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Short communication

Rapid and sensitive simultaneous determination of ezetimibe and simvastatin from their combination drug products by monolithic silica high-performance liquid chromatographic column

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ABSTRACT

A simple, precise and rapid high-performance liquid chromatography (HPLC) method has been developed and validated for the simultaneous determination of ezetimibe (EZE) and simvastatin (SIM) from their combination drug products. The applicability of monolithic LC phases in the field of quantitative analysis has been evaluated. The existing method with UV detection set at 240 nm was successfully transferred from a conventional silica column to a 10 cm × 4.6 mm i.d. monolithic silica column. By simply increasing the mobile phase flow rate, run time was about five-fold reduced and the consumption of mobile phase was about two-fold decreased, while the chromatographic resolution of the analytes remain unaffected. Ranitidine (RAN) was used as internal standard to guarantee a high level of quantitative performance. The method used a mobile phase consisted of acetonitrile–ammonium acetate (50 mM pH 5.0) (65:35, v/v). It was validated with respect to system suitability, specificity, limit of quantitation (LOQ) and detection (LOD), linearity, precision, accuracy, and recovery, respectively. The described method was linear over the range of 40–1200 ng ml^{-1} (r = 0.999) for both drugs. The LOD for EZE and SIM were 13.2 ± 0.4029 and 13.3 ± 0.4772 ng ml^{-1}, respectively. The LOQ were found to be 39.9 ± 1.221 and 39.5 ± 1.446 ng ml^{-1} for EZE and SIM, respectively. The method is fast (less than 2.0 min) and is suitable for high-throughput analysis of the drug and ones can analyze 700 samples per working day, facilitating the processing of large-number batch samples.

1. Introduction

Simvastatin (SIM; 2,2-dimethylbutanoic acid (1*S*,3*R*,7*S*,8*S*,8a*R*,)-1,2,3,7,8,8a-hexahydro-3,7-dimethyl-8-[2-[(2*R*,4*R*)-tetrahydro-4-hydroxy-6-oxo-2*H*-pyran-2-yl]ethyl]-1-naphthalenyl ester [1]) is a selective 3-hydroxy-3-methyl-glutaryl-coenzyme-A (HMG-CoA) reductase inhibitor [2]. SIM is a prodrug, following oral administration, is quickly hydrolyzed to its β-hydroxy acid which is an inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme-A (HMG-CoA) reductase, an essential enzyme involved in the in vivo synthesis of cholesterol. SIM is a highly effective lipid-regulating agent that is derived synthetically from a fermentation product of *Aspergillus terreus* [3]. It is widely used for the treatment of hypercholesterolemia [4–6].

However, administration of the highest approved statin dose offers only limited additional lowering of LDL cholesterol at the expense of an increased incidence of side effects [7]. Therefore, novel compounds that further reduce LDL cholesterol levels when added to statin therapy are of interest. A recently introduced compound, ezetimibe (EZE) (3*R*,4*S*)-1-(4-fluorophenyl)-3-(3*S*)-3-(4-fluorophenyl)-3-hydroxypropyl]-4-(4-hydroxyphenyl)-2-azetidinone [1]) is a novel lipid-lowering agent, that selectively inhibits cholesterol absorption by binding to the Niemann–Pick C1-like 1 (NPC1L1) protein. The latter is located at the brush-border membrane of the enterocyte, where it contributes substantially to the intestinal uptake and cellular transport of cholesterols and noncholesterol sterols [8,9]. Combined therapy of ezetimibe with a statin provides an incremental reduction in LDL cholesterol levels of 12–19% [10,11].

The ever-increasing need for speed and efficient use of time in the pharmaceutical and other fields places a demand for the development of faster higher throughput analytical procedures. The rapid trace level quantitative determination of drugs and their metabolites remains a challenge, which is often driven by the need for same-day turnaround of results from large numbers of biological samples [12]. For HPLC-based assays, the process of reducing analysis time while adequately resolving analytes from endogenous components is often accomplished with short columns packed with small particles. The theoretical advantages for small packing particles include higher optimum linear velocities as well as

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shallower slopes in the high velocity region of plate height versus linear velocity curves [13].

The utility of monolithic columns has been utilized to determine nicardipine and amlodipine in human plasma [14] and for determination of buprenorphine hydrochloride, naloxone hydrochloride and noroxymorphone in a tablet formulation by HPLC [15]. Also, these columns have been used to evaluate propranolol molecularly imprinted solid-phase microextraction fiber for trace analysis of β-blockers in urine and plasma samples [16]. Moreover, they have been used to determine bexarotene in plasma and the determination of dextromethorphan plus metabolites in urine [17]. In addition, monolithic columns have been used to determine dibucaine and naphazoline in human serum simultaneously [18] and methylphenidate with its de-esterified metabolite in rat plasma [12] and rofecoxib with its metabolites in human plasma [13]. Monolithic columns have been effectively used for simultaneous determination of acetoaminophen–caffeine–butalbital in human serum [19] and benzodiazepines in whole blood by HPLC [20]. High-throughput analysis of lamivudine in pharmaceutical preparations was achieved by using monolithic silica HPLC column [21].

Monolithic stationary phases have attracted considerable attention in liquid chromatography due to their simple preparation procedure, unique properties and excellent performance, especially for separation of drugs in biological samples and in pharmaceutical formulations [22]. As opposed to individual particles packed into chromatographic columns, monolithic supports are cast as continuous homogenous phases. They represent an approach that provides high rates of mass transfer at lower pressure drops as well as high efficiencies even at elevated flow rates. Therefore, much faster separations are possible and the productivity of chromatographic processes can be increased by at least one order of magnitude as compared to traditional chromatographic columns packed with porous particles. This enhances the speed of the separation process and reduces backpressure and unspecific binding without sacrificing resolution [23].

In the literature, there are a number of methods described for the determination of SIM in aqueous samples and human plasma including liquid chromatography (LC) [24], liquid chromatography-tandem mass spectrometry (LC-ESI-MS) [3,25–28], gas chromatography-mass spectrometry (GC–MS) [29], gas chromatography–mass spectrometry (GC–MS) [30], liquid chromatography–UV detection (LC–UV) [31,32] and LC with fluorescence detection [33] have been reported. Micellar electrokinetic chromatography [34], LC–UV [35,36], cerimetric reaction based on redox and complex formation [37] and UV spectrophotometry have been used to determine simvastatin in both pure and dosage forms [36,38,39] and LC/electrospray ionization tandem MS (LC/ESI-MS/MS) has been used for determination of SIM in aqueous samples [25]. A few methods have also been described for determination of EZE in pharmaceutical formulations and biological fluids including LC [40,41], LC-ESI-MS [42–45], in human plasma by LC/tandem MS [46] and a reversed-phase HPLC method for determination of the pharmaceutical form of the drug [47]. Liquid chromatography (LC) methods were developed and validated for the determination of EZE in pharmaceutical dosage forms in a C18 column with isocratic elution, and for stability studies with a C8 column using a gradient mode [41,47]. Bioanalytical methods have been described for the analysis of EZE and its metabolites in human plasma using LC–MS/MS with detection performed by negative electrospray ionization in the multiple reaction monitoring (MRM) modes, and in human serum, urine and feces with atmospheric pressure chemical ionization in the negative-ion mode [43,44,48,49]. The application of LC–MS/MS with detection in the positive electrospray ionization mode was also demonstrated for the determination of EZE in human plasma and pharmaceutical formulations.

The present study describes a rapid and sensitive HPLC method using a monolithic column with UV detection, which enables the determination of EZE and SIM with good accuracy. Separation was performed on a reversed-phase monolithic column, which has lower separation impedance compared to the particulate packings, and therefore, it allows easy optimizing chromatographic conditions to obtain desirable resolution in a short time. The developed method has been validated by evaluation of the system suitability, stability, specificity, linearity, limit of detection and quantitation, precision, accuracy, and recovery. The validated method was applied to the commercially pharmaceutical formulations containing both drugs.

2. Experimental

2.1. Chemicals and reagents

SIM, EZE and ranitidine, IS (RAN; N[2-[[[5-[(dimethylamino)methyl]-2-furanyl] methyl]thio]ethyl]-N-methyl-2-nitro-1,1-ethene-diamine) were purchased from Sigma (St. Louis, MO, USA). The purity of all chemicals was above 99%. Solvents were of HPLC grade and purchased from Merck (Darmstadt, Germany). Potassium dihydrogen orthophosphate was obtained from Merck (Darmstadt, Germany). HPLC-grade acetonitrile and methanol and analytical grade ammonium acetate were purchased from BDH chemicals (Poole, UK). Analytical-grade sodium hydroxide and hydrogen peroxide were purchased from WINLAB (UK). The pharmaceutical formulations containing 10/10 mg, 10/20 mg, 10/40 mg and 10/80 mg of EZE/SIM per tablet as a fixed dose combinations (Inegy®, Merck & Co., Inc., Whitehouse Station, NJ, USA (NYSE: MRK) and Schering-Plough Corporation) were purchased from international pharmacies.

2.2. Instrumentation

The LC method of analysis was carried out on a Waters HPLC system (Milford, MA 01757, USA) equipped with 1500 series HPLC pump, operated in isocratic mode to deliver the mobile phase at flow rate of 3.0 ml min^{-1}, a dual wavelength UV detector and an autosampler (717 plus). The peak areas and the rest data were integrated automatically by Dell computer. The pH of the solutions was measured by a pH meter (Thermo Orion Model 420 A, USA). Ultra pure water of 18 MΩ/cm was obtained from Milli-Q PLUS purification system, Millipore, Waters (Milford, MA, USA). All solutions were degassed by ultrasonication (Tecnal, São Paulo, Brazil) and filtered through a 0.22-μm Millex filter (Millipore).

2.3. Chromatographic conditions

Chromatographic separation was achieved using a reversed-phase Chromolith Performance (RP-18e, 100 mm × 4.6 mm) column from Merck (Darmstadt, Germany). The mobile phase consisted of acetonitrile mixed with 50 mM ammonium acetate in a ratio (65:35, v/v) was filtered and degassed using ultrasonic machine prior to use as mentioned above. A flow rate of 3.0 ml min^{-1} was applied in order to separate RAN (IS), EZE and SIM, respectively. All chromatographic experiments were conducted at ambient temperature.

2.4. Preparation of standard solutions

Stock standard solutions of EZE, SIM and RAN, 1.0 mg ml^{-1} were prepared by dissolving appropriate amounts of the compounds in acetonitrile. These solutions when stored in the dark at −20 °C were found to be stable for at least 4 weeks. A series of working standard solutions of EZE and SIM were prepared by subsequent dilution of the above-mentioned stock standard solution in the mobile phase

solution to reach concentration ranges of 40–1200 ng ml^{-1} for EZE and SIM. A 0.5 mg ml^{-1} working standard solution of the internal standard, RAN, was also prepared by serial dilution in the mobile phase solution. The working standard solutions were freshly prepared every week and stored in the dark at −20 °C.

2.5. Sample preparation

Not fewer than 20 tablets were weighed to obtain the average tablet weight and were then powdered. A sample of the powdered tablets, claimed to contain 10 mg of EZE and 10 mg SIM was mixed with 10 mg of RAN (Internal Standard) in a 10 ml volumetric flask. The contents were dissolved in the volume that was made up with acetonitrile. This mixture was sonicated for 15 min and diluted to mark with the same solvent. An aliquot was then removed and centrifuged at 5000 rpm for 20 min and was then filtered through a 0.45 μm membrane filter. An aliquot of this solution (1.0 ml) was transferred to a volumetric flask and made up to a sufficient volume with mobile phase to get a concentration of 100 μg ml^{-1}. From this, 20 μg ml^{-1} of the solution was prepared. Various volumes of this aliquot were diluted to get concentrations between 80 and 120% of test concentration with mobile phase. All determinations were conducted in triplicate. The same procedure was used to estimate the concentration of the drug in three different strengths of EZE and SIM tablets.

3. Results and discussion

3.1. Method development

Drug quality control, stability, metabolism, pharmacokinetics, and toxicity studies all necessitate the determination of drugs in pharmaceutical formulations and biological samples. Likewise, efficient and validated analytical methods are very critical requirements for all these investigations Fig. 1.

Chromatographic parameters were preliminary optimized to develop a LC method for simultaneous determination of EZE and SIM with short analyses time (<1.8 min), and acceptable resolution (R_s > 1.52). The polarity of EZE and SIM differ greatly, as EZE is less lipophilic than SIM and their log P were found 3.496 ± 0.615 and 4.415 ± 0.409, respectively. The mobile phase was acetonitrile–ammonium acetate buffer [0.05 M] of pH 5.0 (65:35, v/v). We tried to add a small quantity of methanol (0.5 ml) to enhance the resolution between analytes especially EZE and RAN then later on we excluded it due to the result of increasing retention time of SIM (>2.0 min). Retention time of SIM decreased rapidly with the increase of acetonitrile content. The candidate IS included ranitidine, phenytoin, sulfathiazole, metronidazole, theophylline, vitamins B1 and B6. Based on the chromatographic performance of each compound, ranitidine was selected as the best internal standard for the analyses. The UV spectra of both drugs exhibit an overlapping of their UV absorbance at 231–232 nm and at 238.5–240 nm. The optimum wavelength of 240 nm (Fig. 2) represents the wavelength of sufficient absorbance of EZE and SIM drugs and was selected in order to permit the simultaneous determination in fixed dose combinations. Peak areas were measured for the quantitation of the analytes.

Optimum separation of EZE and SIM was carried out by isocratic elution using acetonitrile–ammonium acetate buffer (0.05 M) of pH 5.0 (65:35, v/v) with a flow rate of 3 ml min^{-1} (Table 1). Under the chromatographic conditions selected above, retention time of IS, EZE and SIM were 0.5544 ± 0.00055, 0.7101 ± 0.002345 and 1.629 ± 0.001 min, respectively. The retention factor (k') for EZE and SIM were 1.19 and 5.30, respectively, which indicates a good resolution between the two drugs under

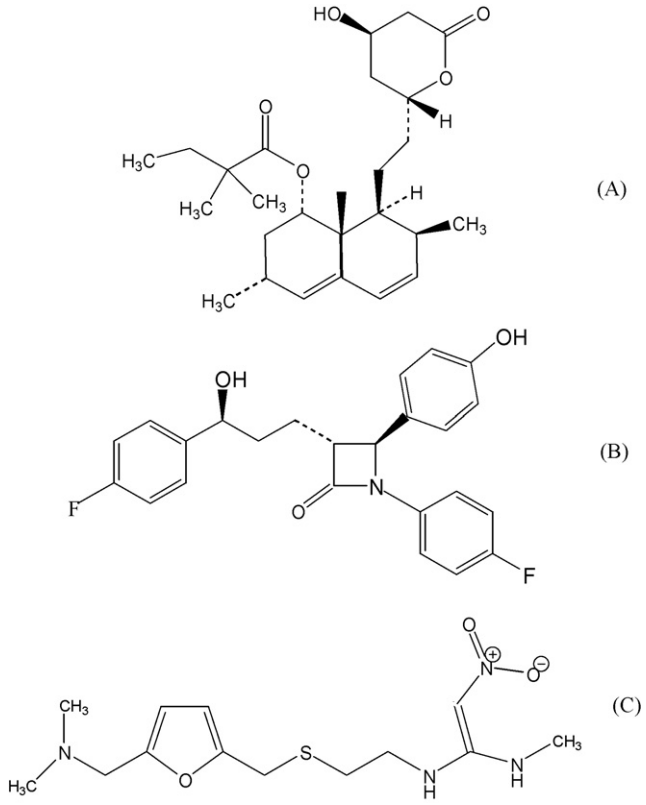

Fig. 1. The chemical structure of: (A) simvastatin, (B) ezetimibe, and (C) ranitidine (IS).

investigation. The HPLC run time for each sample was less than 1.80 min.

In the present study, the flow rate was studied starting from 1.0 to 3.0 ml min^{-1} and we found that the resolution between EZE and IS were not affected excessively as the flow rate increased with a minimum effect on the pump pressure (≈1600 psi). Significant chromatographic parameters have been evaluated and were summarized in Table 1. It appeared that the proposed method was appropriate for regular pharmaceutical applications. As shown in Table 1 retention time of each analyte was very repeatable with relative standard deviations between 0.3303 and 0.0614% (n = 10). The peak area responses were also repeatable with relative standard deviations between 0.1417 and 2.1751% (n = 10).

3.2. Method validation

3.2.1. System suitability

In order to determine the adequate resolution and repeatability of the proposed method, suitability parameters including retention

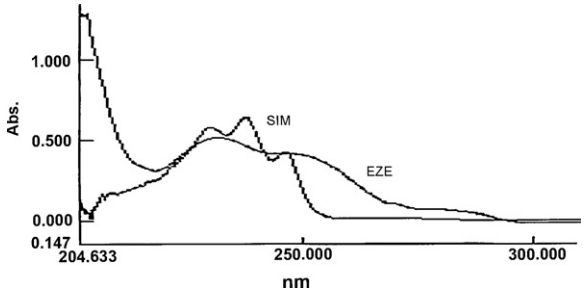

Fig. 2. Overlaid UV spectra of SIM and EZE (10 μg ml^{-1} each).

Table 1
System suitability test parameters for SIM, EZE and RAN by the proposed method.

System suitability test parameters	RAN	EZE	SIM
Retention time (min) (mean ± S.D., $n = 10$)	0.5544 ± 0.00055	0.7101 ± 0.00235	1.629 ± 0.00100
Repeatability of retention time; R.S.D.[a]% ($n = 10$)	0.0989	0.3303	0.0614
Repeatability of peak area; R.S.D.% = (S.D./mean) x 100	0.0033	2.1751	0.1417
Resolution (R_s)[b]	–	1.58	12.3
Tailing factor (asymmetric factor)[c]	1.13	1.18	0.94
Retention factor (k')[d]	0.70	1.19	5.30
Selectivity factor (α)[e]	1.70	4.45	Y[f]

[a] R.S.D.% = (S.D./mean) × 100.
[b] $R_s = 2\,(t_2 - t_1)/(w_{b2} + w_{b1})$. Where t_2 and t_1 are the retention of the second and first peaks w_{b2} and w_{b1} are the peaks widths of the second and first peaks.
[c] Calculated at 5% peak height.
[d] $k' = (t_r - t_0)/t_0$, where t_r is the retention of analytes and t_0 is the column dead-time.
[e] Separation factor, calculated as k_2/k_1.
[f] Y, not calculated.

factor, selectivity, resolution and asymmetry factor were investigated and the results were abridged in Table 1.

3.2.2. Specificity

The specificity of an analytical method may be defined as the ability to obviously determine the analyte in the presence of additional components such as impurities, degradation products and matrix [49–51]. A solution of analytical placebo (containing all the tablet excipients except EZE and SIM) was prepared according to the sample preparation procedure and injected. To identify the interference by these excipients, a mixture of inactive ingredients (placebo), standard solutions, and the commercial pharmaceutical preparations including EZE and SIM were analyzed by the developed method (Fig. 3A and B). The representative chromatograms did not show any other peaks, which confirmed the specificity of the method.

The specificity of the method was also evaluated to ensure there were no interference products resulting from forced degradation.

3.2.2.1. Forced degradation studies. A stock solution containing 5.0 mg of each EZE and SIM and mixture of them in 5.0 ml acetonitrile were prepared. These solutions were used for forced

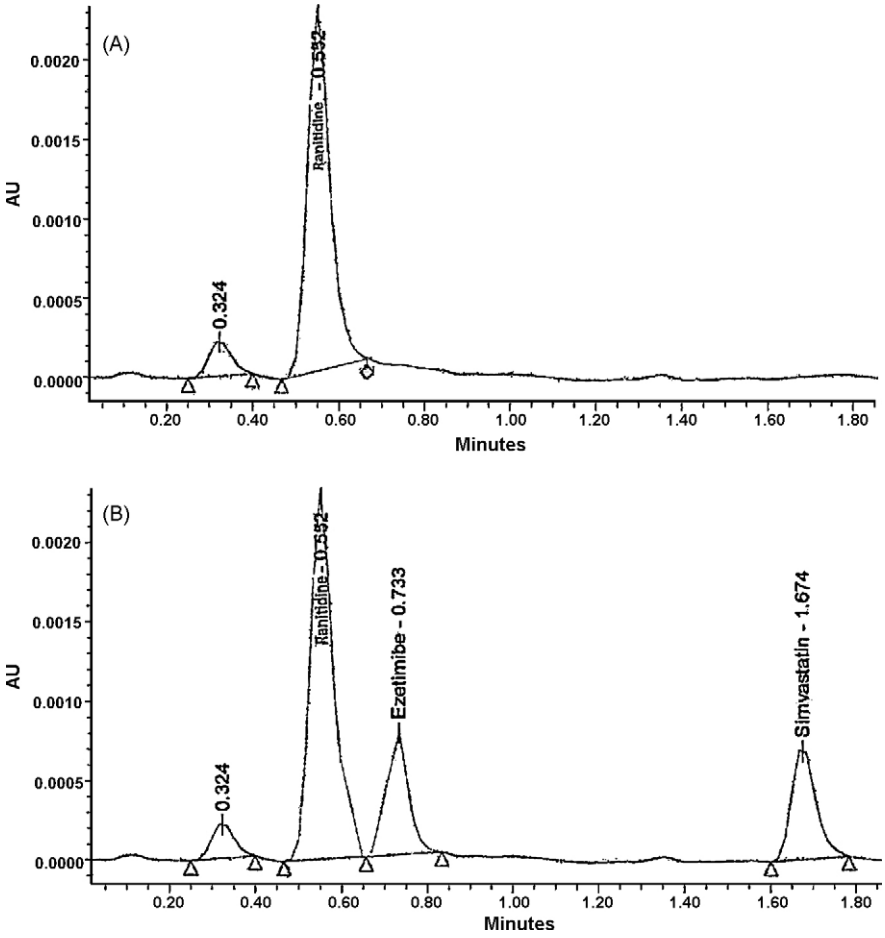

Fig. 3. (A) A representative chromatogram of the tablets blank (placebo) extract spiked with (0.5 μg ml^{-1}) of the IS; RAN. Chromatographic conditions: reversed-phase Chromolith Performance (RP-18e, 100 mm × 4.6 mm) column; mobile phase: acetonitrile–ammonium acetate buffer (50 mM, pH 5.0) (65:35, v/v), flow rate; 3.0 ml min^{-1}, UV detection with λ_{max} = 240 nm. (B) A representative chromatogram of the tablets extract spiked with 40 ng ml^{-1} EZE and SIM and 0.5 μg ml^{-1} of the IS; RAN. Chromatographic conditions were as in (A).

Table 2
Analytical parameters for determination of EZE and SIM by HPLC using monolithic column.

Analyte	Concentration range (ng ml^{-1})[a]	r[b]	S.D.[c]		Sr[d]	$\alpha/S\alpha$[e]
			Slope $\times 10^{-6}$	Intercept $\times 10^{-3}$		
SIM	Run 1					
	40.0–1200	0.9991	4.65	2.40	0.00507	1.57
	Run 2					
	40.0–1200	0.9995	4.54	2.33	0.00507	1.64
	Run 3					
	40.0–1200	0.9994	4.37	2.24	0.00489	1.70
EZE	Run 1					
	40.0–1200	0.9992	3.59	2.49	0.00544	2.09
	Run 2					
	40.0–1200	0.9992	3.55	2.39	0.00521	2.05
	Run 3					
	40.0–1200	0.9993	3.86	2.49	0.00543	2.05

[a] Ratio of the peak area amplitude of the analytes to that of the internal standard versus the corresponding concentration.
[b] Correlation coefficient.
[c] Standard deviation of slope and intercept.
[d] Standard error of the estimate.
[e] Theoretical value of t at $p = 0.05$ level of significance, for $f = n - 2 = 9$ df, 2.62.

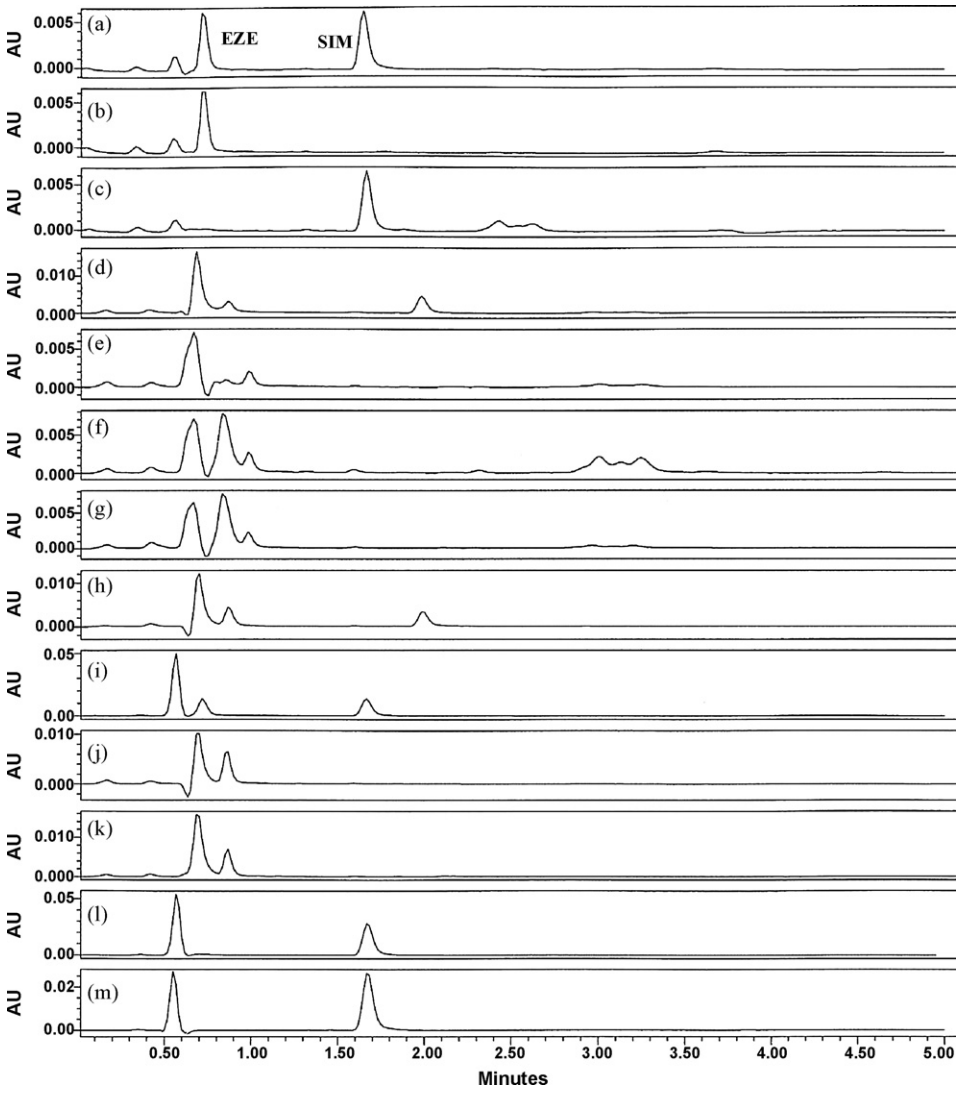

Fig. 4. Overlaid chromatograms obtained from: (a) ezetimibe (EZE) standard, simvastatin (SIM) standard, (b) EZE treated with 0.1 M HCl, (c) SIM treated with 0.1 M HCl, (d) EZE and SIM treated with 0.1 M HCl, (e) EZE treated with 0.1 M NaOH, (f) SIM treated with 0.1 M NaOH, (g) EZE and SIM treated with 0.1 M NaOH, (h) EZE and SIM treated with 3.0% H_2O_2, (i) EZE and SIM treated with 30.0% H_2O_2, (j) EZE powder treated under dry heat, (k) EZE powder treated under wet heat, (l) SIM powder treated under dry heat, and (m) SIM powder treated under wet heat. All of the above mentioned forced degradation experiments were done at room temperature for 2 h each with exception for 3 and 30% H_2O_2 which done for 6 and 24 h, respectively.

degradation to provide an indication of specificity of the proposed method. In all degradation studies (acid- and base-induced degradation, hydrogen peroxide, dry and wet heat degradation) the average peak areas of EZE and SIM after injection of (1000 ng ml^{-1}) of six replicates were obtained.

3.2.2.1.1. Acid-induced degradation. Acid treatment (0.1 M HCl) for 2 h at room temperature did not result in any degradation of EZE but two degradation products were observed for SIM at retention time of 2.41 and 2.62. Similar results were obtained when a mixture of EZE and SIM was treated with 0.1 M HCl. Fig. 4(b–d) shows the chromatograms obtained from acid-treated samples of SIM, EZE, and their mixture. It was reported that, the rate of hydrolysis of EZE was slower when treated with 1 M HCl for 8 h [41], and the lactone ring of SIM is readily hydrolyzed to form a beta hydroxyl acid [53].

3.2.2.1.2. Base-induced degradation. EZE was completely degraded in 0.1 M NaOH with two peaks of degradation products at retention times of 0.6 and 0.9 min. Treatment with 0.1 M NaOH for 2 h at room temperature resulted in partial degradation of SIM. The degradation products, at retention times of 0.9, 1.1, 2.8 and 3.10 min, were well resolved from the main SIM peak. Similar results were also observed when a mixture of SIM and EZE was degraded under alkaline condition (0.1 M NaOH). Fig. 4(e–g) shows chromatograms obtained from samples of SIM, EZE, and their mixture treated with 0.1 M NaOH.

3.2.2.1.3. Hydrogen peroxide-induced degradation. To study hydrogen peroxide-induced degradation, initial studies were performed in 3% hydrogen peroxide at room temperature for 6 h. Subsequently, the drugs were exposed to 30% hydrogen peroxide at room temperature for a period of 24 h and then heated in a boiling water bath for 10 min to completely remove the excess of hydrogen peroxide. Both EZE and SIM were stable in hydrogen peroxide at room temperature as shown in Fig. 4(h and i).

3.2.2.1.4. Dry and wet heat-induced degradation. SIM and EZE drugs powder were placed in oven at 70 °C for 2 h to study the dry heat degradation. A solution of 1000 μg ml^{-1} of SIM and EZE was prepared from the dry heat-degraded sample and the chromatogram was run as described above. No additional peaks of degradation products were observed Fig. 4(j and l). The stability of the sample solutions of EZE and SIM were tested over a period of 7 days. The freshly prepared solution at room temperature and the 7-day stored samples at 30, 50 and 70 °C were analyzed by the proposed HPLC method. The concentrations of EZE and SIM in the stored samples were calculated and compared to that present in the freshly prepared sample. From these results we can conclude that there are no degradation products at elevated temperature and the drugs are stable at 30, 50 and 70 °C for at least 7 days, indicating the possibility of using EZE and SIM samples over a period of 7 days at 70 °C without degradations.

3.2.3. Limits of detection and quantitation

The LOD was calculated using the equations $y - \alpha = 3.3 \times S\alpha$ and $y - \alpha = b \times \text{LOD}$, while the limit of quantitation, LOQ, was attained using the equations $y - \alpha = 10 \times S\alpha$ and $y - \alpha = b \times \text{LOQ}$ (where b is the slope and $S\alpha$ is the standard deviation of the intercept of the regression line) [52]. In particular, LOD and LOQ were calculated taking under consideration data obtained from the calibration equations presented in the previous table (Table 2). Average values of LOD of SIM and EZE were found to be 13.3 ± 0.4772 and 13.2 ± 0.4029 ng ml^{-1}, while average values of LOQ for them were 39.5 ± 1.446 and 39.9 ± 1.221 ng ml^{-1}, respectively.

A Student's t-test was performed to determine whether the experimental intercepts (α) of the regression equations were significantly different from the theoretical zero value. The test is based on the calculation of the quantities $t = \alpha/S\alpha$, where α is the intercept of the regression equations and $S\alpha$ is the standard deviation of α, and their comparison with tabulated data of the t-distribution. The calculated t-values are also presented in Table 2; these values do not exceed the 95% criterion of $t_p = 2.62$ for $f = 9$ degrees of freedom, which denotes that the intercept of all regression lines are not significantly different from zero.

3.2.4. Linearity

Eleven working solutions for each analyte in the range of 40–1200 ng ml^{-1} for EZE, and SIM were simultaneously prepared. All solutions contained 5 μg ml^{-1} IS except the LOQ ones which had 0.5 μg ml^{-1}. Each solution was injected in five replicates. The linear regressions analysis of SIM and EZE were constructed by plotting the peak area of the analytes to the internal standard (y) versus analytes concentration (ng ml^{-1}) in (x) axis. The calibration curves were linear in the range of 40–1200 ng ml^{-1} for each analyte, with a mean correlation coefficient (r) of more than 0.9993 and 0.9992 for SIM and EZE, respectively. A typical calibration curve has

Table 3
Intra-day and inter-day precision and accuracy results of EZE and SIM ($n = 6$).

Analyte	Actual conc. (ng ml^{-1})	Found conc[a]. (ng ml^{-1})	% Recovery	% R.S.D.[b]	% Error[c]
(a) Within-day					
SIM	70	$69.170 \pm 2.81 \times 10^{-4}$	98.82	4.1×10^{-4}	−1.186
	200	$200.75 \pm 5.19 \times 10^{-4}$	100.4	2.6×10^{-4}	0.375
	550	$554.53 \pm 1.71 \times 10^{-3}$	100.8	3.1×10^{-4}	0.824
	1000	$1015.9 \pm 2.97 \times 10^{-3}$	101.6	2.8×10^{-4}	1.590
EZE	70	$69.674 \pm 3.56 \times 10^{-4}$	99.53	5.1×10^{-4}	−0.466
	200	$203.19 \pm 1.80 \times 10^{-3}$	101.6	8.9×10^{-4}	1.595
	550	$554.53 \pm 2.76 \times 10^{-3}$	100.8	4.9×10^{-4}	0.824
	1000	$1005.3 \pm 1.15 \times 10^{-2}$	100.5	1.1×10^{-3}	0.530
(b) Between-day[b]					
SIM	70	$67.606 \pm 5.68 \times 10^{-4}$	96.58	8.4×10^{-4}	−3.42
	200	$201.18 \pm 6.12 \times 10^{-4}$	100.6	3.0×10^{-4}	0.59
	550	$555.07 \pm 1.53 \times 10^{-3}$	100.9	2.8×10^{-4}	0.922
	1000	$1018.8 \pm 2.22 \times 10^{-3}$	100.2	2.2×10^{-4}	1.88
EZE	70	$69.292 \pm 5.68 \times 10^{-4}$	98.99	8.2×10^{-4}	−1.011
	200	$202.78 \pm 1.902 \times 10^{-3}$	101.4	9.4×10^{-4}	1.39
	550	$554.67 \pm 2.77 \times 10^{-3}$	100.9	4.9×10^{-4}	0.849
	1000	$1003.6 \pm 1.06 \times 10^{-2}$	100.4	1.1×10^{-3}	0.360

[a] Mean ± S.D.
[b] (S.D./mean) × 100.
[c] [found conc. − actual conc./actual conc.] × 100.

Table 4
Assay results of combined dosage forms using the proposed HPLC method.

Formulation[a]	Labeled amount (mg/tab)		Amount obtained (mean of mg/tab) ± S.D.		% Recovery	
	EZE	SIM	EZE	SIM	EZE	SIM
A	10	10	9.840 ± 0.3285	9.910 ± 0.135	98.40	99.13
B	10	20	9.895 ± 0.0625	20.02 ± 0.0822	98.95	100.07
C	10	40	10.01 ± 0.0753	39.52 ± 0.111	99.95	98.79
D	10	80	9.980 ± 0.0787	100.02 ± 0.593	99.77	100.02

[a] Tablets are product of Inegy®, Merck & Co., Inc., Whitehouse Station, NJ, USA (NYSE: MRK) and Schering-Plough Corporation.

the mean of regression equations of $y = 0.00059 \times C + 0.00379$ and $y = 0.00062 \times C + 0.00507$ for SIM and EZE, respectively. The mean values (±S.D., $n = 11$) of correlation coefficient, slope, and intercept were summarized in Table 2.

3.2.5. Precision

The precision of the method was evaluated in terms of intermediate precision (intra-day and inter-day) [49–51]. Three different concentrations of EZE and SIM were analyzed in six independent series during the same day (intra-day precision) and six consecutive days (inter-day precision), within each series every sample was injected in triplicate. The R.S.D. values of intra- and inter-day studies for EZE and SIM showed that the precision of the method was satisfactory (Table 3).

3.2.6. Accuracy

The accuracy of an analytical method expresses the nearness between the reference value and found value [49–51]. Accuracy was evaluated as percentage relative error between the found mean concentrations and added concentrations for EZE and SIM. The results obtained are shown in Table 3, from which it is clear that accuracy is excellent for both active ingredients.

4. Application of the LC method for the analysis of commercial formulations

Evaluation of pharmaceutical formulations was performed by using the calibration curve method, since no significant differences between the slopes of the standard calibration curve to that for tablet were observed. Each pharmaceutical preparation was analyzed by performing six independent determinations and each series were injected three times. The same procedure was used to estimate the concentration of the drug in three different strengths of EZE/SIM combined dosage forms (Table 4). The tablet analyses results were abridged in Table 4 for EZE and SIM with their percentage recoveries (99.50% for SIM and 100.34% for EZE). The results indicated that the proposed method was reliable for quantification of SIM and EZE in pharmaceutical formulations. In addition, the slope of the standard calibration curve ($5.887 \times 10^{-4} \pm 2.867 \times 10^{-6}$ for SIM, $6.013 \times 10^{-4} \pm 5.793 \times 10^{-6}$ for EZE) was compared with the slope of the tablets calibration curve ($5.877 \times 10^{-4} \pm 4.028 \times 10^{-6}$ for SIM, $6.15 \times 10^{-4} \pm 4.082 \times 10^{-6}$ for EZE). It was found that there were no significant differences between the slopes which indicated that excipients did not interfere with SIM and EZE.

5. Conclusions

A simple, rapid, and accurate LC method was developed for the simultaneous determination of EZE and SIM in pharmaceutical formulations by isocratic mode using monolithic column. The analytical conditions and the solvent system developed provided good resolution for the analytes (RAN, EZE and SIM) within a short analysis time. The LC method was validated and demonstrated good linearity, precision, accuracy and specificity. Thus, the developed LC method can be proposed for routine analysis laboratories and quality control purposes because of the speed of analysis and simple extraction procedure. Owing to use of the monolithic column, which has lower separation impedance compared to the particulate packings, much faster separations are possible and the productivity of chromatographic processes can be increased by at least one order of magnitude as compared to traditional chromatographic columns packed with porous particles. Accordingly, the chromatographic elution step is undertaken in a short time (<1.80 min) with good resolution.

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