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Development and Validation of Stability Indicating RP-UPLC Method for Simultaneous Determination in Fixed Dose Combination of Ezetimibe and Simvastatin

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Abstract

A stability indicating UPLC method was developed and validated for the simultaneous determination of fixed dose combination of ezetimibe and simvastatin in bulk drug. The developed method was successfully applied to the simultaneous quantitative analysis of the title drugs in tablet dosage form. The chromatographic separation was performed on Kromasil Eternity TM C 18 UHPLC column (2.5 μm, 2.1 mm x 50 mm) using gradient elution of acetonitrile and ammonium acetate buffer (pH 6.70; 0.01 M) as mobile phase at a flow rate of 0.35 mL/min and column oven temperature of 40ºC. UV detection was carried out using a UV-PDA detector at 235 nm. Total run time was 3.5 min within which main compounds and their degradation products were separated. The method was validated for accuracy, repeatability, reproducibility and robustness. Linearity, LOD and LOQ were established for ezetimibe and simvastatin.

Keywords: Hypolipidaemic; Stability indicating assay; UPLC; validation; Ezetimibe; Simvastatin

Introduction

Ezetimibe (EZE), chemically 1-(4-fluorophenyl)-3(R)-[3-(4-fluorophenyl)-3(S)-hydroxypropyl]-4(S)-(4-hydroxyphenyl)-2-azetidinone, is a white crystalline powder that is freely soluble in ethanol, methanol, and acetone and practically insoluble in water. It melts at about 163°C and is reported to be stable at ambient temperature. It is one of the first new classes of lipid-lowering compounds that selectively inhibit the intestinal absorption of cholesterol and related phytosterols [1]. Chemical structure of ezetimibe is given in Figure 1A.

Clinical studies have shown that co-administration of EZE with statins could provide an additional reduction in LDL cholesterol as well as total cholesterol. The combined use of these agents offers a well tolerated lipid management strategy for patients with mixed hyperlipidemia.

Simvastatin(SIM) chemically Butanoic acid,2,2-dimethyl-,1,2,3,7,8,8a-hexahydro-3,7-dimethyl-8-[2-(tetrahydro-4-hydroxy-6-oxo-2H-pyran-2-yl)ethyl]-1-naphthalenylester,[1S-[1a,3a,7b,8b(2S^*,4S^*),8ab]]. 2,2-Dimethylbutyric acid, 8-ester with (4R,6R)-6-2-[(1S,2S,6R,8S,8aR)-1,2,6,7,8,8a-hexahydro-8-hydroxy-2,6-dimethyl-1-naphthyl]ethyl]tetrahydro-4-hydroxy-2H-pyran-2-one is a is a synthetic lipid- lowering agent.

SIM is an inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase. This enzyme catalyzes the conversion of HMG-CoA to mevalonate, an early and rate-limiting step in cholesterol biosynthesis. Simvastatin is a white to off-white crystalline powder that is insoluble in aqueous solutions of pH 4 and below. Simvastatin is very slightly soluble in distilled water, pH 7.4 phosphate buffer, and acetonitrile; slightly soluble in ethanol; and freely soluble in methanol. Chemical structure of simvastatin is shown in Figure 1B [2].

EZE and its pharmaceutical formulation with SIM is not official in any pharmacopoeia yet. Several analytical methods such as UPLC, HPLC [3-5] spectrophotometry [6], HPTLC and LC-MS [7] are reported for the determination of SIM individually and in combination with other drugs. Literature indicates spectrophotometry, HPLC and HPTLC methods for determination of EZE in pharmaceutical formulations, drug substance and biological matrices [8]. HPLC, HPTLC and derivative ratio spectrophotometry methods are reported for the simultaneous determination of EZE and SIM. It should be taken into consideration that EZE and SIM should be monitored together with their degradation compounds preferably in a single chromatographic run.

Because of the very nature of requirement of separation of multiple components during analysis of stability samples, chromatographic methods have taken precedence over the conventional methods of analysis like titrimetry and spectrophotometry [9,10]. Other than separation of multiple components, the advantage of chromatographic methods is that, these possess greater accuracy and sensitivity for even
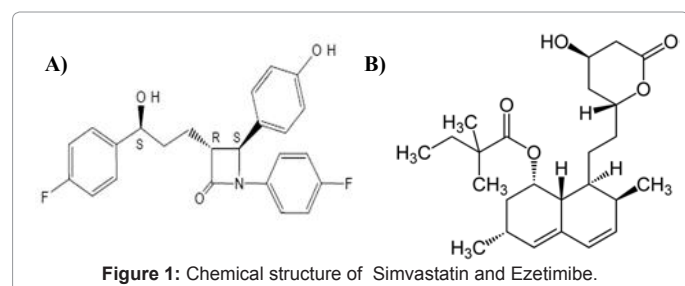
Figure 1: Chemical structure of Simvastatin and Ezetimibe.

small quantities of degradation products produced [11]. Such methods include Thin Layer Chromatography (TLC), High Performance Thin Layer Chromatography (HPTLC), Gas Chromatography (GC), capillary electrophoresis, High Performance Liquid Chromatography (HPLC) and Ultra Performance Liquid Chromatography [12]. UPLC is a recent technique which enables significant reductions in separation time and solvent consumption. It also lifts the backpressure limit up to 15,000 psi from a typical 4,000 psi of HPLC. Due to very narrow and sharp peaks, more number of peaks may appear in less time which may facilitate in analysis of complex mixtures and thus it may give more information regarding sample to be analysed.

The purpose of this study was to develop a stability indicating method for the simultaneous determination of fixed dose combination of EZE & SIM, and to apply the developed method to quantitate amount of the title drugs from tablet. UPLC technique was chosen because of its above mentioned advantages. The proposed method was able to separate the compounds of interest as well as their degradation products within 3.5 mins. Thereafter, this method was validated as per ICH guidelines [13].

Experimental

Chemicals and reagents

Reference standards of EZE and SIM were gifted by Ranbaxy Research Laboratory (Gurgaon, India) with declared purity of 99.5% and 99.50% respectively. Acetonitrile (ACN) for HPLC was obtained from Spectrochem Pvt. Ltd. (Mumbai, India) and ammonium acetate was supplied by Qualigens Fine Chemicals (Mumbai, India). The 0.2 μm filter used to filter standard and sample preparation was mdi SY13VF (PVDF) which was manufactured by Advanced Micro devices (P) Ltd. (Ambala, India). Tablet formulation (simvotin EZ) containing 10 mg each of SIM and EZE was procured from local market in Delhi, India. Milli Q water, obtained from Millipore (Massachusetts, USA), was used in making solutions.

Buffer preparation

Solution of ammonium acetate (0.01 M) was prepared by dissolving about 0.77 g of ammonium acetate in 1 l of water for HPLC. The pH of this solution was adjusted to 6.7 with acetic acid. The buffer preparation was found stable with respect to pH and visual clarity for 48 h.

Chromatographic system

Analysis was performed on Acquity UPLCTM system (Waters, Milliford, USA), consisting of binary solvent manager, sample manager and PDA detector. System control, data collection and data processing were accomplished using Waters EmpowerTM chromatography data software. The analytical column was Kromasil Eternity TM C 18 UHPLC column (2.5 μm, 2.1 mm × 50 mm). The separation of EZE and SIM was achieved by gradient elution using acetonitrile and ammonium acetate buffer (pH 6.70; 0.01 M). The finally selected and optimized conditions were as follows: injection volume 2 μL, gradient elution, at a flow rate of 0.35 mL/min and column oven temperature of 40ºC, detection wavelength 235 nm.

Preparation of standard solution

Standard solution was prepared by dissolving 100 mg each of standard substance in methanol: water(90:10) mixture to obtain solution containing 20 μg/ml each of EZE SIM.

Sample preparation

About 100 mg each of standard substances were taken in 100 mL volumetric flask. About 10 mL of methanol was added to this flask and sonicated for 2 min. This solution was then diluted to the mark with diluent and sonicated for 5 min. It was then filtered through 0.2 μm filter and filtrate was collected after discarding first few millilitres. 2 mL of the filtrate was transferred to 100 mL volumetric flask, diluted to volume with diluent and then sonicated for 5 min resulting in solution containing 20 μg/mL each of EZE and SIM.

Method validation

System suitability: System suitability parameters were measured so as to verify the system performance. System precision was determined on six replicate injections of standard preparations.

Specificity: As per ICH guideline Q1AR and ICH's Common Technical Document, forced degradation studies were performed on drug substance (except photo stability testing on drug product) to establish its inherent stability characteristics in order to demonstrate selectivity and stability indicating capability of the proposed method. The standard substances were exposed to

- Acidic (1N HCl, room temperature),

- Alkaline (0.01N NaOH, room temperature),

- Neutral (water, 80°C, 4 h),

- Strong oxidizing (30% H_2O_2, 80°C, 1 h)

- Thermal degradation conditions (80°C, 1 d)

While photolytic stress study was carried out on tablet sample (254 nm, 1 d). The blank solutions were also subjected to stress in the same manner as the drug solution. All the exposed tablet samples, standards and blank solutions were then analyzed by the proposed method.

Linearity: Linearity was demonstrated from 50% to 150% of standard concentration using minimum six calibration levels (50%, 60%, 80%, 100%, 120% and 150%) for both the title drugs. The method of linear regression was used for data evaluation.

Precision: Precision was investigated using sample preparation procedure for six real samples and analyzed by proposed method. Intermediate precision was studied using different column, performing analysis on different day and also by different analyst.

Accuracy: To confirm the accuracy of the proposed method, recovery experiments were carried out by standard addition technique. Three different levels (80%, 100% and 120%) of standards were added to pre-analyzed tablet samples in triplicate. The percentage recoveries of SIM and EZE at each level and each replicate were determined. The mean of percentage recoveries (n=9) and the relative standard deviation was calculated

LOD & LOQ: The LOD and LOQ of EZE and SIM were determined by using signal to noise approach as defined in ICH guideline. Increasingly dilute solution of each drug was injected into the chromatograph and signal to noise (S/N) ratio was calculated at each concentration.

Robustness: The robustness as a measure of method capacity to remain unaffected by small but deliberate changes in chromatographic conditions was studied by testing influence of small changes in pH of

buffer (± 0.2 units), column temperature (± 5%), organic content of mobile phase (± 2%) and flow rate (± 5%).

Stability of Sample preparation: Stability of sample solution was established by storage of sample solution at ambient temperature for 24 h followed by its assay, which was then compared against fresh sample.

Results and Discussion

Method development

For analysis of EZE and SIM, different chromatographic conditions were tried on HPLC and UPLC and results obtained were compared. . The results from HPLC (Figure 2b) involve few main problems: longer run time, more solvent usage. While with UPLC all these problems are solved and thus gradient run using UPLC provide proper peak with a good baseline in 3.5 min time (Figure 2a) (Table 1,2) was selected for the analysis of SIM & EZE.

Among various columns available for UPLC analysis, Kromasil Eternity TM C 18 UHPLC column (2.5 μm, 2.1 mm x 50 mm) column was preferred, because it provides appreciable peak shape and resolution and absorbance were good and ACN was used as organic phase, because of its favorable UV transmittance. Among different mobile phase used, the mobile phase consisted of ACN and 10 mM ammonium acetate with an apparent pH adjusted to 6.7 ± 0.1 with ammonia was found to be suitable for analysis of SIM and EZE. Further, a flow rate of 0.35 ml/min, an injection volume of 2 μl, and UV detection at 235 nm for drug were found to be suitable for analysis. (Figure 2a) indicates the peak obtained for the sample by the selected method (Table 3).

Analytical parameters and validation

After satisfactory development of method, it was subjected to method validation as per ICH guidelines. The method was validated to demonstrate that it is suitable for its intended purpose by the standard procedure to evaluate adequate validation characteristics (precision, linearity, robustness, stability indicating capability).
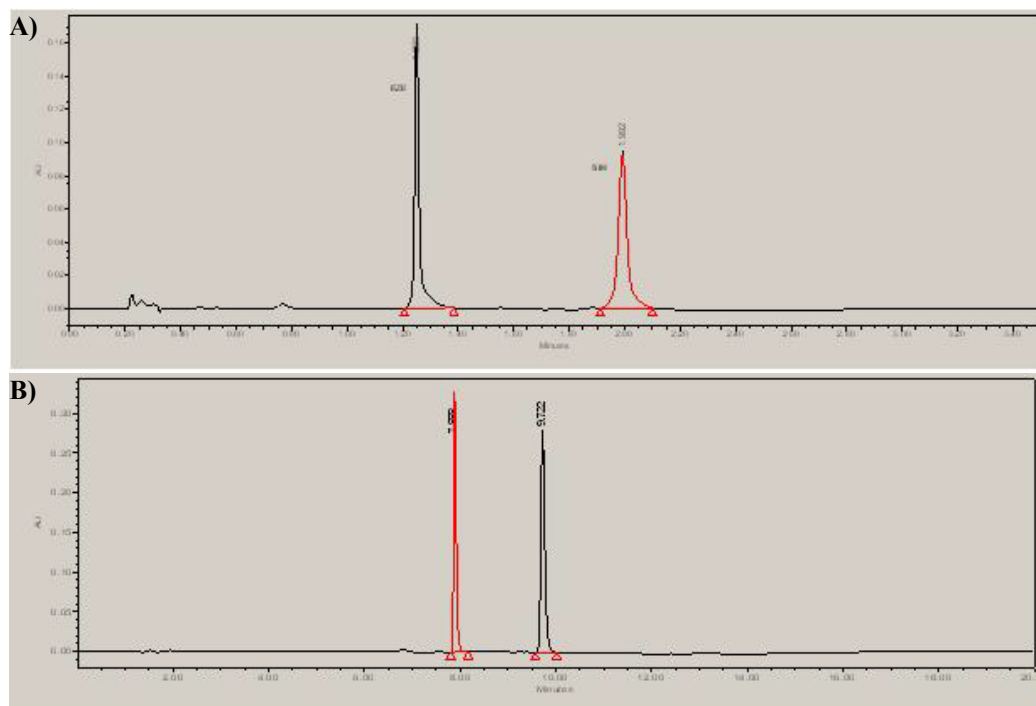

Figure 2: A) UPLC chromatogram of Ezetimibe and Simvastatin. **B)** HPLC chromatogram of Ezetimibe and Simvastatin.

S.No	Column Used	Mobile Phase	Parameter optimised	Inj. Vol.	Observation	Flow rate	Results
2a	Kromasil Eternity C-18 (50×2.1 mm) 2.5 μm	Ammonium acetate (PH 6.7): CAN (gradient)	Flow rate and gradient	5 μl	Pear shape not good, More run time	0.25ml/min	Method rejected
2b	Kromasil Eternity C-18 (50×2.1 mm) 2.5 μm	Ammonium acetate (PH 6.7): CAN (gradient)	gradient	5 μl	Tailing observed	0.35ml/min	Method rejected
2c	Kromasil Eternity C-18 (50×2.1 mm) 2.5 μm	Ammonium acetate (PH 6.7): CAN (gradient)	gradient	3 μl	Longer run time	0.35ml/min	Method rejected
2d	Kromasil Eternity C-18 (50×2.1 mm) 2.5 μm	Ammonium acetate (PH 6.7): CAN (gradient)	gradient	2μl	Peak shape good	0.35ml/min	Method accepted

Table 1: Different chromatographic conditions applied and results obtained.

Recovery level %		Amount taken µg/ml	Amount found µg/ml	Recovery %	Mean*	%RSD
	80	18	18.12	100.60		
	80	18	18.03	100.01	100.10	0.457
	80	18	17.96	99.70		
	100	21	21.10	100.04		
	100	21	21.15	100.71	100.51	0.415
	100	21	21.17	100.80		
	120	23	21.18	100.72		
	120	23	22.99	99.99	100.67	0.564
	120	23	23.23	101.1		

Table 2: Recovery study results for Ezetimibe.

Recovery level %		Amount taken µg/ml	Amount found µg/ml	Recovery %	Mean*	%RSD
	80	18	18.20	100.01		
	80	18	18.11	100.6	100.55	0.481
	80	18	18.01	100.05		
	100	21	20.89	99.4		
	100	21	21.21	100.01	100.43	0.899
	100	21	21.19	100.90		
	120	23	23.02	100.01		
	120	23	23.03	100.30	100.67	0.778
	120	23	23.23	101.41		

Table 3: Recovery study results for Simvastatin.

System suitability parameter	Robustness parameter	EZE	SIM
Resolution	No change (repeatability)	-	17.22
	pH of buffer (+0.2 units)	-	17.13
	pH of buffer (-0.2 units)	-	16.65
	Column temperature (+5%)	-	17.18
	Column temperature (-5%)	-	16.97
	Flow rate (+5%)	-	17.18
	Flow rate (-5%)	-	17.12
	Organic content of mobile phase (+2%)	-	17.72
	Organic content of mobile phase (-2%)	-	16.63
USP Tailing	No change (repeatability)	1.51	1.11
	pH of buffer (+0.2 units)	1.52	1.12
	pH of buffer (-0.2 units)	1.51	1.11
	Column temperature (+5%)	1.51	1.11
	Column temperature (-5%)	1.52	1.13
	Flow rate (+5%)	1.52	1.12
	Flow rate (-5%)	1.50	1.11
	Organic content of mobile phase (+2%)	1.51	1.12
	Organic content of mobile phase (-2%)	1.52	1.11
Column effciency	No change (repeatability)	28021	21342
	pH of buffer (+0.2 units)	28600	21898
	pH of buffer (-0.2 units)	28167	21274
	Column temperature (+5%)	29802	23137
	Column temperature (-5%)	27964	20422
	Flow rate (+5%)	28676	21570
	Flow rate (-5%)	26779	21646
	Organic content of mobile phase (+2%)	26120	22622
	Organic content of mobile phase (-2%)	31419	20946

Table 4: System suitability parameters and robustness.

System suitability: Results of other system suitability parameters such as theoretical plates, purity angle, and purity threshold are presented in (Table 4). The data presented in (Table 4) indicated the acceptable system suitability parameters, as the % RSD is not more than 2%. Tailing factor was not more than 2 and theoretical plates are more than 1000 and purity angle was less than purity threshold.

The percentage (%) RSD of area count of six replicate injections was below 2 which indicated the system precision.

Specificity: The results of forced degradation studies are given in

Degradation Condition	% Assay of EZE	% Assay of SIM
No Degradation (Control)	99.9%	100.0%
Acid hydrolysis (1N HCl, 25°C)	100.1%	68.5%
Alkali hydrolysis (0.01N NaoH, 25°C)	92.6%	61.0%
Neutral hydrolysis (Water, 80°C, 4 h)	69.9%	85.7%
Oxidation (30% H_2O_2, 80°C, 4 h)	88.0%	97.7%
Thermal (80°C, 1d)	101.6%	99.3%
Photolytic (UV at 254 nm, 1d)	100.8%	98.1%

Table 5: Forced degradation data.

(Table 5). SIM was found sensitive to acid hydrolysis. There was less than 10% degradation of SIM using 0.1N HCl. Therefore, 1N HCl was selected for degradation studies by which 32% of the simvastatin is been degraded. Ezetimibe was stable for the acid hydrolysis (figure 3).

EZE & SIM was found sensitive to alkali hydrolysis. The reaction in 0.1N NaOH at 80°C was so fast that whole of the drug of SIM was degraded in few minutes and at room temperature, there was more than 30%degradation of drug. Subsequently, studies were performed in 0.01N NaOH at room temperature (25°C) in which the 7% and 35% of the EZE and SIM was degraded respectively. Degradation of EZE & SIM was found to be directly proportional to the strength of alkali and also, the drug gradually decreased with time on heating at 50°C in 0.01N NaOH (figure 4).

Both EZE and SIM were stable in water at room temperature. Upon heating the solution at 80°C for 4 h, the degradation of the EZE was found to be 29% and SIM was found to be 15.1%. The rate of hydrolysis

of SIM was found to be slower as compared to EZE (figure 5).

Both the drugs were stable to hydrogen peroxide (30%) at room temperature, no degradation products were observed. Upon heating the solution in H_2O_2 at 80°C for 1 h, the degradation of the EZE and SIM was found to be 12% and 3% respectively (figure 6).

Both EZE and SIM were found stable to the effect of temperature. When the mixture of drug powders were exposed to dry heat at 80°C for 1 day, no decomposition of the drugs were seen.

Both EZE and SIM were found stable to the effect of light. When the tablet sample was exposed to ultraviolet light (UV chamber) for 1 day, no decomposition of EZE is seen and very minor degradation of SIM was observed.

Linearity: The response was found linear from 70% to 130% standard concentration. The correlation coefficient (R^2) was greater than 0.99 (Table 5) (figures 7,8).

Precision: The % assay was calculated taking standard sample solution as control. Low values of RSD 0.79% and 0.54% for EZE and SIM respectively indicate that the method is precise.

Intermediate precision was studied using different equipment and column and performing the analysis on different day, the %RSD values of the intermediate precision are 0.89% and 0.78% for EZE and SIM respectively. Low value of %RSD indicates the method is precise (Table 5).
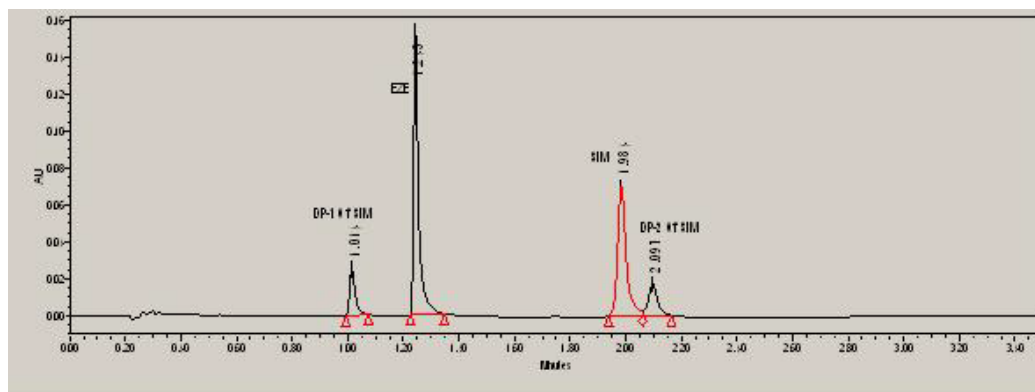

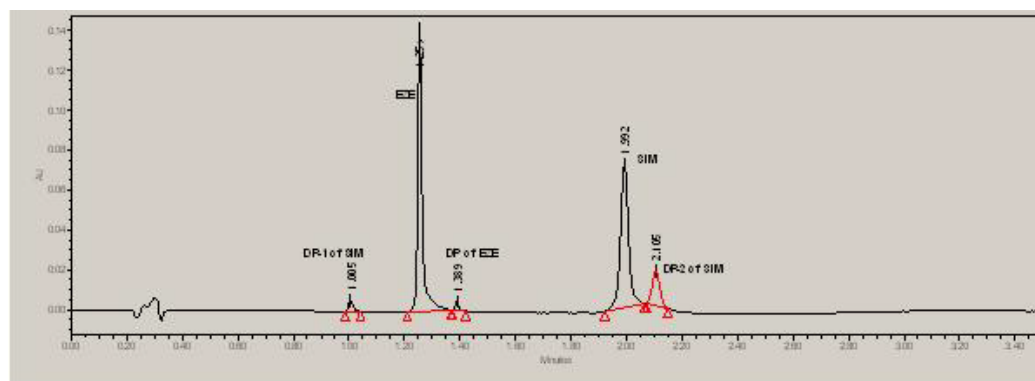**Figure 3:** Chromatogram of acid degraded sample containing EZE and SIM.

Figure 4: Chromatogram of base degraded sample containing EZE and SIM.
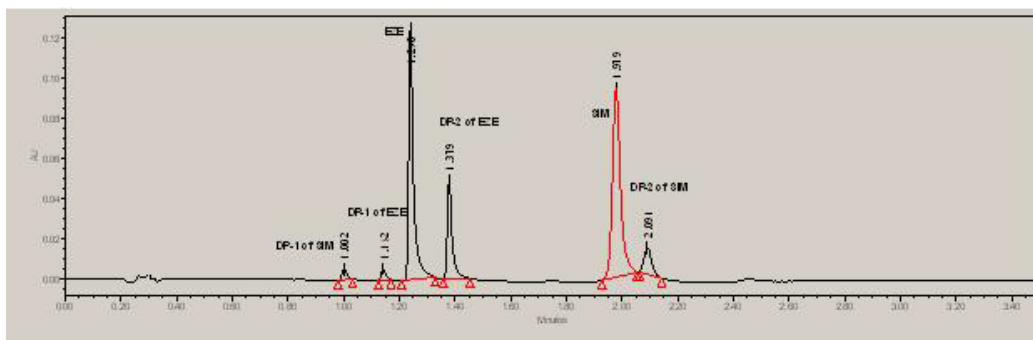
Figure 5: Chromatogram of water degraded standard solution containing both title drugs.
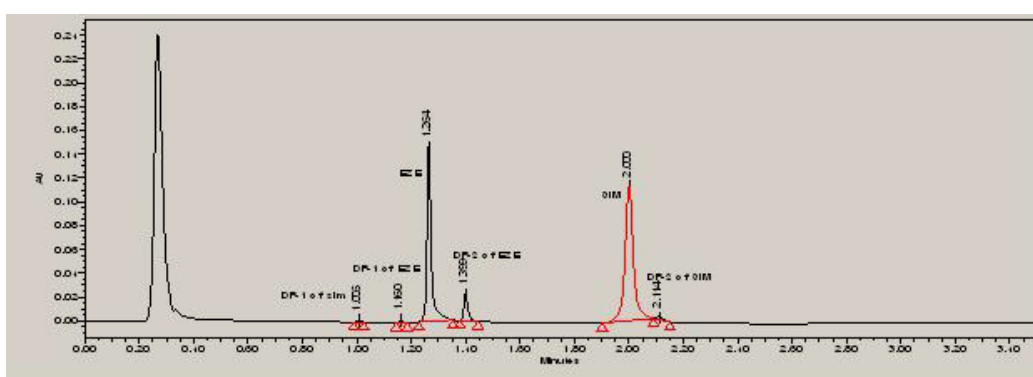

Figure 6: Chromatogram of peroxide degraded sample containing EZE and SIM.

Robustness: No significant effect was observed on system suitability parameters such as theoretical plates, purity angle, and purity threshold, when small but deliberate changes were made for chromatographic conditions such as change in flow rate (± 5%), temperature (± 5 units), pH (± 0.2 units), and organic content (± 2%). The results are presented in (Table 3), along with system suitability parameters of normal methodology. Thus, the method was found to be robust with respect to variability in above condition.

Stability in sample solution: Sample solution did not show any appreciable change in assay value when stored at ambient temperature up to 24 h. Assay results are presented in (Table 5).

Conclusion

A novel UPLC method was successfully developed and validated for simultaneous determination of EZE and SIM. The total runtime was 3.5 min within which both the drugs and their degradation products were separated. Method validation results have proved the method to be selective, precise, accurate, robust and stability indicating. This method can be successfully applied for the routine analysis as well as stability study by the industry. It can also be utilized for determination of content uniformity and dissolution profiling of this product where sample load is higher and high throughput is essential for faster deliver of results.

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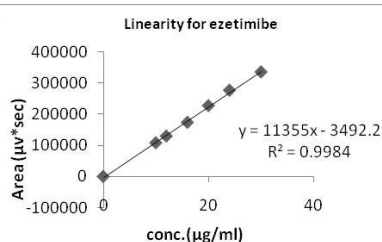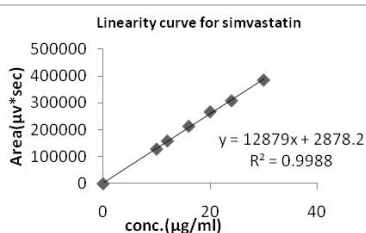
Figure 7: Linearity curve for Ezetimibe.

$$y = 11355x - 3492.2$$
$$R^2 = 0.9984$$

Figure 8: Linearity curve for Simvastatin.

$$y = 12879x + 2878.2$$
$$R^2 = 0.9988$$

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