DRUG FORMULATIONS AND CLINICAL METHODS

Stability-Indicating Reversed-Phase Liquid Chromatographic Method for Simultaneous Determination of Simvastatin and Ezetimibe from Their Combination Drug Products

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A simple, precise, and rapid stability-indicating reversed-phase column liquid chromatographic (RP-LC) method has been developed and subsequently validated for simultaneous estimation of simvastatin (SIM) and ezetimibe (EZE) from their combination drug product. The proposed RP-LC method utilizes a LiChrospher® 100 C_{18}, 5 μm, 250 × 4.0 mm id column at ambient temperature; optimum mobile phase consisting of acetonitrile–water–methanol (60 + 25 + 15, v/v/v) with apparent pH adjusted to 4.0 ± 0.1; mobile phase flow rate of 1.5 mL/min; and ultraviolet detection at 238 nm. SIM, EZE, and their combination drug product were exposed to thermal, photolytic, hydrolytic, and oxidative stress conditions, and the stressed samples were analyzed by the proposed method. There were no other coeluting, interfering peaks from excipients, impurities, or degradation products due to variable stress conditions, and the method is specific for the estimation of SIM and EZE in the presence of degradation products. The described method was linear over the range of 1–80 and 3–80 μg/mL for SIM and EZE, respectively. The mean recoveries were 99.17 and 100.43% for SIM and EZE, respectively. The intermediate precision data were obtained under different experimental conditions, and the calculated value of the coefficient of variation was found to be less than the critical value. The proposed method can be useful in the quality control of bulk manufacturing and pharmaceutical dosage forms.

Simvastatin (SIM; 1) is a synthetic lipid-lowering agent that inhibits 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG–CoA) reductase; it is an inactive pro-drug that undergoes in vivo hydrolysis in order to produce its effects. Ezetimibe (EZE; 2) inhibits the absorption of cholesterol, decreasing the delivery of intestinal cholesterol to the liver. The combination (3) drug product of SIM and EZE, which has recently been introduced in the market, increases the likelihood of therapeutic success in patients with dyslipidemia. Chemically, SIM is butanoic acid, 2,2-dimethyl-,1,2,3,7,8,8a-hexahydro-3,7-dimethyl-8-[2-(tetrahydro-4-hydroxy-6-oxo-2*H*-pyran-2-yl)-ethyl]-1-naphthalenyl ester, [1*S*-[1α,3α,7β,8β,(2*S**,4*S**),-8aβ]] (4), and EZE is (3*R*,4*S*)-1-(4-flurophenyl)-3-[(3*S*)-3-(4-flurophenyl)-3-hydroxypropyl]-4-(4-hydroxyphenyl)-2-azetidinone (5).

Stability testing is an important part of the process of drug product development. The purpose of stability testing is to provide evidence on how the quality of a drug substance or drug product varies with time under the influence of a variety of environmental factors, such as temperature, humidity, and light, and enables recommendation of storage conditions, retest periods, and shelf lives to be established. The 2 main aspects of a drug product that play an important role in shelf life determination are assay of the active drug and degradation products generated during the stability study. The drug product in a stability test sample needs to be determined using a stability-indicating method, as recommended by the International Conference on Harmonization (ICH) guidelines (6) and U.S. Pharmacopeia (USP) 26 (7). Although stability-indicating methods have been reported for assay of various drugs in drug products, most of them describe assay procedures for drug products containing only one active drug substance. Only few stability indicating methods are reported for assay of combination drug products containing 2 or more active drug substances. The objective of this work was to develop a simple, precise, and rapid column liquid chromatographic (LC) procedure that would serve as a stability-indicating assay method for combination drug product of SIM and EZE.

USP 28 (8) describes an LC method for determination of SIM, but it does not involve simultaneous determination of EZE. Detailed survey of the literature for SIM revealed several methods based on different techniques, viz, LC (9–11) and LC/tandem mass spectrometry (LC/MS/MS; 12) for its determination in human plasma, UV spectrometry (13) for its determination in human serum and pharmaceutical

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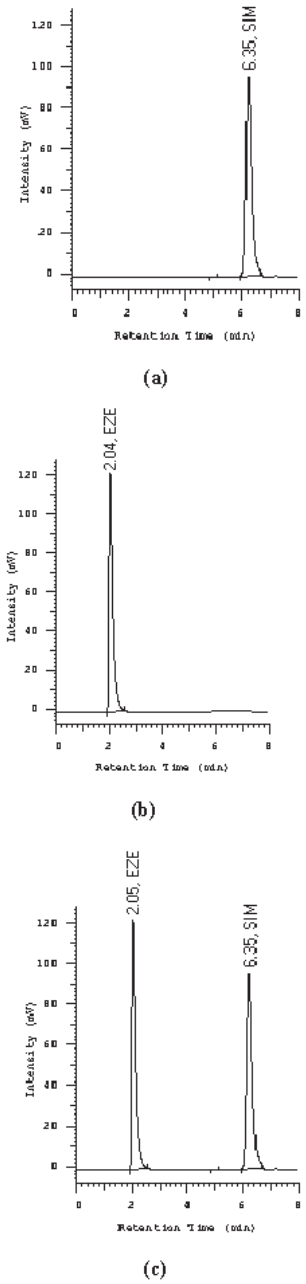

Figure 1. Chromatograms of untreated (a) SIM, (b) EZE, and (c) tablet solutions having concentrations of 40 μg/mL for both the drugs.

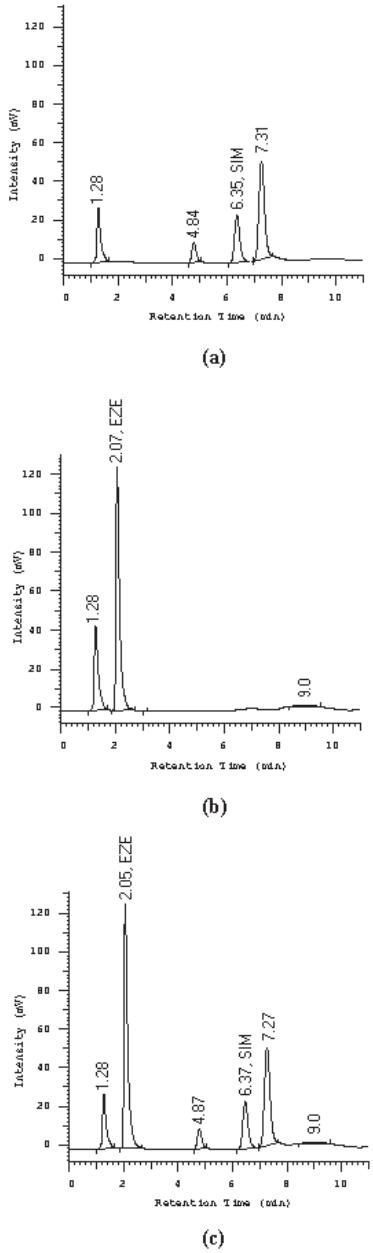

Figure 2. Chromatograms of acid hydrolysis-degraded (a) SIM, (b) EZE, and (c) tablet solutions having concentrations of 40 μg/mL for both the drugs.

formulations, LC (14–16) for its determination from pharmaceuticals in combination with other drugs, and UV spectrometry (17) for its determination in pharmaceuticals. Similarly, survey of literature for EZE revealed methods based on LC (18) for its determination in pharmaceuticals and LC/MS/MS (19, 20) for its determination in human plasma or serum. A stability-indicating LC method with gradient elution has been reported for determination of EZE (21). None of the reported analytical procedures describes a stability-indicating method for simultaneous determination of SIM and EZE in the presence of their degradation products.

This paper describes the development and subsequent validation (22) of a stability-indicating isocratic reversed-phase LC (RP-LC) method for simultaneous determination of SIM and EZE in the presence of their degradation products. To establish the stability-indicating nature of the method, forced degradation of drug substances and drug product was performed under stress conditions (thermal, photolytic, acid/base hydrolytic, and oxidative), and stressed samples were analyzed by the proposed method. The proposed LC method was able to separate both drugs from degradation products generated during forced degradation

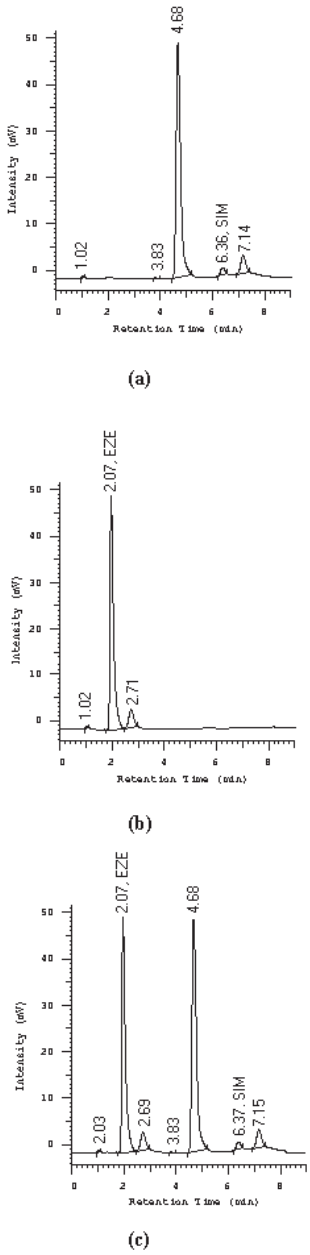

Figure 3. Chromatograms of base hydrolysis-degraded (a) SIM, (b) EZE, and (c) tablet solutions having concentrations of 40 μg/mL for both the drugs.

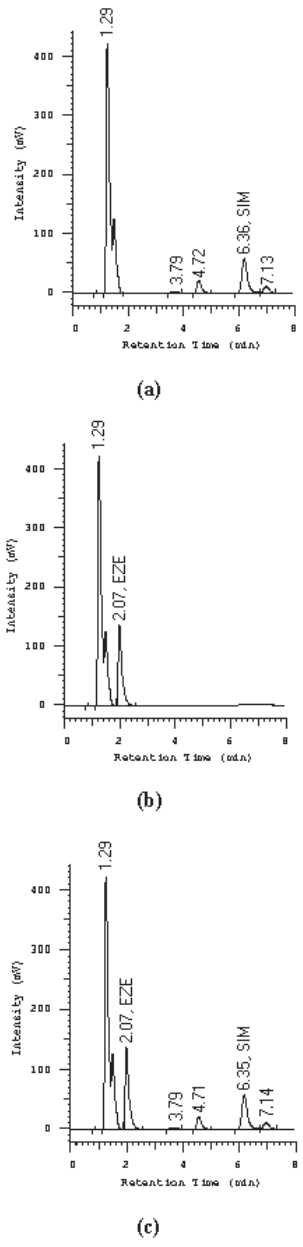

Figure 4. Chromatograms of oxidative-degraded (a) SIM, (b) EZE, and (c) tablet solutions having concentrations of 40 μg/mL for both the drugs.

studies. The linearity of response, accuracy, and intermediate precision of the described method for assay of SIM and EZE has been validated.

Experimental

Chemicals and Reagents

SIM and EZE working standards were generous gifts from Torrent Research Centre, Ahmedabad, India. Combination drug products of SIM and EZE (label claim SIM 10 mg and EZE 10 mg) and Simvas EZ Tablets (Micro Labs Ltd., Bangalore, India) were purchased from a local pharmacy. Rankem acetonitrile, methanol, and water (Ranbaxy Fine Chemicals Ltd., New Dehli, India) were of LC grade. *o*-Phosphoric acid used was of analytical reagent grade (S.D. Fine Chemicals Ltd., Mumbai, India). Sodium hydroxide, hydrochloric acid, and hydrogen peroxide were from Qualigens Fine Chemicals (GlaxoSmithKline, Mumbai, India).

LC Instrumentation and Conditions

The LC system consisted of a Hitachi (San Jose, CA) pump L-7110, Rheodyne (Cotati, CA) universal injector 77251, and Hitachi L-7420 UV-Vis detector. Chromatographic separations were performed using a LiChrospher® 100 C_{18},

Table 1. Results of forced degradation study of samples using the proposed method, indicating percentage degradation of SIM and EZE

	Degradation, %	
Stress condition/duration/state	SIM	EZE
Thermal/80°C/48 h/solid	10.72	5.48
Photo/UV 254 and 366 nm/48 h/solid/room temperature	15.17	10.21
Acidic/0.5 M HCl/48 h/solution/room temperature	74.8	4.11
Alkaline/0.01 M NaOH/0 h/solution/room temperature	94.92	7.91
Oxidative/3.0% H_2O_2/48 h/solution/room temperature	46.46	13.7

5 μm, 250 × 4.0 mm id column (Merck, Darmstadt, Germany) at ambient temperature, eluted with mobile phase at a flow rate of 1.5 mL/min. The mobile phase consisted of acetonitrile–water–methanol (60 + 25 + 15, v/v/v); the apparent pH was adjusted to 4.0 ± 0.1 with 10% phosphoric acid solution. The mobile phase was filtered through a 0.45 μm nylon filter and degassed in an ultrasonic bath prior to use. The detection wavelength was selected by scanning standard solutions of both drugs from 200 to 400 nm using a Shimadzu (Columbia, MD) Model 1601 double-beam UV-Vis spectrophotometer with a pair of 10 mm matched quartz cells. Measurements were made with an injection volume of 20 μL and UV detection at 238 nm, as both components showed reasonably good response at this wavelength.

Standard and Sample Preparation

The standard stock solutions containing 1 mg/mL each of SIM and EZE were prepared separately by dissolving working standards in methanol and diluting with the same solvent. Aliquots (10 mL) from the above standard stock solutions of SIM and EZE were transferred into a 100 mL volumetric flask, and the volume was made up to the mark with mobile phase to prepare a mixed standard stock solution having a concentration of 100 μg/mL of each drug. From this solution, aliquots of 0.1, 0.3, 0.5, 1, 2, 3, 4, 5, 6, 7, and 8 mL were transferred into different 10 mL volumetric flasks, and the volumes were made up to mark with mobile phase to obtain mixed standard calibration solutions of SIM and EZE having concentrations in the range of 1–80 μg/mL.

Analysis of Dosage Forms

Twenty tablets were weighed, their mean weight was determined, and they were crushed in mortar. An amount of powdered mass equivalent to 25 mg each of SIM and EZE was weighed and transferred into a conical flask. The drugs from the powder were dissolved and extracted with 15 mL methanol. To ensure complete extraction of the drugs, the solution was sonicated for 30 min. The extract was filtered through Whatman No. 41 paper (Gelman Laboratory, Mumbai, India), and the residue was washed with 7 mL methanol. The extract and washing were pooled and transferred to a 25 mL volumetric flask, and the volume was made up to the line with methanol. A 5 mL aliquot from this solution was transferred into a 50 mL volumetric flask, and the volume was adjusted with mobile phase up to mark (sample stock solution). From the sample stock solution, 2 mL aliquots were suitably diluted with mobile phase to achieve concentrations of 20 μg/mL for both drugs and used for injection onto the LC column.

Procedure for Forced Degradation Study

Forced degradation of each drug substance and the drug product was carried out under thermolytic, photolytic, acid/base hydrolytic, and oxidative stress conditions. Thermal and photodegradation of drug substances and drug product were carried out in the solid state. After the degradation, stock solutions were prepared by dissolving in methanol to achieve a concentration of 1 mg/mL. From these solutions, aliquots were diluted with mobile phase to achieve a concentration of 40 μg/mL each of SIM and EZE.

For hydrolytic and oxidative degradation, solutions were prepared in 50 mL volumetric flasks by dissolving 10 mg of drug substance or drug product in 10 mL methanol and diluting with methanolic solutions of hydrochloric acid, sodium hydroxide, or hydrogen peroxide solution to achieve a concentration of 200 μg/mL each of SIM and EZE. After the degradation, aliquots from these solutions were diluted with mobile phase to achieve a concentration of 40 μg/mL each of SIM and EZE. Based on the labeled strength of SIM and EZE in tablets, the nominal concentrations of SIM and EZE in solution were 40 μg/mL.

For thermal stress, samples of drug substances and drug product were placed in a controlled-temperature oven at 80°C for 48 h. For photolytic stress, samples of drug substances and drug product, in solid state, were irradiated for 48 h at 254 and 366 nm in a cabinet with a dual-wavelength UV lamp (Camag, Muttenz, Switzerland).

Acid hydrolysis of drug substance and drug product in solution state was conducted with 0.5 M hydrochloric acid at ambient temperature (25°C) for 48 h. During the initial forced degradation experiments, it was observed that basic hydrolysis was a fast reaction for both drugs, and almost complete degradation occurred when 0.5 M sodium hydroxide solution was used. In the next experiment, SIM

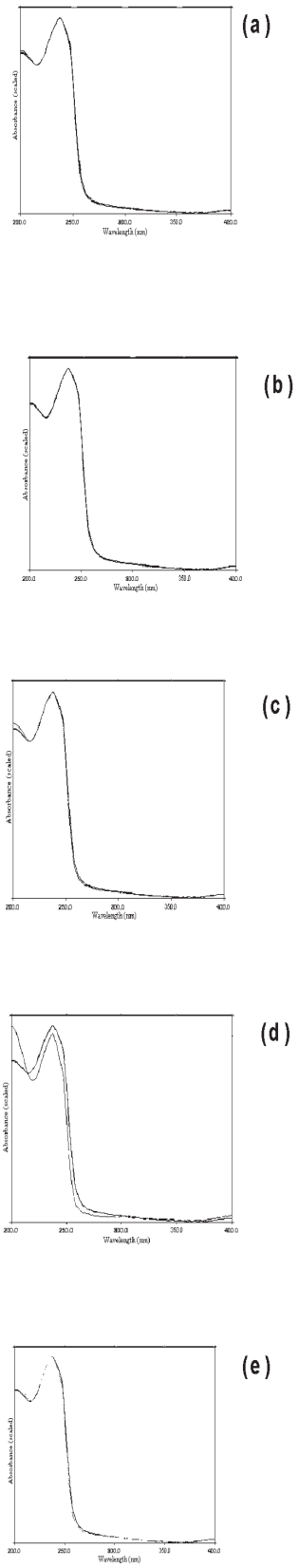

Figure 5. Spectral overlay of standard SIM with (a) thermal degraded sample, (b) photodegraded sample, (c) acid degraded sample, (d) base degraded sample, and (e) oxidation degraded sample of SIM.

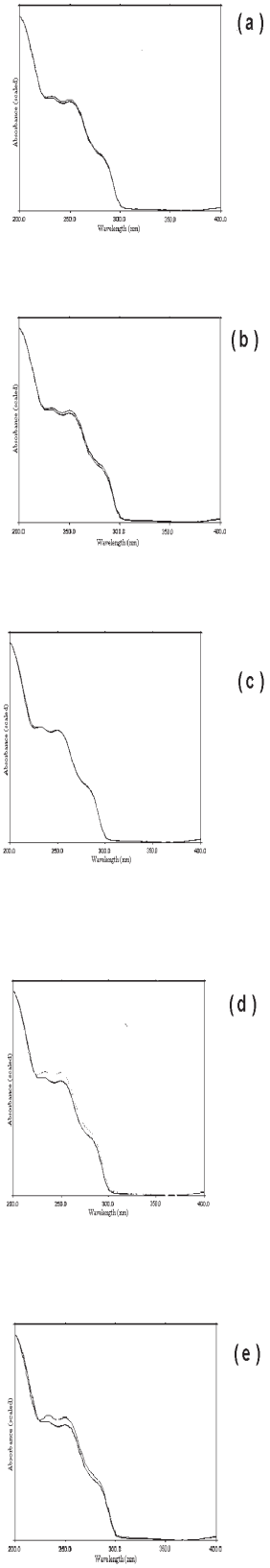

Figure 6. Spectral overlay of standard EZE with (a) thermal degraded sample, (b) photodegraded sample, (c) acid degraded sample, (d) base degraded sample, and (e) oxidation degraded sample of EZE.

Table 2. Regression characteristics and system suitability parameters of the proposed RP-LC method

Parameters	SIM	EZE
Retention time, min	6.35	2.04
Tailing factor	1.29	1.31
Asymmetry	1.36	1.38
Theoretical plates	4225.6	3409.4
Linearity range, μg/mL	1–80	3–80
Limit of detection, μg/mL	0.5	0.7
Limit of quantification, μg/mL	1.0	3.0
Regression equation $y = a + bc$[a]		
Slope (b)	37867	31607
Intercept (a)	–45929	–39366
Correlation coefficient (r)	0.9996	0.9996
Repeatability; CV, % (n = 7)	0.678	0.851

[a] c = Concentration.

showed almost complete degradation within a few minutes even in 0.01 M sodium hydroxide solution. Thus, in a later experiment, base hydrolysis of drug substances and drug product in solution state was conducted by 0.01 M sodium hydroxide solution at ambient temperature, and chromatography was carried out immediately.

For oxidative stress, sample solutions of drug substance and drug product in 3% hydrogen peroxide were kept at ambient temperature for 48 h. All the samples solutions for acid/base hydrolysis and oxidation were kept in the dark to prevent the effect of light.

Results and Discussion

Method Development

The USP 28 monograph (8) for SIM specifies an RP-LC method using a C_{18} column for its assay. The mobile phase composition in this method is acetonitrile–dilute (0.1%) phosphoric acid (60 + 40, v/v) with a flow rate of 3.0 mL/min for drug substance, and acetonitrile–buffer solution (65 + 35, v/v) with a flow rate of 1.5 mL/min for tablet formulation. This method does not describe simultaneous determination of SIM and EZE in the presence of their degradation products generated during forced degradation studies. In case of RP-LC various columns are available, but as the main aim of the method is to resolve both the compounds in the presence of degradation products and impurities, a C_{18} column (250 mm × 4.0 mm id, 5 μm particle size) was preferred over other columns. A LiChrospher® 100 C_{18} column was preferred as it has high carbon loading with very closely packed material to give high resolution compared to other C_{18} columns. To develop a precise, accurate, specific, and suitable stability indicating RP-LC method for the simultaneous estimation of SIM and EZE, different mobile phases and chromatographic conditions were tested for the quantitative determination of SIM and EZE in the presence of degradation products and impurities. The optimum mobile phase consisted of acetonitrile–water–methanol (60 + 25 + 15, v/v/v), with the apparent pH adjusted to 4 ± 0.1 with 10% phosphoric acid solution. This was selected because it was found to ideally resolve the peaks of SIM (retention time, t_R = 6.35 ± 0.03 min) and EZE (t_R = 2.04 ± 0.01 min) and give complete separation of their degradation products and impurities at a flow rate of 1.5 mL/min. UV detection at 238 nm, an injection volume of 20 μL, and ambient temperature for the column were found to be best for analysis.

Singh and Bakshi, in their article on stress testing (23), suggested a target degradation of 20–80% for establishing the stability-indicating nature of the assay method, as even intermediate degradation product should not interfere with any stage of drug analysis. Though conditions used for forced degradation were adjusted to achieve degradation in the range of 20–80%, this could not be achieved in some cases even after exposure for prolonged duration. Figure 1 (a–c) shows the chromatograms of untreated drugs in pure form as well as in tablet solution. SIM showed extensive degradation in acidic hydrolytic conditions, as depicted in Figure 2 (a–c). Both drugs showed extensive degradation in basic hydrolytic conditions, particularly complete degradation of SIM as depicted in Figure 3 (a–c). Both drugs showed reasonable degradation in oxidative conditions as depicted in Figure 4 (a–c). Table 1 indicates the extent of degradation of

Table 3. Intraday precision data of the proposed RP-LC method

Concentration		Intraday precision			
		SIM		EZE	
SIM, μg/mL	EZE, μg/mL	Peak area ± SD[a] (n = 7)	CV, %	Peak area ± SD[a] (n = 7)	CV, %
10	10	309670 ± 3879.89	1.253	267433 ± 3233.19	1.209
20	20	730584 ± 5515.57	0.755	613349 ± 4954.40	0.808
30	30	1068903 ± 14509.8	1.357	900662 ± 6937.74	0.770
40	40	1440777 ± 17446.7	1.211	1179566 ± 13870.8	1.176

[a] SD = Standard deviation.

Table 4. Interday precision data of the proposed RP-LC method

Concentration		Interday precision			
		SIM		EZE	
SIM, μg/mL	EZE, μg/mL	Peak area ± SD (*n* = 7)	CV, %	Peak area ± SD (*n* = 7)	CV, %
10	10	306792 ± 4769.07	1.554	267696 ± 3061.93	1.144
20	20	726105 ± 3368.27	0.464	613477 ± 4883.8	0.796
30	30	1071467 ± 13032.1	1.216	898284 ± 7716.78	0.859
40	40	1427994 ± 18803.2	1.317	1192866 ± 10999.6	0.922

SIM and EZE under various stress conditions. The peak purities of SIM and EZE responses were assessed by comparing the shape of the spectra of standard drugs and degraded samples at the upslope, apex, and downslope of the peaks (24) as shown in Figures 5 (a–e) and 6 (a–e). No other coeluting peak was found with the main peaks, suggesting the specificity of the method for the simultaneous estimation of SIM and EZE in the presence of degradation products and impurities.

Method Validation

The described method has been validated, in addition to its specificity, for linearity, system suitability, accuracy, and intermediate precision. The standard solutions for linearity were prepared 5 times at different concentration levels. The limit of detection (LOD) and the limit of quantification (LOQ) were determinated according to a signal-to-noise (S/N) ratio of 3:1 and 10:1, respectively. Characteristic parameters for the regression equation and system suitability are given in Table 2. Repeatability of measurements of peak area was evaluated using 7 replicates of the same concentration (20 μg/mL for each drug). The intraday and interday variation for the determination of SIM and EZE were evaluated at 4 different concentration levels (10, 20, 30, and 40 μg/mL). The low coefficient of variation (CV) values of within-day and day-to-day variations for SIM and EZE revealed that the proposed method is precise (Tables 3 and 4). Accuracy of the method was checked by a recovery study using the standard addition method at 3 different concentration levels, i.e., a multilevel recovery study. The preanalyzed samples were spiked with an extra 50, 100, and 200% of the standard SIM and EZE, and the mixtures were analyzed by the proposed method. Results of the recovery study are shown in Table 5.

Assay of SIM and EZE from Its Tablet Dosage Forms

The assay results of SIM and EZE in tablet dosage forms were comparable with the value claimed on the label. The obtained results, presented in Table 6, indicate the suitability of the method for routine analysis of SIM and EZE from their combination drug products.

Conclusions

Based on the results obtained from the analysis of forced degraded samples using the described method, it can be concluded that there is no other coeluting peak with the main peaks and the method is specific for the estimation of SIM and EZE in the presence of degradation products. The method has linear response in the stated range and is accurate and precise. Although no attempt was made to identify the degradation products, the described method can be used as a stability-indicating method for assay of SIM and EZE in their combination drug products.

Table 5. Recovery data for the proposed RP-LC method[a]

Drug	Level	Amount of sample taken, μg/mL	Amount of standard spiked, μg/mL	Amount of standard recovered, μg/mL	Recovery, %	CV, %
SIM	I	10	5	4.98	99.70	0.442
	II	10	10	9.89	98.91	1.592
	III	10	20	19.78	98.91	1.439
EZE	I	10	5	5.16	103.13	1.268
	II	10	10	10.01	100.11	1.552
	III	10	20	19.61	98.05	1.043

[a] *n* = 5.

Table 6. Analysis of SIM and EZE from their combination drug products by the proposed RP-LC method[a]

	Labeled amount, mg		Amount found, mg		Assay, % ± CV, %	
Formulations	SIM	EZE	SIM	EZE	SIM	EZE
Set 1	10	10	9.84	9.83	98.43 ± 1.51	98.36 ± 1.27
Set 2	10	10	9.93	10.04	99.33 ± 0.68	100.42 ± 0.57

[a] $n = 5$.

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