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Stability Indicating Method Development for Simultaneous Estimation of Ezetimibe and Atorvastatin in Pharmaceutical Formulations by RP-HPLC

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Abstract

Simple, specific, economical and precise high performance liquid chromatographic method for the simultaneous determination of ezetimibe and Atorvastatin in API (active pharmaceutical ingredient) and formulation has been developed and validated. Chromatography was carried out at 30°C on a prepacked Zorbax SB C18 (5 mm, 250×4.6 mm) column with the 0.02 M Potassium dihydrogen phosphate: Acetonitrile: Methanol (10:40:50, v/v/v) was used as the mobile phase. The UV detection was carried at 236 nm. The results obtained showed good agreement with the declared contents. Ezetimibe and Atorvastatin separated in less than 10 min with good resolution and minimal tailing and without interference of excipients. The retention times of ezetimibe and Atorvastatin were 5.7 min and 9.1 min, respectively. The method was linear in the range of 5–50 µg/ml for Ezetimibe concentration with a correlation co-efficient 0.9992 and in the range 5–60 µg/ml for Atorvastatin concentrations having correlation co-efficient 0.9994 and the recovery was 99-102%. The method was validated according to ICH guidelines and the acceptance criteria for accuracy, precision, linearity, specificity and system suitability were met in all cases. The proposed method can be used for quantitative determination of Ezetimibe and Atorvastatin combination from API and formulations.

Keywords: Content uniformity; Method validation; Ezetimibe; Atorvastatin; Stability-indicating; ICH guidelines

Introduction

Atorvastatin (ATV) is chemically [R-(R*,R*)]-2-(4-flurophenyl)-β,δ-dihydroxy-5- (1-methylethyl)-3-phenyl-4-[(phenylamino) carbonyl]-1H-pyrrole-1-heptanoic acid, calcium salt trihydrate. Atorvastatin calcium is an inhibitor of 3-hydroxy-3-methylglutaryl Coenzyme A (HMG-CoA) reductase. This enzyme catalyses the conversion of HMG-CoA to mevalonate, an early and rate limiting step in cholesterol biosynthesis [1,2]. Ezetimibe (EZE) is [(3*R*,4*S*)-1-(4-fluorophenyl)-3-[(3*S*)-3-(4-fluorophenyl)-3-hydroxypropyl]-4-(4-hydroxyphenyl)-2-azetidinone] . It is a selective cholesterol absorption inhibitor used in the treatment of primary hypercholesterolemia. It inhibits the absorption of biliary and dietary cholesterol from small intestine without affecting absorption of fat soluble vitamins, triglycerides and bile acids. After oral administration, Ezetimibe is metabolized into its glucuronide in the liver and small intestine, which is also active in prevention of absorption of cholesterol. Ezetimibe does not have significant pharmacokinetic interactions with other lipid lowering drugs as it does not influence the activity of cyotochrome P450. EZE is administered at the dose of 10 mg with and without Atorvastatin [3]. A literature survey regarding quantitative analysis of these drugs revealed that attempts were made to develop analytical methods for Atorvastatin using extractive spectrophotometry [4], HPLC [5-9], GC-MS [10], LC-MS [11], LC-electrospray tandem mass spectrometry [11-15] and HPTLC [16] methods. HPLC method has been reported for the determination of atorvastatin and its impurities in bulk drug and tablets [10].

No analytical method has so far been reported for the simultaneous determination of atorvastatin calcium and ezetimibe in pharmaceutical dosage forms. The objective of the present work was to develop an accurate, specific and reproducible method for the simultaneous estimation of Atorvastatin and ezetimibe in pharmaceutical dosage forms. The objective of this work was to develop a simple, precise, reliable and rapid stability-indicating liquid chromatographic analytical method for assay of Ezetimibe and Atorvastatin for determination of the content uniformity of a tablet formulation, to validate the method in accordance with ICH [17] guidelines. The validation procedure followed the guidelines of USP 30 [18]. The present work is aimed at development of a sensitive, specific and validated reverse phase high performance liquid chromatographic method for simultaneous determination of Atorvastatin and Ezetimibe from the dosage form and its degradation products formed under stress degradation of both Atorvastatin and Ezetimibe.

Materials and Methods

Atorvastatin and Ezetimibe reference standard (label claim 99.8% pure) was provided by Ranbaxy Pharmaceuticals Ltd. Tablets of, Atorvastatin and Ezetimibe Tonact-EZ with 10 mg each label claim manufactured by Lupin Pharmaceutical Pvt. Ltd, Ahmedabad, India were procured from a local pharmacy. HPLC grade acetonitrile, Orthophosphoric acid water, methanol and sodium phosphate acid were obtained from Merck India Limited, Mumbai, India. Analytical grade hydrochloric acid, sodium hydroxide pellets and hydrogen peroxide solution 30% (v/v) were obtained from Ranbaxy Fine Chemicals, New Delhi, India and 0.45 µm nylon membrane filter was obtained from Pall Life Sciences, Mumbai.

Chromatography

The chromatographic system used to perform development and

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validation of this assay method consisted of a perkin elemer series 200 LC pump, and turbochrom series 200 UV\VIS detector (Perkin elmer). Chromatographic analysis was performed on Zorbax SB C18 ((250 mm×4.6 mm), 5 µm particle size) column. Separation was achieved using a mobile phase consist of 0.02 M Potassium dihydrogen phosphate: Acetonitrile: Methanol (10:40:50, v/v/v) solution at a flow rate of 1.1 ml/min. The eluent was monitored using UV detector at a wavelength 236 nm. The column was maintained at ambient temperature and injection volume of 20 µl was used. The mobile phase was filtered through 0.45 µm filter prior to use.

Preparation of mobile phase

Mobile phase consist of 0.02 M Potassium dihydrogen phosphate: Acetonitrile: Methanol (10:40:50, v/v/v). The mobile phase was filtered using 0.45 µ nylon filters (Millipore, USA) and was degassed by sonication before use.

Preparation of standard solution

A stock standard solution containing 0.5 mg per ml each of Ezetimibe and Atorvastatin was prepared by dissolving 10 mg each of Ezetimibe and Atorvastatin in mobile phase in 50 ml volumetric flask and raising the volume up to the mark. To prepare the working standard solution (500 µg/ml for both Ezetimibe and Atorvastatin), the stock standard solution was diluted with mobile phase.

Specificity

Placebo of the tablets, equivalent to the sample weight was taken and solution prepared similarly to the sample solution. The solution was analyzed as per the proposed method. Sample solution was also analyzed as per the proposed method. No interference from placebo was observed at the retention time of the drugs peaks. Peak purity plots also indicated that the peaks of the Ezetimibe and Atorvastatin are pure and don't have any co eluting peaks. Therefore, it is concluded that the method is specific. Chromatogram of placebo and sample along with peak purity plots are given in Figure 1.

Linearity and range

Different aliquots, 0.5, 1, 2, 3, 4, 5, and 6 ml of the stock solution were taken in a series of 10 ml volumetric flasks and diluted up to the mark with the diluent to get required concentration range of 70% to 130%. The solutions were then filtered through 0.2 µm glass nylon filters and injected into the HPLC system.

Accuracy

6, 8 and 10 ml of the stock solution was taken in triplicate in nine 10 ml volumetric flasks containing about 0.230 g of placebo in it. Then sufficient diluent was added to the flasks and sonicated for 20 to 25 min to dissolve. The volume was made up with diluent and mixed. The solutions were filtered through 0.2 µm glass nylon filters and injected into the HPLC system. The chromatograms were recorded and the percentage recovery was calculated (Table 2).

Precision

For the precision study, precision of the assay was determined by repeatability (intra-day) and intermediate precision (inter-day) in triplicate. Repeatability refers to the use of the analytical procedure over a short period of time that was evaluated by assaying the QC samples during the same day. Intermediate precision was assessed by comparing the assays on different days (3 days).The mean and % RSD was calculated.

Robustness

The Robustness was determined by injecting duplicate injections of standard and three-sample solutions in single at each different condition with respect to control condition (20 µg/ml). Robustness of the method was checked by varying the instrumental conditions such as flow rate (± 10%), organic content in mobile phase ratio (± 2%), wavelength of detection (± 5 nm), column oven temperature (± 5°C) and change in pH of buffer (± 0.2). Sample solution was injected in each condition and assayed for Ezetimibe and Atorvastatin. The mean, standard deviation, and RSD are shown in Table 3. Robustness of the method is indicated by the overall RSD value between the data of set-1 and data at each variable condition.

Stability in analytical solution

A sample solution 20 µg/ml of the drugs was prepared by taken 4 ml of standard stock solution and kept at room temperature. It was analyzed initially and at different time intervals. As the cumulative RSD up to 780 min meets the acceptance criteria, it is concluded that sample is stable in analytical solution for at least 13 hr at room temperature. Results are shown in Table 5.

System suitability data

Standard solution was injected on different days during the validation studies. Using the system suitability software, the USP Tangent and USP Tailing ezetimibe and Atorvastatin peaks were calculated (Table 1).

Stress degradation study

A stress degradation study was carried out on the Ezetimibe and Atorvastatin. Hydrolytic and Oxidative degradation sample solutions of the drugs was prepared in 1N hydrochloric acid, 1N sodium hydroxide and 30% hydrogen peroxide solutions and immediately was followed by analysis as per the proposed method. Thermal degradation sample solutions of the drugs was prepared and subjected to thermal degradation by keeping at 105°C for 1 hr, followed by analysis as per the proposed method. Photolytic degradation study was carried out by exposing the sample to light in a photolytic chamber at 2600 lux for 24 hr, followed by analysis as per proposed method. Using the peak purity test, the purity of the drugs peaks were checked at every stage of above-mentioned studies.

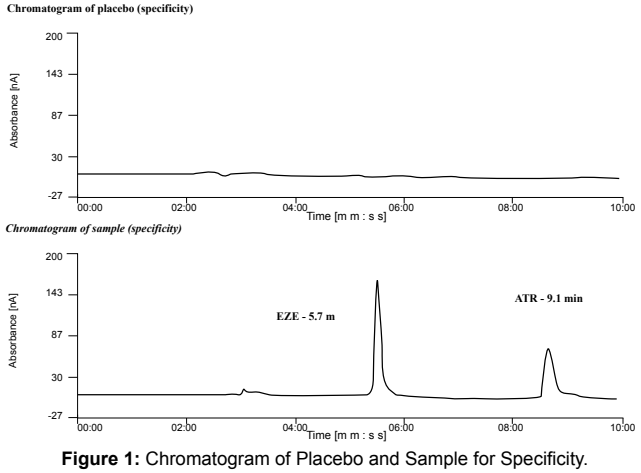

Figure 1: Chromatogram of Placebo and Sample for Specificity.

Sample ID	Ezetimibe				Atorvastatin			
	Conc. (µg/mL)	Area Counts (µV*sec)			Conc. (µg/mL)	Area Counts (µV*sec)		
		Inj#1	Inj#2	Mean		Inj#1	Inj#2	Mean
L-1	5	121621	123065	122343	5	157448	152342	154895
L-2	10	156212	152272	154242	10	194844	192876	193860
L-3	20	198578	199536	199057	20	267616	264398	266007
L-4	30	251045	252645	251845	30	337876	372324	355100
L-5	40	307624	305644	306634	40	408234	408114	408174
L-6	50	359221	351439	355330	50	482234	483422	482828
L-7	--	---	**---**	**--**	60	533214	543212	538213
Ezetimibe			**Intercept**	**98601**	**Atorvastatin**		**Slope**	**122644**
			r	**5147**			**Intercept**	**7112**
			Intercept	**0.9992**			**r**	**0.9994**

Table 1: Data for Calibration Curve for Ezetimibe.

SAMPLE	ACTUAL AMT. ADDED (mg)	EZETIMIBE		ATORVASTATIN	
		AMT. RECOVERED (mg)	% RECOVERY	AMT. RECOVERED (mg)	% RECOVERY
80%-Rec-1	20	20.12	100.60	20.08	100.40
80%-Rec-2	20	20.35	101.75	20.15	100.75
80%-Rec-3	20	20.02	100.10	20.10	100.50
100%-Rec-1	20	20.10	100.50	20.16	100.80
100%-Rec-2	20	19.96	99.80	20.12	100.60
100%-Rec-3	20	20.18	100.90	19.98	99.90
120%-Rec-1	20	20.20	101.00	20.20	101.00
120%-Rec-2	20	20.16	100.80	20.14	100.70
120%-Rec-3	20	19.90	99.50	19.96	99.80
		Mean ± SD	**100.55 ± 0.68**	**Mean SD**	**100.49± 0.405**

Table 2: Accuracy for Ezetimibe and Atorvastatin.

PARAMETERS	OBSERVATION	
	Ezetimibe	**Atorvastatin**
SPECIFICITY	No Interference was found w.r.t. excipients	
LINEARITY (R)[5]	0.9992	0.9994
RANGE	70 – 130 % of test concentration	
PRECISION (RSD)*		
a) Repeatability (n=6)	0.63	0.25
(system precision)		
b) Intermediate Precision	1.27	
(inter-analyst) (n=6)		0.53
c) Method Precision (n=6)	1.70	
		1.26
ACCURACY (% Recovery)**	100.67 ± 1.70	100.11 1.8
STABILITY IN ANALYTICAL SOLUTION	Stable	
STRESS DEGRADATION	The Peaks were **Pure** without any interference	
ROBUSTNESS (Overall RSD)***	Less than 2%	
a) Change in Wavelength	0.81	0.35
• 245 nm	1.37	0.36
• 235 nm		
b) Change in Flow rate	0.48	0.159
• 1 ml/min	0.92	0.189
• 1.2 ml/min		
c) Change in Organic Conc.	0.09	0.10
• - 2%	0.049	0.08
• + 2%		
d) Change in Column Temp.	0.49	0.21
• 25°C	0.39	0.125
• 35°C		
e) Change in pH of Buffer	0.39	0.028
• 3.8	0.16	0.16
• 4.2		
RUGGEDNESS(Overall RSD)***	0.94	0.90

Acceptance Criteria: RSD ≤ 2 %. ** Acceptance mean recovery: for 80 – 120 %.
*** Acceptance Criteria: RSD ≤ 2 %

Table 3: Summary of Validation Parameters.

Application of method to dosage form

The developed and validated HPLC method was applied for determination of Ezetimibe and Atorvastatin from dosage forms. Ezetimibe and Atorvastatin tablets of 10 mg strength from Ranbaxy Labs (Storrvas - EZ) were evaluated for Ezetimibe and Atorvastatin content in tablets. The tablets were powdered and powder equivalent to 10 mg of drugs was weighed. The weighed samples were placed in extraction flask and methanol was added to extract the drug. The suspension was sonicated for 10 min. The supernatant was diluted suitably to obtain 30 µg/ml concentrations of both drugs. The solutions were injected into HPLC and analyzed for drug content.

Results and Discussion

In this work an analytical HPLC method for assay and determination of content uniformity of Ezetimibe and Atorvastatin in a tablet formulation was developed and validated. The basic chromatographic conditions were designed to be simple and easy to use and reproduce and were selected after testing the different conditions that affect HPLC analysis, for example column, aqueous and organic components of the mobile phase, proportion of mobile phase components, detection wavelength, diluents and concentration of analyte. The on Zorbax SB C18 (250 mm×4.6 mm), column was used because of its advantages of high resolving capacity, better reproducibility, low-back pressure, and low tailing. For mobile phase selection, preliminary trials using mobile phases of different composition containing water adjusted to acid pH by addition of orthophosphoric acid and methanol resulted in poor peak shape. When methanol was replaced by acetonitrile better peak shape was obtained. The proportion of the mobile phase components was optimized to reduce retention times and enable good resolution of Ezetimibe and Atorvastatin from the degradation products. A detection wavelength of 236 nm was selected after scanning the standard solution over the range 200-400 nm by use of the UV detector. Detection at 236 nm resulted in good response and good linearity. The drug substance was easily extracted from the pharmaceutical dosage form using 0.02 M Potassium dihydrogen phosphate: Acetonitrile: Methanol (10:40:50, v/v/v). The tablet dispersed readily in water and the drug substance was freely soluble in acetonitrile. Solutions of standard and test preparations were found to be stable in this solvent mixture. By using the same concentration of analyte for assay and for determination of content uniformity both methods could be validated simultaneously except for determination of precision. To determine linearity a calibration graph was obtained by plotting Ezetimibe and Atorvastatin concentration against peak area. The method was linear in the range of 5–50 µg/ml for Ezetimibe concentration with a correlation co-efficient 0.9992 and in the range 5–60 µg/ml for Atorvastatin concentrations having correlation co-efficient 0.9994. The result was shown in Table 1, Figures 2 and 3. The accuracy of the method was assessed by determination of recovery for three concentrations covering the range of the method. The amount of Ezetimibe and Atorvastatin was recovered, in the presence of placebo interference, was calculated. The mean recovery of Ezetimibe and Atorvastatin was 100.55%, 100.49% respectively which is satisfactory and result were shown in Table 2.

Precision of the method were done in the % RSD for repeatability was found 0.63, 0.25 and Intermediate Precision was found 1.27, 0.53 for Ezetimibe and Atorvastatin respectively. The method precision was found 1.70 and 1.23 for both drugs respectively. From the data obtained the developed RP-HPLC method was found to be precise. The accuracy of the method was assessed by determination of recovery for three concentrations covering the range of the method. Known amounts of rosuvastatin (30, 40, and 50 µg/ml) were added to a placebo preparation and the amount of rosuvastatin recovered, in the presence of placebo interference, was calculated. The mean recovery of Ezetimibe and Atorvastatin was found 100.55%, 100.49 %, respectively which is satisfactory (Table 3). The robustness of the method was

Experiment	Ezetimibe			Atorvastatin		
	USP Tangent	USP Tailing	%RSD for replicate inj.	USP Tangent	USP Tailing	RSD for replicate inj.
Method precision	57812	1.09	0.13	71985	1.03	0.12
System precision	57234	1.12	0.58	71164	1.04	0.42
Accuracy	56594	1.21	0.12	71256	1.13	0.68
Ruggedness	57975	1.18	0.41	71624	1.16	0.47
Stability	57295	1.11	0.45	71985	1.12	0.23
Linearity	57866	1.13	0.54	71353	1.02	0.36
Degradation	57791	1.12	0.55	71619	1.34	0.34
Robustness	57866	1.10	0.77	71353	1.27	0.23

Table 4: System Suitability Data for Ezetimibe and Atorvastatin.

Sample	Ezetimibe Area counts (µv*sec)	Atorvastatin Area counts (µv*sec)	Ezetimibe		Atorvastatin	
			Assay 25mg	Percent Degradation	Assay 25mg	Percent Degradation
Sample (1 N HCl, 5ml)	197865	242001	22.63	9.48	22.12	11.52
Sample(1 N NaOH, 5ml)	195122	262735	23.75	5	22.09	11.64
Sample (H_2O_2 30%, 5ml)	196802	260685	21.88	12.48	2407	3.72
Sample Thermal Deg.(105°C/1hr)	185449	253395	20.63	17.48	23.79	4.84
Sample photolytic Deg. (2600 Lux/24 hr)	187514	452202	24.19	13.34	23.76	4.96

Table 5: Degradation Study for Ezetimibe and Atorvastatin.

Drug	n	Amount claimed (mg per tablet)	Amount Found (mg per tablet)	Mean Recovery %	RSD
Ezetimibe	6	10	10.165	101.65	1.02
Atorvastatin	6	10	10.025	100.25	0.95

Table 6: Result of Analysis of Ezetimibe and Atorvastatin in Tablet.

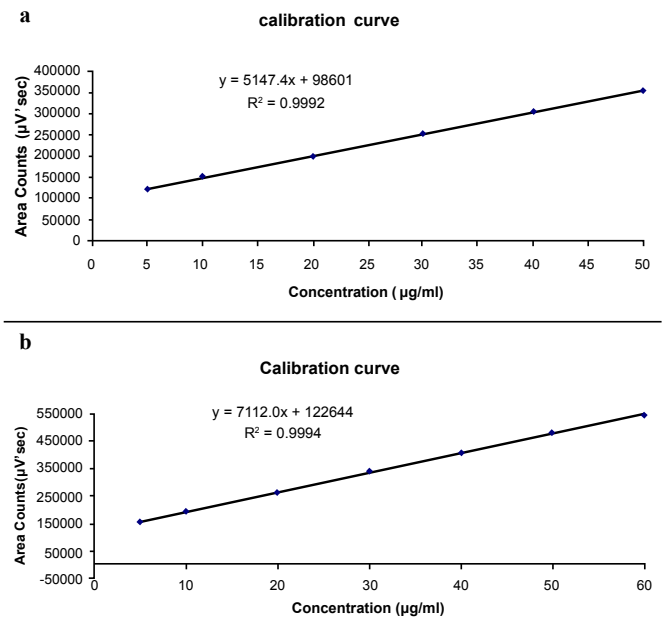

Figure 2: Calibration Curve of Ezetimibe and Atorvastatin.

assessed by assaying test solutions under different analytical conditions deliberately changed from the original conditions. For each different analytical condition the standard solution and test solution were prepared separately. The result obtained from assay of the test solution was not affected by varying the conditions and was in accordance with the true value (Table 3). System suitability data were also found to be satisfactory during variation of the analytical conditions (Table 3). The analytical method therefore remained unaffected by slight but deliberate changes in the analytical conditions. During study of the stability of stored solutions of standards and test preparations for assay determination the solutions were found to be stable for up to 13 h. Assay values obtained after 13 h were statistically identical with the initial value without measurable loss result were shown in Table 3. This furnished evidence that the method was suitable for its intended purpose. The specificity of the method was determined by checking for interference with the drug from placebo components. The specificity of the method was also evaluated by checking the peak purity of the analyte peak during the forced degradation study. The peak purity of the Ezetimibe and Atorvastatin peak under different stress conditions was 1.00, which is satisfactory and indicates there was no interference with the analyte peak from degradation products result were shown (Table 4). Major degradation up to 11.52% occurred under acidic conditions (Figure 3.5a). Under alkaline conditions the drug was degraded by approximately 11.64% (Figure 3.5b). The drug was approximately 3.72% degraded under oxidizing conditions (Figure 3.5c). The drug was degraded 4.84% under thermal condition and 4.96% degradation occurred under photolytic conditions (Figures 3.5d and 3.5e). Table 5 shows the degradation of drugs.

Conclusion

The intensive approach described in this manuscript was used to develop and validate a liquid chromatographic analytical method that can be used for both assay and determination of content uniformity of Ezetimibe and Atorvastatin in a pharmaceutical dosage form. Degradation products produced as a result of stress did not interfere

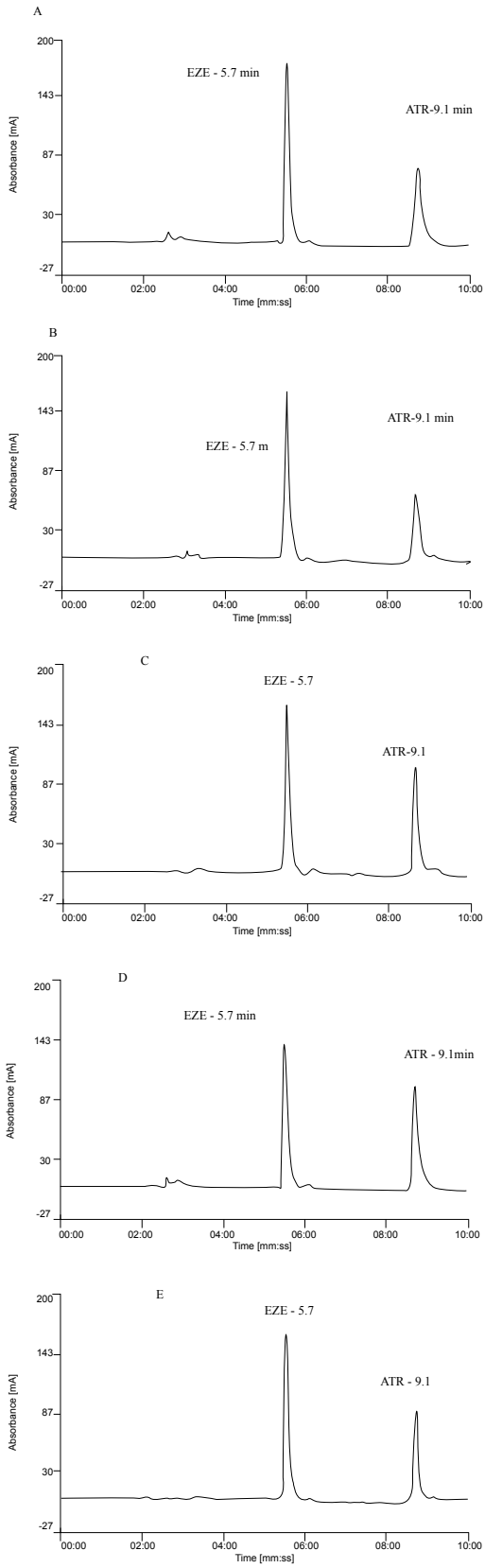

Figure 3: Chromatogram of Forced Degradation Study. Chromatographic profiles of rosuvastatin tablets after subjecting them to (a) acidic, (b) alkaline, (c) oxidative, (d) thermal and (e) photolytic degradation.

with detection of Ezetimibe, Atorvastatin and the assay method can thus be regarded as stability indicating. This HPLC method for assay and determination of content uniformity of Ezetimibe and Atorvastatin in a tablet formulation was successfully developed and validated for its intended purpose. The method was shown to specific, linear, precise, accurate, and robust. Because the method separates Ezetimibe and Atorvastatin and all the degradation products formed under variety of stress conditions it can be regarded as stability indicating. Because there is no pharmacopeial method for assay and determination of content uniformity of Ezetimibe and Atorvastatin in pharmaceutical dosage forms, this method is recommended to the industry for quality control of drug content in pharmaceutical preparations.

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