

Original Article

A VALIDATED STABILITY INDICATING RP-HPLC METHOD FOR THE SIMULTANEOUS DETERMINATION OF ATORVASTATIN CALCIUM AND EZETIMIBE HYDROCHLORIDE IN BULK AND TABLET DOSAGE FORM

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ABSTRACT

Objective: To develop a simple, selective and precise stability indicating reverse-phase high performance liquid chromatography method for the simultaneous estimation of atorvastatin calcium and ezetimibe hydrochloride in bulk and tablet dosage form.

Methods: The chromatographic separation was performed by Agilent Zorbax column (250×4.6 mm, 5 μm) using methanol: 0.1 % v/v orthophosphoric acid in water (65:35) as mobile phase at flow rate of 1.0 ml/min with injection volume 20 μl and the detection was carried out using UV detector at 240 nm. The method was validated as per ICH guidelines.

Results: The retention time for atorvastatin calcium (ATV) and ezetimibe hydrochloride (EZT) was found to be 6.81 min and 4.96 min respectively. The linear regression analysis data for the calibration plots showed good linear relationship in the concentration range of 5-50 μg/ml for both ATV and EZT. The percentage recoveries of ATV and EZT in the marketed dosage form were found to be 100.82 and 94.27 respectively. The correlation coefficients for ATV and EZT were 0.9983 and 0.998 respectively. The percentage degradation at different stress conditions like acid, alkaline, oxidative and photolytic for atorvastatin calcium were found to be 14.91, 8.26, 8.02 and 2.65 respectively and for ezetimibe hydrochloride, found to be 9.70, 32.18, 2.51 and 0.16 respectively.

Conclusion: The developed method was successfully validated as per ICH guidelines. This method is simple, selective, linear, precise, accurate and sensitive, and can be used for routine analysis of tablet dosage forms containing both the drugs.

Keywords: Atorvastatin calcium, Ezetimibe hydrochloride, RP-HPLC, Stress degradation

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INTRODUCTION

Anti-hyperlipidemic drugs are classified into various categories each acting through different mechanism enhancing each other's effect such as bile acid sequestrates reduce plasma low-density lipoprotein (LDL) levels by indirectly increasing the rate of LDL clearance, as a result of increased LDL receptors, from the bloodstream. These drugs are not orally absorbed and act locally in the gastrointestinal tract by binding with glycocholic acid and taurocholic acid, thereby increasing fecal excretion. HMG-CoA reductase inhibitors inhibit the rate-limiting step in cholesterol biosynthesis, enhance receptor-mediated LDL uptake and reduce very low-density lipoprotein (VLDL) precursors [1, 2]. Acyl Coenzyme A-Cholesterol Acyltransferase (ACAT) inhibitors bind to a specific transport protein located in the wall of the small intestine resulting in a reduction of cholesterol transport and absorption [3]; thereby lower plasma cholesterol levels by inhibiting the absorption of cholesterol at the brush border of the intestine [1]. Fibrates decrease plasma cholesterol levels and significantly decrease VLDL levels and has variable effect on LDL, such as in patients with hypertriglyceridemia, it raises LDL level while in patients with normal triglyceride levels, it lowers LDL level; nicotinic acid inhibits lipolysis in adipose tissue, initially resulting in sequential events that cause reduction in plasma triglycerides and cholesterol, and also decreases mobilization of free fatty acids, thus reducing plasma levels.

Atorvastatin calcium (ATV), is (βR, 8R)-2-(4-fluorophenyl)-α, δ-dihydroxy-5-(1-methylethyl)-3-phenyl-4-[(phenylamino)carbonyl]-1-H-pyrrole-1-heptanoic acid calcium salt (2:1) trihydrate (fig. 1). It is a member of the class of statins used primarily for reducing the blood cholesterol and to prevent associated events of cardiovascular diseases. Atorvastatin is a 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase which catalyses the conversion of HMG-CoA to mevalonate, in the rate-determining step in biosynthesis of cholesterol [4, 5]. ATV calcium typical dose is 10-80 mg per day, which reduces LDL levels by 40-60%.

Ezetimibe hydrochloride (EZT), is ((3R, 4S)-1-(4-fluorophenyl)-3-[(3S)-3-(4-fluorophenyl)-3-hydroxypropyl]-4-(4-hydroxyphenyl)-2-azetidione hydrochloride (fig. 1). It acts by reducing the absorption of cholesterol through the intestinal wall, from dietary and biliary sources. This leads to the reduction in delivery of cholesterol to the liver and hence promotes the synthesis of LDL receptors resulting in reduction in serum LDC-C [6]. Co-administration of ATV and EZT produces similar effect as that of high dose ATV (80 mg) monotherapy. Both ATV and EZT act by different mechanisms to reduce cholesterol levels in blood. Several analytical methods have been reported in the literature for the analysis of ATV [7-11] and EZT [12-16] individually and in combination [17-26] in pharmaceutical dosage forms.

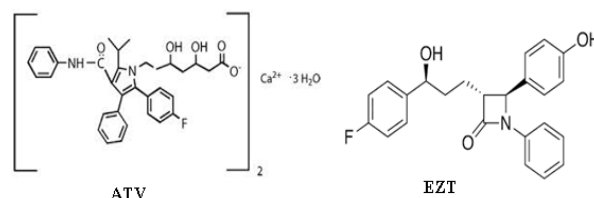


Fig. 1: Chemical structures of atorvastatin calcium and Ezetimibe

Some of the reported methods require SPE, evaporation, and other time-consuming preparation steps or expensive equipment that are not economically feasible for routine use in pharmacokinetic and pharmaceutical studies in which more number of samples are analyzed. Frequent clinical use of these two agents in combination provides a provision or the development of an analytical method for simultaneous analysis of ATV and EZT in pharmaceutical dosage form. The Liquid Chromatography with UV detection offers an

important advantages such as rapid set-up of instrumentation, versatility, and low cost and it has been proved to be a valuable method in the QC of drug compounds [27].

The literature survey revealed that there is no sensitive, simultaneous determination method on ATV and EZT in combined dosage forms. Till date no detailed report on the forced degradation studies on this combination under the mild stress conditions to resolve the drug from its potential impurities and degradation products. No interference of impurities and degradation products was observed during method development. Therefore, the aim of the present study was the development and validation of a simple, accurate, precise, stability-indicating, reliable and cost-effective RP-HPLC method for the simultaneous estimation of ATV and EZT in a combined commercial tablet form. Stability tests are performed on both drug substances as per International Conference on Harmonization (ICH) norms.

MATERIALS AND METHODS

Chemicals and reagents

ATV and EZT, in its pure form, were gifted by Biocon Ltd., Bangalore, India. Methanol and water of HPLC grade were purchased from S D-Fine Chemicals Ltd., Mumbai, India. Orthophosphoric acid and triethylamine were of analytical reagent grade. Methanol and water as a mixture, in the ratio of 1:1, was used as diluent to prepare working solutions of required concentrations. All the stock and working solutions were protected from sunlight and stored in a refrigerator at 4 °C.

Equipment and chromatographic conditions

A Shimadzu Separation Module LC-10AT HPLC, used for method development, forced degradation studies and method validation, equipped with VP Binary pump LC-10ATvp pump, SIL-10ADvp auto-sampler, CTO-10Avp column temperature oven, SPD-10Avp UV-Visible detector. All the components of the system are controlled using SCL-10Avp system controller. Data acquisition was carried out using LC solutions version 1.23 SP 1 software. Chromatographic separations were carried out using Agilent Zorbax column (150×4.6 mm, 5 µm), at room temperature (25 °C). The chromatograms were recorded, and the peaks were quantified using an automatic integrator. In this method, 1.0 ml/min flow rate was used for the separation with detection at 240 nm. The injection volume was 20 µl.

Preparation of standard solutions

Stock standard solutions of ATV and EZT were prepared with diluent at concentrations of approximately 1000 µg/ml for ATV and EZT, each. Both solutions were confined to the dark and used within 24 h to avoid decomposition. The concentrations of ATV and EZT varied in the range of 5-50 µg/ml each. The calibration curves for LC analysis were constructed by plotting the peak area of the drug to that corresponding drug concentration.

Generation of stressed samples for establishment of the stability-indicating assay

Degradation studies were performed with stress conditions of acid and base hydrolysis, oxidation and photolysis, to evaluate the ability of the proposed method to separate ATV and EZT from their

degradation products [28]. The optimized method was used to study the stress degradation behavior of ATV and EZT and may be applicable in stability testing of pharmaceutical dosage forms of routine analysis. The reactions were carried out at drug concentrations each 25 µg/ml of ATV and EZT. The stressed conditions were as follows: hydrolytic conditions-acidic hydrolysis, drug solution in 0.1 M HCl was exposed for 24 h; alkaline hydrolysis, drug solution in 0.1 M NaOH was exposed for 24 h; oxidative condition-drug solution in 3% H₂O₂ stored for 24 h; photolytic conditions-drug solution was exposed to UV light for 6 h.

Validation of the developed method

A similar method validation protocol was followed for both drugs.

System suitability

The system suitability was assessed by six replicates analyses of both drugs at concentrations 25 µg/ml each of ATV and EZT. The % CV of peak area and retention time for both drugs ATV and EZT are within 2% indicating that the suitability of the system (fig. 2 and table 1) [29].

Specificity

Specificity is the ability of the method to measure the analyte response in the presence of other drugs, excipients and their potential impurities (fig. 3) [30-34].

Linearity

Linearity of the method was established by triplicate injections of the solutions containing the drugs in the range of 5-50 µg/ml for each drug. The calibration curves were constructed and the acceptable fit to the linear regression was demonstrated and reported by the necessary parameters (fig. 4 and 5, table 2) [30-34].

Accuracy and precision

To determine the precision, triplicate injections of selected concentrations were analyzed, and the values of %RSD were calculated. In order to demonstrate applicability and accuracy of the proposed method, recovery tests are also carried out by analyzing the synthetic mixtures of the ATV and EZT. After five repeated experiments, the recoveries from these synthetic mixtures were calculated for each compound (table 3).

Assay of Statix-Ez® tablets

Ten tablets labeled to contain 10 mg of ATV, 10 mg of EZT and excipients, were weighed and finely powdered. An accurate amount of the powder equivalent to the content of one tablet was weighed, transferred into a 10 ml volumetric flask, diluted with diluent, stirred for 10 min, and then the volume was adjusted with the same diluent. After filtration, appropriate solutions were prepared by taking suitable aliquots from clear filtrate solution and were diluted with the diluent in order to obtain the final solutions. The content amounts of ATV and EZT were calculated from the corresponding regression equations. Recovery experiments from tablets also showed the reliability and suitability of the method. Accuracy was established across the specified range of the analytical procedure. The percentage of recovery and RSD were calculated for both the drugs.

Table 1: System suitability studies results of the proposed RP-LC method

Parameters	ATV	EZT	Recommended value
Retention time, min	6.81	4.96	-
Resolution (R _s)	4.65	0.00	>2
Theoretical plates (N)	3600.17	3355.17	>2000
Tailing factor	1.60 (avg)	1.70 (avg)	<2
Peak area	1028365.00 (avg)	1267154.17 (avg)	--
% RSD (for retention time)	1.80	1.70	<2

* Average of six injections each

RESULTS

Method validation

The U. S. Pharmacopeia (USP) suggests that system suitability tests be performed prior to analysis. System suitability for the proposed

method was evaluated. A system suitability test can be defined as a test to ensure that the method can generate results of acceptable accuracy and precision. The requirements for system suitability are usually designed after method development. And also the method developed has been validated as per ICH guidelines. The parameters

include tailing factor, retention factor, theoretical plate number, retention time, asymmetry factor, selectivity factor and RSD of peak height or area for repeated injections. Typically, at least two of these criteria are required to demonstrate system suitability for the

proposed method. Some of the tests were carried out on the freshly prepared standard solutions including two drugs. System suitability test results are reported in table 1 and fig. 2, and found to satisfy the USP requirements.

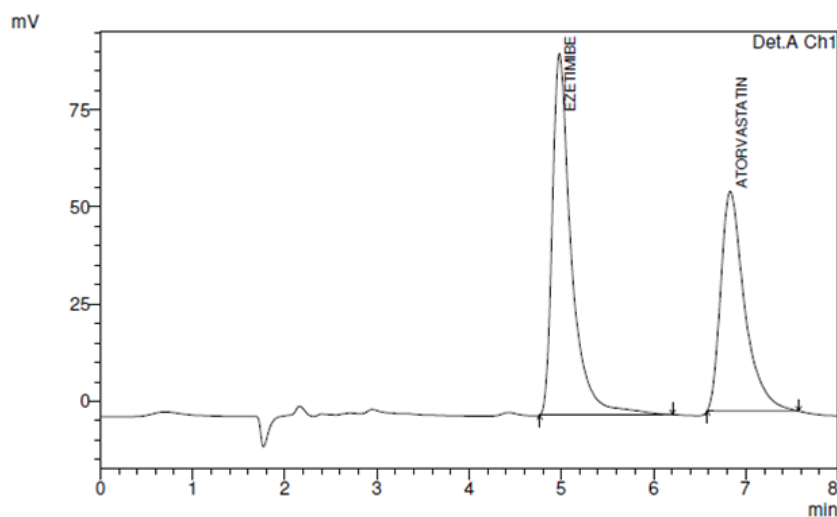


Fig. 2: System suitability

Specificity

The specificity of the method is performed by separate injections of the blank, ATV, EZT and combined ATV & EZT samples.

The specificity chromatogram was shown in fig. 3, where the retention time of ATV does not interfere with the retention time of the EZT.

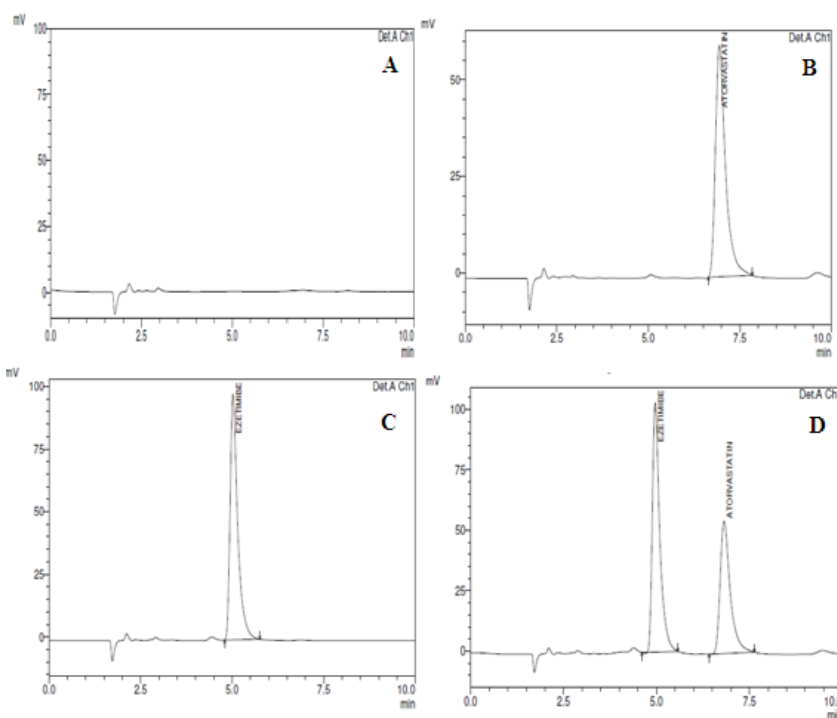


Fig. 2: Typical specificity chromatograms: A) blank sample: B) EZT: C) ATV: D) Both ATV and EZT

Linearity

Linearity was established by least squares linear regression analysis of the calibration curve. The constructed calibration curves were linear over the concentration range of 5-50 $\mu\text{g/ml}$ for both the

drugs. Peak areas of ATV and EZT were plotted versus their respective concentrations in the mobile phase, and linear regression analysis performed on the resultant curves and was confirmed by the high value of the correlation coefficients 0.9983 & 0.998 for ATV and EZT respectively (table 2). Calibration curves of ATV and EZT

were shown in fig. 4 and 5. The LOD and LOQ were calculated from the following equations using the standard deviation (SD) of the response of the analyte (low concentration) and the slope (m) of the corresponding calibration curve:

$$\text{LOD} = 3.3 \times s + m$$

$$\text{LOQ} = 10 \times s + m$$

Limit of detection and quantification

Limit of detection (LOD) and quantification (LOQ) was estimated from signal-to-noise ratio. The limit of detection (LOD) value was found to be 0.0035 µg/ml and 0.0078 µg/ml, and limit of quantification (LOQ) values was 0.0107 µg/ml and 0.0235 µg/ml for ATV and EZT respectively.

Table 2: Statistical evaluation of the calibration data of ATV and EZT by LC

Compounds	ATV	EZT
Retention time, min	6.81	4.96
Linearity range, µg/ml	5-50	5-50
Slope	48147	63168
Intercept	65564	52660
Correlation coefficient	0.9983	0.998
LOD, µg/ml	0.0035	0.00776
LOQ, µg/ml	0.01069	0.0235
Precision (RSD %)	1.206	0.93

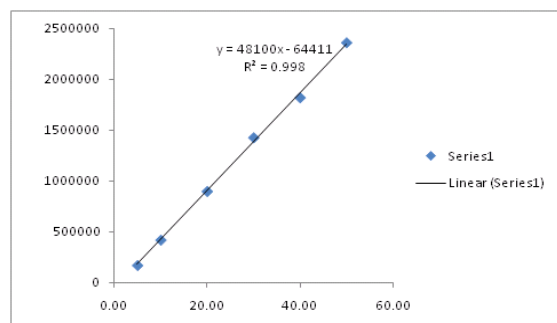


Fig. 4: Linearity graph for ATV

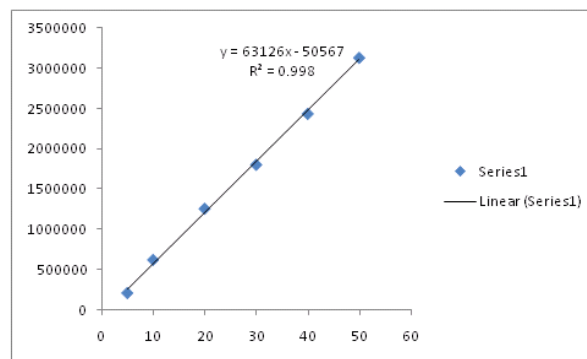


Fig. 5: Linearity graph for EZT

Precision

Precision data is summarized in table 3 and was assessed by using quality control samples prepared at three different concentrations (LQC, MQC and HQC) in the mobile phase. Precision was investigated by injecting triplicate aliquots of the samples. All the data were within the acceptance criteria of 5%.

Accuracy

In order to demonstrate the accuracy, validity, and applicability of the proposed LC method, recovery tests were carried out by analyzing synthetic mixtures of ATV and EZT with different concentrations. Accuracy data for the assay following the determination of each of the compounds of interest are summarized in table 3. In each case, %RSD and accuracy (recovery) were calculated for each drug. All the data were within the acceptance criteria of 5% except in EZT concentrations of 12.54 and 25.08 µg/ml, the % recovery was found to be 93.60 and 92.65 respectively.

Recovery

The recovery analysis results were shown in table 3. The results of the recovery assay indicated that the method was selective, accurate and precise for both ATV and EZT, without the interference of excipients, used to formulate and produce the tablet dosage form studied.

Table 3: Accuracy and precision data

	LQC		MQC		HQC	
	ATV	EZT	ATV	EZT	ATV	EZT
Mean	13.24	11.74	24.62	23.24	37.49	36.33
SD	0.22	0.09	0.25	0.24	0.29	0.36
% RSD	1.68	0.78	1.01	1.04	0.76	0.98
% Recovery	105.26	93.60	97.84	92.65	99.37	96.56

* Average of three injections each

Table 4: Ruggedness studies of ATV and EZT

	Drug	Retention time (min)	Tailing factor	Theoretical plates
Analyst 1	ATV	6.79	1.64	3402
	EZT	4.94	1.70	3209.33
Analyst 2	ATV	6.81	1.63	3465
	EZT	4.97	1.69	3277

* Average of three injections each

Ruggedness

Ruggedness was determined by using the data obtained by the analysis performed by two different analysts. Each

Robustness

Robustness is the measure of method capacity to retain unaffected by deliberate small changes in the chromatographic conditions. The

impact of flow rate (± 0.1 ml) and effect of mobile phase composition ($\pm 5\%$) was evaluated on the important system suitability factors such as retention time, theoretical plates and tailing factor were studied. Results are shown in table 5.

Stress testing studies

The degradation product(s) of each of the parent compound was found to be same for mild stress conditions [35, 36]. Typical

chromatograms obtained for the control samples and stressed samples of ATV and EZT, using mild conditions are shown in fig. 6 A-E and 7 A-E, respectively. Possible degradation products were observed in ATV and EZT stressed samples that were subjected to light, oxidation and acid and base hydrolysis under mild conditions. ATV and EZT were stable when exposed to UV light. ATV and EZT were clearly degraded during stress by oxidation, acid and alkaline

hydrolysis (table 6) leading to the formation of some unknown degradation peaks (fig. 6 & 7). Peak purity test results derived from the UV detector confirmed that the ATV and EZT peaks were homogeneous and pure in nearly all analyzed stress samples. Both ATV and EZT responses were successfully resolved from degradation products with the proposed method, as shown in fig. 6 and 7, respectively.

Table 5: Robustness studies of ATV and EZT

	Parameters	Variation	Retention time (min)	Tailing factor	Theoretical plates
ATV	Flow rate	0.9 ml/min	7.23	1.68	3666
		1.1 ml/min	6.06	1.65	3351
	Mobile phase	60 % organic phase	14.52	1.71	4180.33
		70 % organic phase	3.91	1.62	2743.67
EZT	Flow rate	0.9 ml/min	5.25	1.75	3372.67
		1.1 ml/min	4.42	1.72	3073.33
	Mobile phase	60 % organic phase	9.04	1.77	3820.33
		70 % organic phase	3.26	1.69	2517

* Average of three injections

Table 6: Results of hydrolytic (acidic and alkaline), oxidizing and photolytic mild stress conditions of ATV and EZT

Response to mild conditions	Peak area		Degradation of compounds, % ^a	
	ATV	EZT	ATV	EZT
HCl (0.1M)	945790	1242175.5	14.91	9.70
NaOH (0.1M)	1019736.5	932926.5	8.26	32.18
H ₂ O ₂ (3 %)	1022459.5	1341167	8.02	2.51
UV (24 h)	1082105.5	1377870.5	2.65	0.16
Control sample	1111576	1375683	-	-

^aEach value is the mean of three experiments

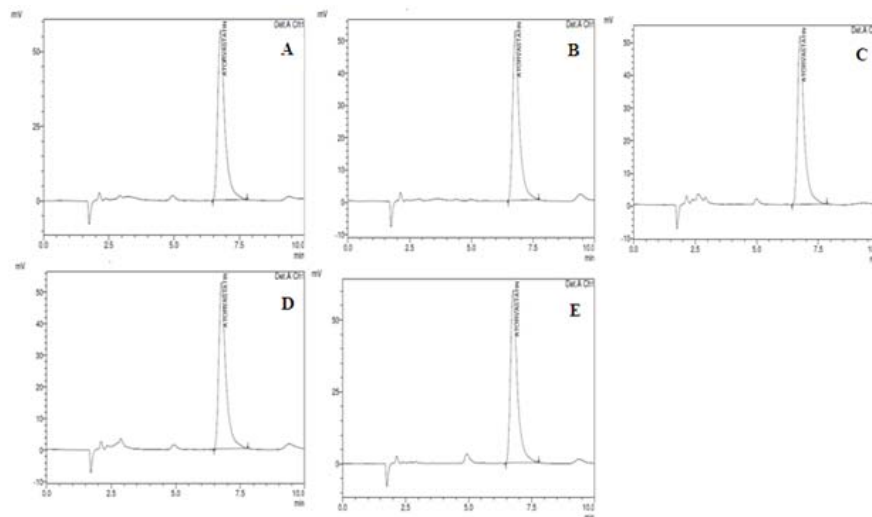


Fig. 6: Typical degradation chromatograms of ATV; A) Control sample; B) 0.1 N HCL at 25 °C after 24 h; C) 0.1 N NaOH at 25 °C after 24 h; D) 3 % H₂O₂ at 25 °C after 24 h; E) UV light (210 nm) after 24 h

Assay of Statix-Ex® tablets

After working on synthetic mixtures, results encouraged the use of the proposed method for the simultaneous assay of ATV and EZT in a commercial tablet dosage form. The results corresponding to the tablet dosage form of ATV and EZT are shown in table 3. The proposed RP-LC method could be used for the simultaneous determination of ATV and EZT in the presence of each other and without prior separation of the excipients. Each tablet contained 10.0 mg ATV as atorvastatin calcium, 10.0 mg EZT as ezetimibe hydrochloride, and the inactive ingredients. Removal of the excipients before analysis was found to be unnecessary. fig. 8 shows a typical chromatogram obtained for the analysis of ATV and EZT in

the tablets with well-shaped, symmetrical single peaks, well-separated from the solvent front. No interfering peaks were obtained in the chromatogram due to tablet excipients. The determined amount of drugs indicates that the active ingredients in samples were present at level included within the requirements with respect to the label claimed by the manufacturer. The utility of the proposed method was verified by means of triplicate analyzes of the pharmaceutical preparation.

Results obtained from the proposed method of the analysis of both drugs in the tablet formulation indicated that the proposed technique can be used for simultaneous quantification and routine QC analysis of this binary mixture in the pharmaceutical product.

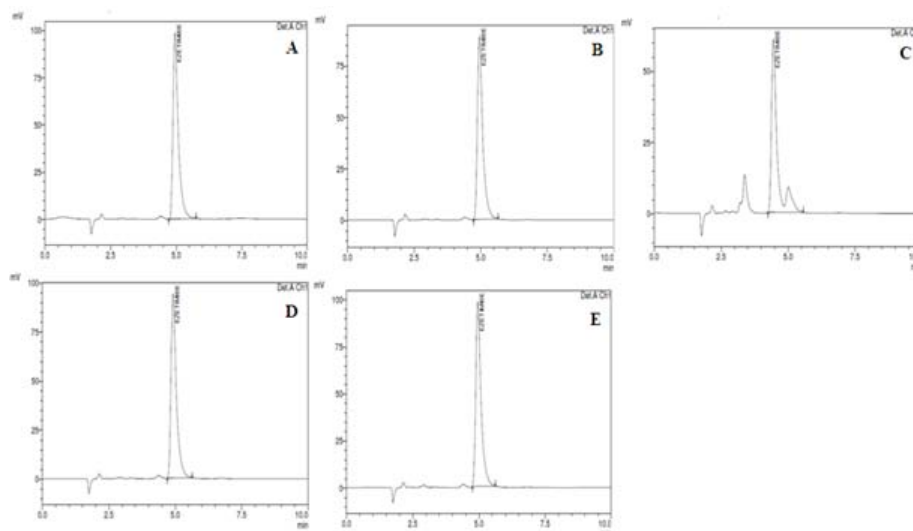


Fig. 7: Typical degradation chromatograms of EZT; A) Control sample; B) 0.1 N HCL at 25 °C after 24 h; C) 0.1 N NaOH at 25 °C after 24 h; D) 3 % H₂O₂ at 25 °C after 24 h; E) UV light (210 nm) after 24 h

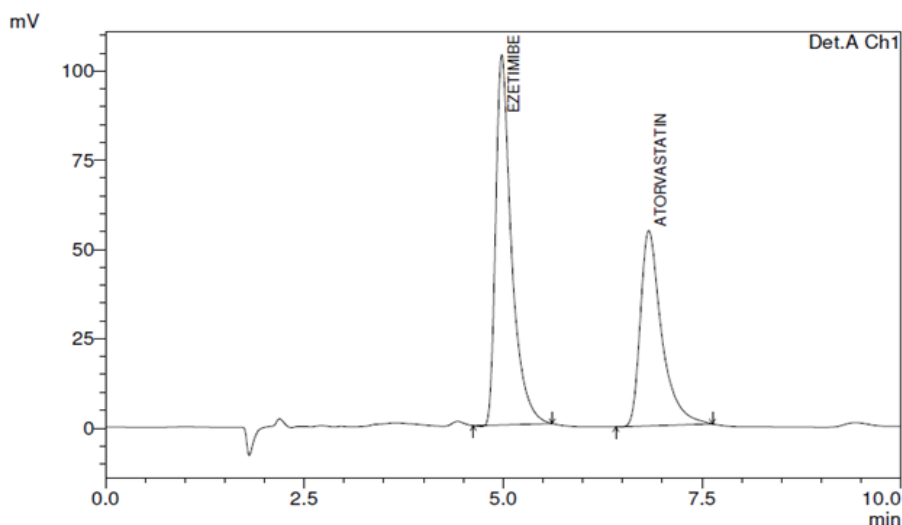


Fig. 8: Assay of Statix-Ez®

DISCUSSION

The composition, pH and flow rate of the mobile phase were changed to optimize the separation conditions using standard substances of the two drug analytes. In order to effect the simultaneous separation of the working compounds, mixtures of methanol and 0.1% v/v OPA in different combinations with different ratios and at various flow rates were evaluated. Finally, an RP Agilent Zorbax column (150 x 4.6 mm, 5 µm) and the mobile phase methanol: 0.1% v/v OPA (65:35) was found to be most suitable for LC analysis. A flow rate of 1.0 ml/min was selected for further studies after several preliminary investigatory chromatographic runs. The selected operating conditions were found to be optimal for sharp and symmetric peak shapes as well as to achieve minimal background noise. Under the described experimental conditions, all the peaks were well-defined and free from tailing. The proposed method was successfully used for the simultaneous determination of ATV and EZT in a tablet dosage form.

With the optimized operating conditions, the retention times corresponding to ATV and EZT were 6.81 min and 4.96 min, respectively and were stable among injections. However, the analysis time was set to 10 min, allowing elution of possible excipients and degradation products that could be retained, without the need of a

further stabilization time between injections. The proposed RP-LC method provided simple, simultaneous determination of the ATV and EZT in the drug by UV detection at 240 nm. After determining the optimum conditions, a satisfactory resolution was obtained in a short analysis time (about 7 min). For both the compounds, sharp, symmetrical and well-resolved peaks were obtained (fig. 2).

Having optimized the efficiency of a chromatographic separation the quality of the chromatography was monitored by applying the system suitability tests. The acceptance criterion was $\pm 2\%$ for the percent coefficient of variation for the peak area and retention times for ATV and EZT. The number of theoretical plates should not be less than 2500 and the tailing factor should not be more than 2.0. The peak purity of AZT and EZT were assessed by comparing the retention time (Rt) of standard and sample. A chromatogram obtained from reference substance solution is presented. The linearity range for AZT and EZT were found to be 5-50 µg/ml. The results of linearity studies are shown in table 2. Both precision and accuracy were determined with standard quality control samples prepared in triplicates at different concentration levels covering the linearity range. The repeatability and intermediate precision are reported as % RSD in table 3 and the minimum variation in the % RSD indicate that the present method is precise. The accuracy of the proposed method was assessed by adding known amount of the

drug to a drug solution of known concentration and subjecting the samples to the proposed HPLC method. All solutions were prepared and analyzed in triplicate. The above procedure is adopted for AZT and EZT with high recovery values obtained (table 3) indicate that the proposed method is highly accurate. To evaluate the robustness of the developed RP-HPLC method, small deliberate variations in the optimized method parameters were done. The effect of a change in flow rate, pH and mobile phase ratio on the retention time and tailing factor were studied. The values for proposed method are well within acceptance limits with a % RSD of less than 2.0. Above experiments indicated that the method is rugged and provides consistent and reliable results. The method specificity was assessed by studying the chromatograms obtained for a mixture of the drugs and the common excipients. As none of the excipients interfered with the analytes of interest, the method was found to be suitable for analyzing the commercial formulation of these drugs.

CONCLUSION

A simple, rapid, accurate, and precise stability-indicating RP-LC method has been developed and validated for routine analysis of ATV and EZT standard samples and a tablet dosage form. Using the described chromatographic conditions, ATV and EZT were well-separated. The proposed method gives good resolution between selected compounds within short analysis time. The method developed would serve as a versatile analytical tool suitable for the simultaneous analysis of the drugs in the tested formulation and would be of interest for QC and clinical monitoring laboratories. Recovery studies exhibited that the method was free from interference by the excipients in the tablet dosage form. The results of stress testing undertaken according to the ICH guidelines revealed that the method is selective and stability-indicating. The proposed RP-LC method can be used for the routine analysis of production samples and to check the stability of bulk samples of ATV and EZT.

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CONFLICT OF INTERESTS

Authors do not have any conflict of interest in this research work

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