation.

For simvastatin

Standard stock solutions of 'acid and alkaline-degradates' were prepared separately, by mixing 5 ml of the stock standard solution of (II), separately, with 10 ml of '0.1M HCl and 1.0 M NaOH', heating in water-bath at 80°C for '4.5 and 3.5' hours, respectively, cooling, then neutralizing the media with '0.1M NaOH and 0.1M HCl' and making the volume to 50 ml with acetonitrile, to obtain a concentration of 200 $\mu\text{g}\cdot\text{ml}^{-1}$.

Standard solution of oxidative-degradates was prepared by mixing 5 ml of the stock solution of (II) with 5 ml 30% H_2O_2 , leaving at room temperature for 48 hrs and then making volume to 50 ml with acetonitrile to obtain a concentration of 200 $\mu\text{g}\cdot\text{ml}^{-1}$.

For ezetimibe

Stock and working standard solutions of (III) 'acid and alkaline-degradates' were prepared separately, by the same way like that used for Simvastatin.

Complete degradation was checked by using

HPTLC system; silica gel 60 F_{254} plates and diethyl ether: chloroform: galacial acetic acid (9: 1, v/v) as a developing system or HPLC system; Supelcosil C18 5 μm column and acetonitrile: acetic acid (pH 3.0) (60: 40, v/v) as a mobile phase.

Then, all the prepared standard stock degraded solutions, used for HPLC technique were further diluted with the mobile phase to prepare the working stock solutions having a concentration of 40 $\mu\text{g}\cdot\text{ml}^{-1}$.

PROCEDURES

HPLC method

Pravastatin sodium

Stationary phase, C18 Supelcosil column (5 μm , 250 \times 4.6 mm), acetonitrile: diluted acetic acid 'pH 3' in a ratio (50:50, v/v) with a flow rate of 1.3 $\text{ml}\cdot\text{min}^{-1}$ as 'degassed and filtered' mobile phase, and UV detection at 237 nm, were the chromatographic conditions adopted. Construction the calibration curve was performed by transferring aliquots of (I)-working standard solution into a series of 25 ml volumetric flasks and diluting with the mobile phase to the volume, having a concentration range of 0.4-30 $\mu\text{g}\cdot\text{ml}^{-1}$. Under the previously mentioned chromatographic conditions, 50 μl -volume from each solution was injected in triplicate, the average peak area obtained for each concentration was plotted versus concentration and the regression equation was then computed.

Simvastatin and ezetimibe

The adopted chromatographic conditions were degassed and filtered mobile phase consists of acetonitrile: diluted acetic acid 'pH 3' in a ratio (60:40, v/v), with a flow rate of 1.3 $\text{ml}\cdot\text{min}^{-1}$, C18 Supelcosil column (5 μm , 250 \times 4.6 mm) as a stationary phase and UV detection at 247 nm. Calibration curves were constructed by transferring aliquots of (II) and (III) working standard solutions into a series of 50 ml volumetric flasks and diluting with the mobile phase to the volume having concentration ranges of 1-90 $\mu\text{g}\cdot\text{ml}^{-1}$ and 0.5-90 $\mu\text{g}\cdot\text{ml}^{-1}$, respectively. Under the above mentioned chromatographic conditions, 50 μl -volume from each solution was injected in triplicate, the average peak area obtained for each concentration was plotted versus concentration and then the regression equation was computed.

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TABLE 1 : Validation report of the proposed HPLC and HPTLC methods for the determination of Pravastatin sodium

Parameters	Methods	
	HPLC	HPTLC
Linearity	0.4-30 $\mu\text{g.ml}^{-1}$	0.4-10 $\mu\text{g.spot}^{-1}$
Intercept	0.0262	20.99
Slope(b) ^a	1.9426	-
Coefficient1(b1) ^b	-	382.58
Coefficient2(b2) ^b	-	-15.291
Correlation coefficient (r)	0.9998	0.9997
Accuracy ^c	100.28 \pm 0.615	99.89 \pm 0.983
Precision		
Repeatability ^c	100.87 \pm 0.910	100.10 \pm 0.913
Intermediate precision ^c	100.90 \pm 1.008	99.39 \pm 0.936

^aRegression equation = "A = a + bc" for HPLC; where "A" = peak area and "c" = the concentration ($\mu\text{g.ml}^{-1}$). ^bRegression equation = "A = a + b1c + b2c2" for HPTLC; where "A" = the peak area and "c" = the concentration ($\mu\text{g.spot}^{-1}$). ^cMean \pm S.D

TABLE 2 : Validation report of the proposed HPLC and HPTLC methods for the determination of Simvastatin (I) and Ezetimibe (II)

Parameters	Methods			
	HPLC		HPTLC	
	(I)	(II)	(I)	(II)
Linearity	1-90 $\mu\text{g.ml}^{-1}$	0.5-90 $\mu\text{g.ml}^{-1}$	0.4-5 $\mu\text{g.spot}^{-1}$	0.2-5 $\mu\text{g.spot}^{-1}$
Intercept	-0.524	0.182	26.867	74.509
Slope(b) ^a	1.192	1.448	-	-
Coefficient1(b1) ^b	-	-	372.59	620.89
Coefficient2(b2) ^b	-	-	-18.885	-48.608
Correlation coefficient (r)	0.9998	0.9997	0.9997	0.9997
Accuracy ^c	100.16 \pm 0.936	99.75 \pm 0.959	99.99 \pm 0.794	100.09 \pm 0.913
Precision				
Repeatability ^c	100.45 \pm 0.833	99.79 \pm 0.723	99.46 \pm 0.949	99.47 \pm 0.645
Intermediate precision ^c	99.31 \pm 0.930	99.85 \pm 1.023	100.05 \pm 0.679	99.49 \pm 0.856

^aRegression equation = "A = a + bc" for HPLC; where "A" = peak area and "c" = the concentration ($\mu\text{g.ml}^{-1}$). ^bRegression equation = "A = a + b1c + b2c2" for HPTLC; where "A" = the area and "c" = the concentration ($\mu\text{g.spot}^{-1}$). ^cMean \pm S.D

TABLE 3 : Statistical comparison between the proposed methods and the official method* for the determination of Pravastatin sodium

Methods	Parameters					
	Mean	S.D.	n	Variance	Student's t-test	F test
B.P.	99.75	0.997	5	0.994	-	-
HPLC	100.28	0.615	6	0.378	0.427(2.23)	2.629(5.19)
HPTLC	99.99	0.983	7	0.967	0.744(2.20)	1.028(4.53)

Values in parenthesis are the theoretical values of *t* and *F* at *P*=0.05. *Official B.P. (2007) HPLC method^[48]

TABLE 4 : Statistical comparison between the proposed methods and the manufacturer's method* for determination of Simvastatin (I) and Ezetimibe (II)

Parameters	(I)			(II)		
	Manufacturer's method	HPLC	HPTLC	Manufacturer's method	HPLC	HPTLC
Mean	99.87	100.16	99.99	100.46	99.75	100.09
S.D.	0.86	0.936	0.794	0.848	0.959	0.913
n	5	6	8	5	8	8
Variance	0.739	0.877	0.629	0.719	0.919	0.833
Student's t test	-	0.693 (2.23)	0.659 (2.18)	-	0.239 (2.18)	0.436 (2.18)
F test	-	1.187 (6.26)	0.851 (4.12)	-	1.187 (6.09)	1.159 (6.09)

Values in parenthesis are the theoretical values of *t* and *F* at *P*=0.05. *Manufacturer's HPLC method^[49]

TABLE 5 : Determination of Pravastatin sodium in laboratory prepared mixtures containing its acid-degradates by the proposed HPLC and HPTLC methods

Sample no.	% Degradates	% Recovery*	
		HPLC*	HPTLC*
1	10	100.08	100.29
2	50	100.19	98.94
3	100	100.35	99.02
Mean		100.21	99.42
R.S.D.%		\pm 0.136	\pm 0.76

*Mean of three determinations

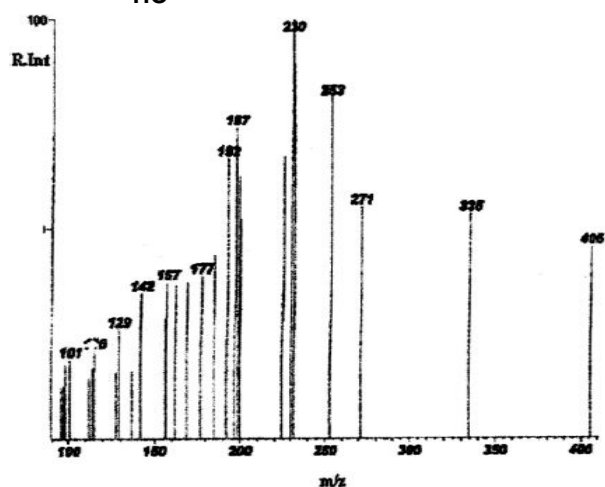
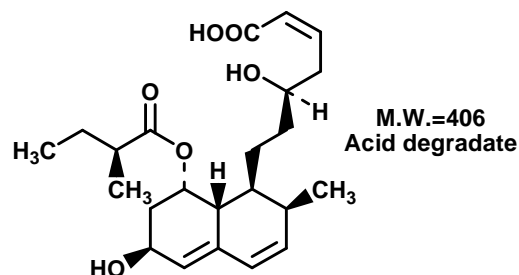


Figure 5 : Mass spectrum of first acid-degrade of Pravastatin sodium

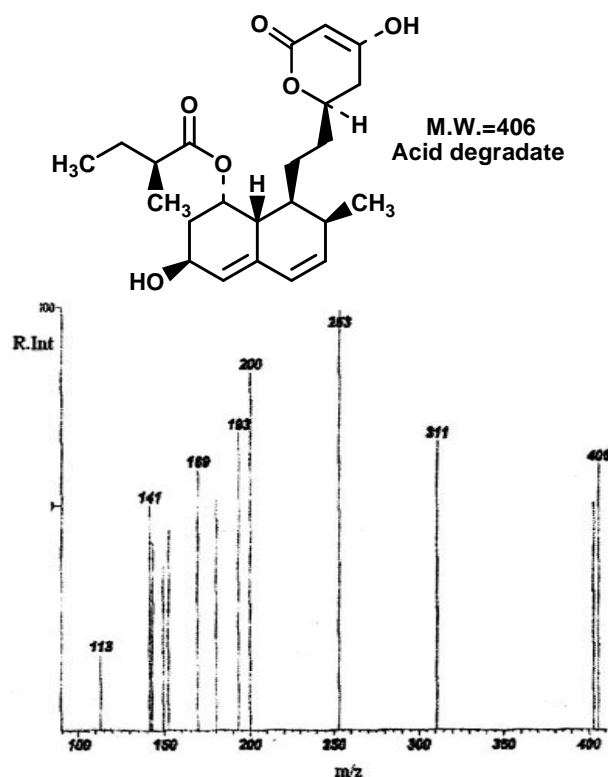


Figure 6 : Mass spectrum of second acid-degradata of Pravastatin sodium

HPTLC method

Pravastatin sodium

Calibration curve was performed by applying different aliquots (0.4-10 μl) of (I)-stock standard solution as separate compact spots 10 mm apart and 15 mm from the bottom of HPTLC plates. The plates were developed in chloroform: ethanol: glacial acetic acid (9:1:0.2, v/v/v) system, by ascending chromatography to a distance of about 80 mm in a chromatographic tank pre-saturated for 30-minutes then removed, dried in air, scanned at 237nm and then the peak area corresponding to each concentration was measured. The average peak area obtained for each concentration was plotted versus concentration and the regression equation was then computed.

Simvastatin and ezetimibe

Different aliquots (0.8-10 μl and 0.4-10 μl) of (II) and (III) working standard solutions were applied as separate compact spots 10 mm apart and 15 mm from the bottom of HPTLC plates and the above mentioned steps used for construction the calibration curve of (I) were adopted, but using diethyl ether: chloroform (9:1,

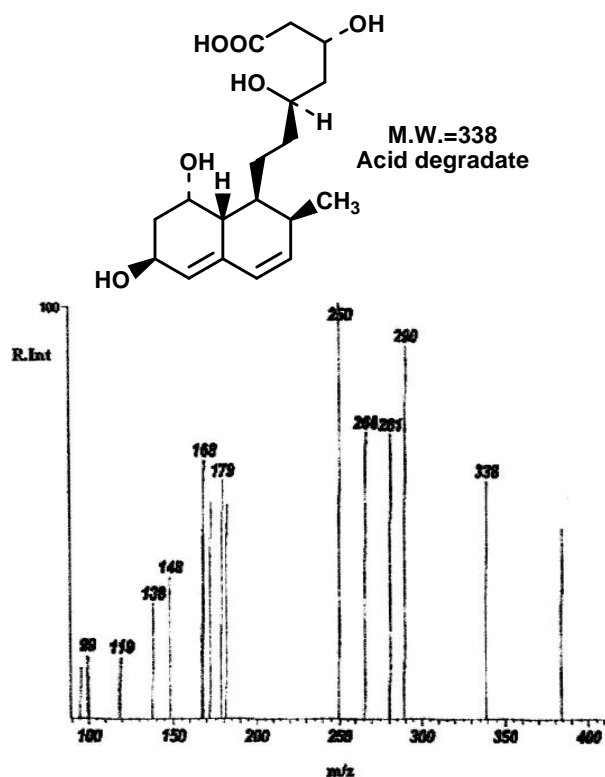


Figure 7 : Mass spectrum of the alkaline-degradata of Pravastatin sodium

v/v) system and scanning at 231 nm.

Assay of the pharmaceutical formulations

Pravastatin sodium

Twenty tablets were accurately weighed and finely powdered. A portion of the powder equivalent to one tablet of (I) was accurately weighed, transferred to 100 ml volumetric flask, shaken for 15-minutes with 50 ml water, filtered and then completed to the volume with water, to obtain a concentration of 200 $\mu\text{g}\cdot\text{ml}^{-1}$. Solutions having concentration of 10 $\mu\text{g}\cdot\text{ml}^{-1}$ were prepared by further diluted with the mobile phase and methanol, respectively to be used for Pravastatin determinations by HPLC and HPTLC techniques, as mentioned under 2.4.1. and 2.4.2.

Simvastatin and ezetimibe

Twenty tablets were accurately weighed and finely powdered. A portion of the powder equivalent to one tablet of (II) and (III) was accurately weighed, transferred to 100 ml volumetric flask, shaken for 15-minutes with 50 ml acetonitrile, filtered and then completed to volume with acetonitrile. Solutions having the concentrations of 20 $\mu\text{g}\cdot\text{ml}^{-1}$ (II) and 10 $\mu\text{g}\cdot\text{ml}^{-1}$ (III) were

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TABLE 6a : Determination of Simvastatin and ezetimibe in laboratory prepared mixtures containing their acid-degradates* by the proposed HPLC method

Mixtures no.	Simvastatin ($\mu\text{g.ml}^{-1}$)	Ezetimibe ($\mu\text{g.ml}^{-1}$)	% Recovery ^a	
			Simvastatina	Ezetimibe
1	2	20	99.05	100.79
2	20	20	99.99	100.24
3	20	2	100.53	101.09
Mean			99.86	100.71
R.S.D.%			± 0.750	± 0.428

^aMean of three determinations. *Each sample contains 20 $\mu\text{g.ml}^{-1}$ acid degradates of each drug

TABLE 7 : Results from robustness testing of the proposed HPLC method for Pravastatin sodium

Conditions	Rt	N	T	RS*
Flow rate				
1.2 ml.min ⁻¹	2.89	2404.51	1.9	4.82
1.4 ml.min ⁻¹	2.50	2637.22	1.9	4.51
Mobile phase composition				
Acetonitrile: acetic acid (52: 48, v/v)	2.59	2681.36	1.9	4.84
Acetonitrile: acetic acid (48: 52, v/v)	2.87	2815.96	2	4.29
pH				
2.8	2.40	2815.97	2	4.03
3.2	2.93	2861.57	1.5	4.13
Column				
Thermo Hypersil C18 (5 μm , 250 \times 4.6 mm)	2.62	2725.86	1.8	4.13

*Resolution of the nearest acid-degradate relative to Pravastatin sodium

prepared by appropriate dilutions with the mobile phase and acetonitrile, respectively to be used for (Simvastatin and Ezetimibe) determinations by HPLC and HPTLC techniques, as mentioned under 2.4.1. and 2.4.2.

RESULTS AND DISCUSSION

Method development

HPLC methods

Pravastatin sodium

The separation of Pravastatin from its degradation-products has been performed on Supelcosil C-18 column. The proportion of the mobile phase components was optimized to reduce each of 'retention time and tailing' and to enable good resolution from its-degradates. At high acetonitrile ratio, retention time of different components decrease but with excessive tail-

TABLE 6b : Determination of Simvastatin and Ezetimibe in laboratory prepared mixtures containing their acid-degradates* by the proposed HPTLC method

Mixtures no.	Simvastatin ($\mu\text{g.spot}^{-1}$)	Ezetimibe ($\mu\text{g.spot}^{-1}$)	% Recovery ^a	
			Simvastatina	Ezetimibe
1	1	3	100.13	99.98
2	3	3	99.83	99.11
3	3	1	100.68	100.07
Mean			100.21	99.72
R.S.D.%			± 0.430	± 0.531

^aMean of three determinations. *Each sample contains 2 $\mu\text{g.spot}^{-1}$ acid-degradates of each drug

TABLE 8a : Results from robustness testing of the proposed HPLC method for determination of Simvastatin

Conditions	Rt	N	T	RS*
Flow rate				
1.2 ml.min ⁻¹	14.42	3040.89	1.9	6.54
1.4 ml.min ⁻¹	13.07	2751.45	1.7	5.79
Mobile phase composition				
Acetonitrile: acetic acid (52: 48, v/v)	12.74	2822.46	1.9	5.58
Acetonitrile: acetic acid (48: 52, v/v)	14.01	2985.51	1.9	6.22
pH				
2.8	13.91	2967.18	1.6	6.44
3.2	13.97	2894.36	1.9	6.11
Column				
Thermo Hypersil C18 (5 μm , 250 \times 4.6 mm)	13.99	2930.66	1.5	6.82

* Resolution of the nearest acid-degradate relative to Simvastatin

ing of its peak. High resolution was obtained by using acetonitrile: diluted acetic acid (50:50, v/v) as a mobile phase, with a flow rate 1.3 ml.min⁻¹, and detection at 237 nm, where the maximum sensitivity was observed. The average retention time was 2.65 ± 0.05 min for 10 replicates as shown in (Figure 1a, 1b and 1c).

Simvastatin and ezetimibe

At high acetonitrile ratio, retention time of different components decrease but with excessive tailing of Simvastatin peak and poor resolution of Ezetimibe from its-degradates. Thus, proportion of the mobile phase components was optimized, by using acetonitrile: acetic acid pH 3 (60:40, v/v) as a mobile phase system with flow rate 1.3 ml.min⁻¹, and detection at 247 nm, where a high resolution was obtained. The average retention times for Simvastatin and Ezetimibe were 13.95 ± 0.05 min and 4.22 ± 0.01 min, respectively as shown

TABLE 8b : Results from robustness testing of the proposed HPLC method for determination of Ezetimibe

Conditions	Rt	N	T	RS*
Flow rate				
1.2 ml.min ⁻¹	4.45	3059.47	2	3.06
1.4 ml.min ⁻¹	4.07	2930.66	1.9	2.36
Mobile phase composition				
Acetonitrile: acetic acid (52: 48, v/v)	3.98	2804.62	1.8	2.36
Acetonitrile: acetic acid (48: 52, v/v)	4.39	3191.04	1.6	3.25
pH				
2.8	4.22	3124.91	2	3.15
3.2	4.28	3325.39	1.6	3.39
Column				
Thermo Hypersil C18 (5µm, 250×4.6 mm)	4.25	3325.39	1.6	3.54

*Resolution of the nearest acid-degradate relative to Ezetimibe

in (Figure 2a, 2b and 2c). The system suitability tests were used to verify that the resolution and reproducibility of the chromatographic systems are adequate for analysis^[47].

HPTLC method

Experimental conditions, such as developing system, scan mode and wavelength of detection were optimized to provide accurate, precise and reproducible results. The chosen scan mode was the zigzag mode and the wavelength of scanning was chosen to be 237nm for **(I)** and 231nm for **(II)** and **(III)**. A variety of developing systems were evaluated and good resolution with minimum tailing of these drugs from their-degradates was obtained by using developing system consists of chloroform: ethanol: glacial acetic acid (9:1:0.2, v/v/v) for **(I)** and diethyl ether: chloroform (9:1, v/v) for **(II)** and **(III)**. For **(I)**, the tailing factor was 1.3 while R_f -values were 0.29 and '0.39, 0.05 and 0.03' for **(I)** for its main acid, alkaline and oxidative-degradates, respectively (Figure 3). While, tailing factors for **(II)** and **(III)**, were 1.1 for both, and R_f -values were 0.39 and 0.66 for **(II)** and **(III)**, 0.18 for both acid, alkaline and oxidative-degradates of **(II)**; and 0.79 for nearest acid and alkaline-degradates of **(III)** (Figure 4a, 4b and 4c) respectively.

Methods validation

ICH-guidelines³ for method validation were followed. All validation parameters are shown in (TABLE 1 and 2).

TABLE 9 : Determination of Pravastatin sodium in pharmaceutical formulations using the proposed HPLC and HPTLC methods and application of standard addition technique

Items							
Pharmaceutical formulation*				Standard addition technique			
Claimed		%Found± S.D.		HPLC		HPTLC	
HPLC	HPTLC	HPLC	HPTLC	Added (µg.ml ⁻¹)	% Recovery ^a	Added (µg.spot ⁻¹)	% Recovery ^a
				2.00	99.07	0.40	99.40
				5.00	98.66	0.80	99.64
	20 mg	101.49± 0.268	99.43± 1.052	10.00	99.41	1.00	101.01
				15.00	100.55	2.00	99.11
				20.00	99.31	4.00	100.99
Mean					99.40		100.03
±R.S.D.					±0.709		±0.903

*Lipostat® tablets (Batch no: J42992) (labeled to contain 20 mg Pravastatin sodium per tablet). ^aMean of four determinations

Linearity

Pravastatin sodium

For HPLC method, a linear correlation was obtained between peak area and concentration of **(I)** in a range of 0.4-30 µg.ml⁻¹ with correlation coefficient (r) = 0.9998. While, for HPTLC method, linear and polynomial regression between **(I)** concentrations and peak areas of the spots was investigated and the correlation value was found to be, (r = 0.9709) in a concentration range of 0.4-10.0 µg.spot⁻¹. Thus, the second order polynomial fit was found to be more suitable. Moreover, the ICH-guidelines^[3] mentioned that for some analytical procedures which do not demonstrate linearity, the analytical response should be described by an appropriate function of the concentration of an analyte sample. The regression equation showed correlation coefficient (r) of 0.9997 in the same concentration range.

Simvastatin and ezetimibe

For HPLC method, linear correlations were obtained between peak area and concentration in ranges of '1-90 µg.ml⁻¹ and 0.5-90 µg.ml⁻¹' with correlation coefficients (r) of 0.9998 and 0.9997 for **(II)** and **(III)**, respectively. While, For HPTLC method, the linear regression between concentrations and peak areas of the spots was investigated and the correlation coefficients (r) were found to be 0.9944 and 0.9810, over the concentration ranges of 0.4-5.0 µg.spot⁻¹ and 0.2-5.0 µg.spot⁻¹, for **(II)** and **(III)**, respectively. Thus, the sec-

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TABLE 10a : Determination of Simvastatin in pharmaceutical formulations using the proposed HPLC and HPTLC methods and application of standard addition technique

Items							
Pharmaceutical formulation*				Standard addition technique			
Claimed		%Found± S.D.		HPLC		HPTLC	
HPLC	HPTLC	HPLC	HPTLC	Added (µg.ml ⁻¹)	% Recovery ^a	Added (µg.spot ⁻¹)	% Recovery ^a
				10.00	100.34	0.40	100.13
				20.00	99.12	0.60	98.39
20 mg		100.54 ±0.646	99.91 ±0.719	30.00	100.09	1.00	99.08
				40.00	101.54	2.00	99.32
				50.00	99.78	3.00	98.94
Mean					100.17		99.17
±R.S.D.					±0.889		±0.640

*Inegy[®] tablets (Batch no: NE16760) (labeled to contain 20 mg Simvastatin & 10mg Ezetimibe per tablet). ^aMean of four determinations

second order polynomial fit was found to be more suitable. The regression equations showed correlation coefficient (r) of 0.9997 in the same concentration ranges for both drugs.

Accuracy

Accuracy of the proposed methods was tested by analyzing freshly prepared solutions of the studied drugs in triplicate. The recovery percent and standard deviations (S.D.) revealed excellent accuracy. The results obtained by applying the proposed chromatographic methods were statistically compared with those results obtained by the official B.P. method^[48] for (I) and the manufacturer's method^[49] for (II) and (III). It was concluded that with 95% confidence, there is no significant difference between them since the calculated t and F values are less than the theoretical values^[50] (TABLE 3 and 4).

Repeatability and reproducibility

The intra- and inter-day precision was evaluated by assaying freshly prepared solutions in triplicate, as shown in (TABLE 1 & 2).

Specificity

The specificity of the HPLC and HPTLC methods was illustrated by the complete separation of the studied drugs from their different-degradates, as shown in (Figure 1, 2, 3 & 4). The Rs-values from main acid, alkaline and oxidative-degradates were always above

TABLE 10b : Determination of Ezetimibe in pharmaceutical formulations using the proposed HPLC and HPTLC methods and application of standard addition technique

Items							
Pharmaceutical formulation*				Standard addition technique			
Claimed		%Found±S.D.		HPLC		HPTLC	
HPLC	HPTLC	HPLC	HPTLC	Added (µg.ml ⁻¹)	% Recovery ^a	Added (µg.spot ⁻¹)	% Recovery ^a
				5.00	100.59	0.20	101.17
				10.00	100.39	0.40	99.40
10 mg		100.54 ±0.646	99.66 ±0.929	15.00	101.35	1.00	100.15
				20.00	101.64	2.00	99.24
				25.00	100.46	3.00	99.22
Mean					100.89		99.84
±R.S.D.					±0.569		±0.838

*Inegy[®] tablets (Batch no: NE16760) (labeled to contain 20 mg Simvastatin & 10mg Ezetimibe per tablet). ^aMean of four determinations

2, which ensured complete separation. Furthermore, the studied drugs were determined in solutions of laboratory prepared mixtures containing their acid-degradates by the proposed methods. The Recovery % and R.S.D. % proved the high specificity of these methods (TABLE 5, 6a & 6b).

Robustness and system suitability of the HPLC method

The robustness of an analytical procedure is a measure of its capacity to remain unaffected after slight but deliberate changes in the analytical conditions. Separation of the studied drugs from their different-degradates was performed under these conditions. There was slight decrease or increase in the Rs-values of all peaks. However, the calculated Rs-values were always above 2, ensuring complete separation. The system suitability parameters of HPLC method were evaluated^[47] (TABLE 7, 8a & 8b).

Standard addition technique

The proposed methods were applied for the determination of the studied drugs in the commercial tablets. The results shown in (TABLE 9, 10a & 10b), were satisfactory and with good agreement with the labeled amount. Moreover, to check the validity of the adopted proposed methods, the standard addition method was applied by adding known amounts of the studied drugs to the previously analyzed tablets. The recoveries were calculated by comparing the concentration obtained from

the spiked samples with that of the pure drug. The results of the commercial tablets analysis and the standard addition method (recovery study) (TABLE 9, 10a & 10b) suggested that there is no interference from any excipients, which are normally present in tablets.

Identification of acid and alkaline-degradates

(I) was influenced by the reaction with 0.1 M HCl for 2-hrs at 70°C giving two acid-degradates (II) and (III). First degradate is formed through the dehydration of the secondary alcoholic -OH at C₃, because secondary and tertiary alcohols can easily undergo dehydration by acid-catalyzed elimination reaction^[50]. While, second degradate is formed through intra-molecular esterification of (I), resulting in the lactone-form^[51-54].

Also, (I) was influenced by the reaction with 1.0 M NaOH for 3.5-hrs at 70°C giving the following alkaline-degradate that is formed through the cleavage of the ester linkage.

The identity of acid and alkaline-degradates was confirmed by separating these degradates on HPTLC plates and using chloroform: ethanol: glacial acetic acid (9.0: 1.0: 0.2 v/v/v) as a developing system, and then applying mass spectroscopy for each one. (Figure 5 and 6) show the parent peak at m/z 406 which is the molecular weight of each acid-degradate, while (Figure 7) shows the parent peak at m/z 338 which is the molecular weight of the alkaline-degradate. These results confirm the proposed mechanisms of the acid and alkaline-degradation.

The acid and alkaline-degradates of (II) exhibited the same R_f-values and the same retention times as mentioned under the HPLC and HPTLC methods. This concurs with previous reports in which only one-degradate was obtained after (II) hydrolysis. These reports concluded that, the degradate either from acid or alkaline hydrolysis, corresponds to the opening of the lactone-ring Simvastatin-hydroxy acid^[55].

(III) has β-lactam ring in its structure like penicillins, so it is very labile to alkaline hydrolysis giving one-degradate that corresponds to the opening of the lactam-ring. This degradate is also formed by acid hydrolysis but with slower rate, this was indicated by the appearance of a peak for one of the acid-degradates at the same R_f and R_t-values of the alkaline-degradate.

(III) was stable to 3 and 20% H₂O₂ at room tem-

perature for 24-hrs, and more drastic conditions (30% H₂O₂ at room temperature for 48-hrs and at 40°C for 5- hrs) were tried but it was stable.

CONCLUSION

The proposed methods are precise, specific, accurate and stability-indicating ones. Pravastatin sodium, Simvastatin and Ezetimibe can be determined in bulk powder and in pharmaceutical formulations without interference from excipients present, as well as in the presence of their different-degradates by the proposed HPLC and HPTLC methods. ICH-guidelines were followed throughout the study for method validation and the suggested methods can be applied for routine quality control analysis and stability studies.

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