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Impurity Estimation with Structural Elucidation and Correlation of X-Ray Diffraction Pattern with Dissolution Study of Atorvastatin-Ezetimibe Combination Marketed Formulation with Degradation



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ABSTRACT

Estimation of unknown impurities in a pharmaceutical formulation meant for commercial purpose is of utmost importance because it directly contributes to the safety of drug therapy and efficacy of proposed formulation for the desired treatment to the patients. Atorvastatin and Ezetimibe both are administered as solid dosage form in combination therapy to achieve maximum and effective drug activity, excellent efficacy. In order to determine Impurities, a simple, accurate, economical and reproducible procedure for quantitative HPLC analysis of Atorvastatin and Ezetimibe as in the tablet solid dosage form was developed and validated. Furthermore, forced degradation studies were carried out and structural elucidation of impurities by Fourier Transform Infra-Red spectroscopy and Mass spectroscopy with impurity profiling exhibited good analytical techniques to estimate nature of related substances or impurities present in the manufactured formulation (solid dosage form). Also, dissolution studies carried out using calibrated dissolution apparatus (USP II) paddle 60 rpm & bath temp at $37 \pm 1^\circ\text{C}$. Nine hundred milliliter freshly prepared and degassed 0.1N HCl solution was used as dissolution medium. X-ray diffraction pattern correlates with the dissolution and solubility aspects of the combination. Thus overall study showed method development and validation for estimation of impurities in marketed formulation with reproducible results.

INTRODUCTION

Atorvastatin is selective as well as competitive HMG-CoA reductase inhibitor and Ezetimibe exhibits lipid-reducing mechanism of action as an active medicament as sole administration or in combined dosage form. Thus both are potentially used in combination formulation in order to reduce the amount of Cholesterol and triglycerides in systemic circulation. Available literature review reveals that several methods have been reported for analysis of Atorvastatin by high-performance liquid chromatography (HPLC) and high-performance thin layer chromatography (HPTLC) as well as for estimation of Ezetimibe by HPLC, either as a solo drug or combination with other drugs but no precise and reproducible HPLC method has yet been reported for the estimation of unknown impurities in Atorvastatin- Ezetimibe marketed tablet formulation. Also, no simple and sensitive isocratic RP-HPLC method with PDA detection has been reported for the determination of unknown impurities in desired anti-hyper-lipidaemic drug combination treatment. The purpose of this research was to establish and validate, as well as to elucidate structural aspect as well as correlation of X-ray Diffraction for crystallinity and solubility in Dissolution apparatus; in accordance with International Conference on Harmonization (ICH) guidelines, a simple, accurate, economical and reproducible procedure for quantitative HPLC analysis of Atorvastatin and Ezetimibe as in the tablet solid dosage form.

MATERIALS AND METHODS:

HPLC grade Acetonitrile, (Batch no. R054E11) was purchased from Rankem Chemicals (Mumbai, India). Analytical reagent grade Ammonium acetate and Glacial acetic acid were purchased from Merck Chemicals (Mumbai, India). HPLC grade Tetrahydrofuran was supplied by "LOBA Chemie Pvt. Ltd., Mumbai, India". HPLC grade Water was obtained from JK Labs, Mumbai, India. Pure drug sample of Atorvastatin, % purity 99.37 and Ezetimibe, % purity 99.14 was obtained as a gift sample from Aarti Drugs Pvt. Ltd. and Zim laboratories, Mumbai respectively. These samples were used without further purification. Tablet formulations named GRASALIP FORTE containing labeled amount of 20 mg of Atorvastatin and 10 mg of Ezetimibe was obtained from (Gen Pharma, Pune). The X-ray diffraction analysis was carried out with a fifth generation Rigaku Miniflex 600 X-ray powder diffractometer equipped with a 600 watt X-ray tube, a copper anode operating in reflectance mode at wavelength $k\alpha \lambda$ 1.5418 Å, voltage of 40 kV and current 15 mA.

Selection of stationary phase:

It is clear from the molecular structure that all compounds do not possess a functional group that can readily ionize indicating non-polar in nature. But the presence of hydroxyl, carbonyl, and amine groups shows some polar nature. Hence we started the development activity with C₈ stationary phase of various manufacturers using different mobile phases. The poor resolution between impurities and broad peak shape for Atorvastatin and Ezetimibe implies that C₈ stationary phase is not suitable for this application. Hence C₁₈ stationary phase was chosen to improve resolution among the peaks and peak shape for Atorvastatin and Ezetimibe. The peak shape for Atorvastatin and Ezetimibe as well as resolution among all components improved with Inertsil C₁₈, 250mm×4.6mm, 5μ columns. But the stationary phase is not only the parameter, which can give better separation among all impurities. Mobile phase, pH and organic modifies also plays very important role which leads to the best separation. For stationary phase selection; trials on following columns were conducted:

Table No. 1 HPLC columns screened during method development

Sr. No.	Column	Observation	Chromatograph Ref. No.
1.	C 18 Hypersil BDS, 250X4.6mm, 5μ	Potassium phthalimide peak is not separated API peak tailing was seen Stage A peak was well separated	1
2.	Inertsil ODS 250X4.6mm, 5μ	All peaks are well resolved	2

Reference Chromatograph for Column Selection

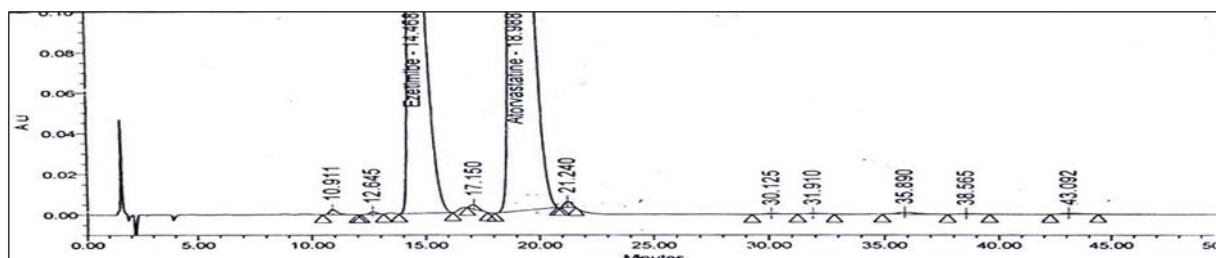


Fig. No. 1 Hypersil BDS Column

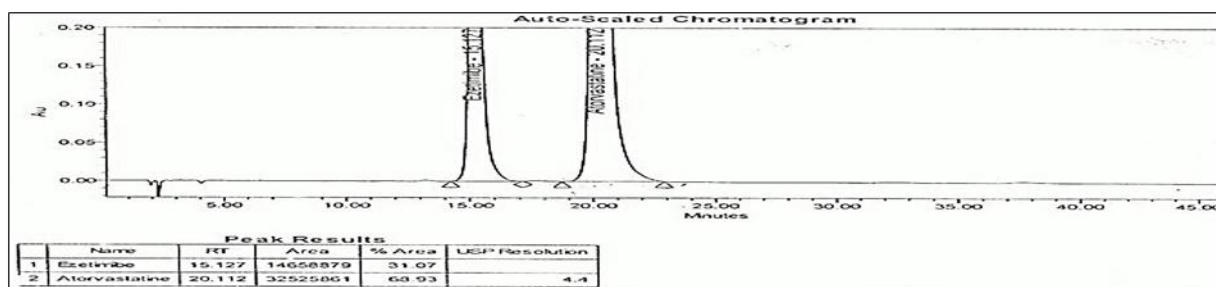


Fig No. 2 Inertsil ODS Column

Thus the column showing optimum selectivity was found out to be Inertsil ODS 250X4.6mm, 5 μ .

Gradient Selection

In gradient elution, the composition of the mobile phase changes during the run. Binary-solvent mobile phases A and B generally are used in gradient elution, with the concentration of the strong solvent A (% A) increasing during the run. Optimized time program for gradient elution was as follows and observed in Fig.No.3.

Table No. 2 Selection of Gradient

Time (min.)	% Mobile Phase A	% Mobile Phase B
0.0	60	40
2.0	60	40
7.0	50	50
14.0	60	40
28	60	40

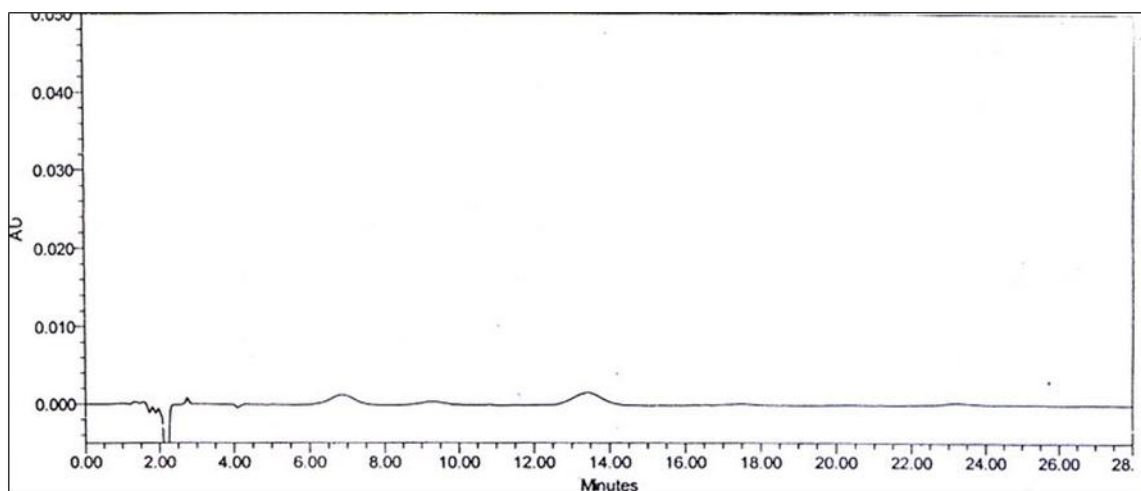


Fig. No.3 Reference Chromatograph for gradient selection (Diluent)

Chromatographic Conditions

The isocratic elution of mobile phase composition (Buffer: Acetonitrile: THF) (60: 35: 5 v/v/v) pH 4.0 at the flow rate of 1.5 ml min^{-1} was carried out. The runtime was set at 25.0 minutes. The column oven temperature was maintained at 50°C ($\pm 3^{\circ}\text{C}$). While the sampler temperature was 10°C ($\pm 2^{\circ}\text{C}$). The volume of injection was $20 \mu\text{L}$, prior to injection of analyte; the column was equilibrated for 30-40 min with mobile phase. UV Detector signal was monitored at a wavelength of 244 nm.

Standard Solutions and Calibrations Graphs

Stock solution of Atorvastatin and Ezetimibe ($1000 \mu\text{g/ml}$) were separately prepared in the diluents i.e. Water and Acetonitrile in the ratio of (60:40). To study the linearity range of each component, serial dilutions of ATV and EZT each were made from 1.0 to $15.0 \mu\text{g/ml}$ and 0.50 to $7.50 \mu\text{g/ml}$, respectively in mobile phase and injected on to column. Calibration curves were plotted as concentration of drugs versus peak area response. From the standard stock solutions, a mixed standard solution was prepared containing the analytes in the given ratio and injected on to column. The system suitability test was performed from six replicate injections of mixed standard solution. A typical chromatogram obtained from a standard solution is shown in Fig. No. 4.

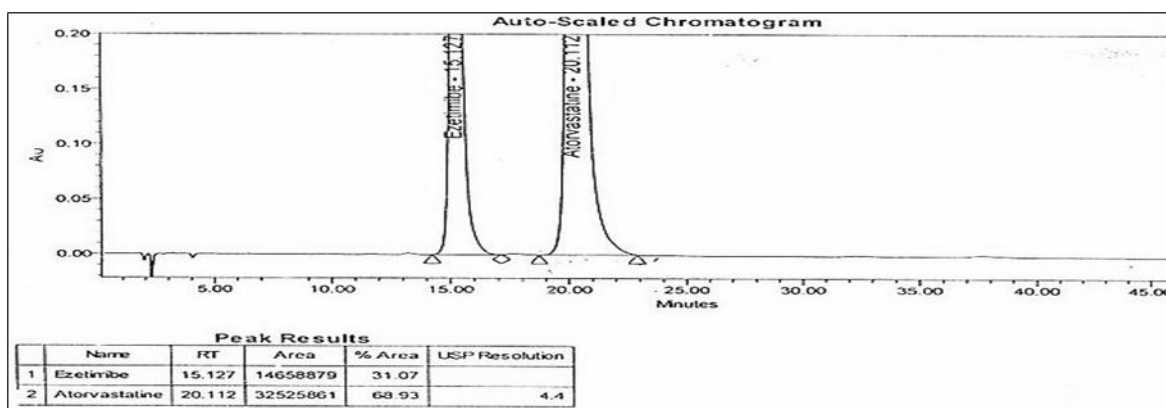


Fig. No. 4 Typical Chromatogram of the formulation consisting ATV and EZT

Analysis of Tablet Formulation:

Twenty tablets were weighed accurately and a quantity of tablet powder equivalent to 20 mg of ATV (10 mg of EZT) was weighed and dissolved in 10 ml of diluents i.e. Water and Acetonitrile mixture (60:40) with the aid of ultra-sonicator for 10 min. and solution was filtered through GF/C filter paper into a 1000 ml volumetric flask. The above solution was centrifuged at 2500 rpm in the research centrifuge for 10 minutes and was filtered through 0.45- μ m nylon filter. Filter paper was washed with the solvent, adding washings to the volumetric flask and volume was made up to the mark. The solution was suitably diluted with the diluents to get of 10 μ g/ml of ATV (5 μ g/ml of EZT), filtered through 5 microns, nylon66 membrane filter and injected on to column. The first 10 ml of the filtrate was discarded and subsequent filtrate was utilized as sample solution for assay and was analyzed as given under the described chromatographic conditions. Chromatogram was recorded and the amounts of drugs were calculated.

Details of Marketed Tablet Formulation Used for Analysis:

Contents: Atorvastatin 20 mg. & Ezetimibe 10 mg.

Manufacturer: Gen Pharma, India.

Brand Name: GRASILIP FORTE

Selection of analytical wavelength:

From the standard stock solution further dilutions were prepared using mobile phase and scanned over the range of 200-400 nm and the spectra were overlain. λ_{\max} observed for ATV and EZT were 244.0 nm and 235.8 nm respectively. With reference to Fig.5.5, 244 nm wavelengths were selected for simultaneous determination of Atorvastatin and Ezetimibe.

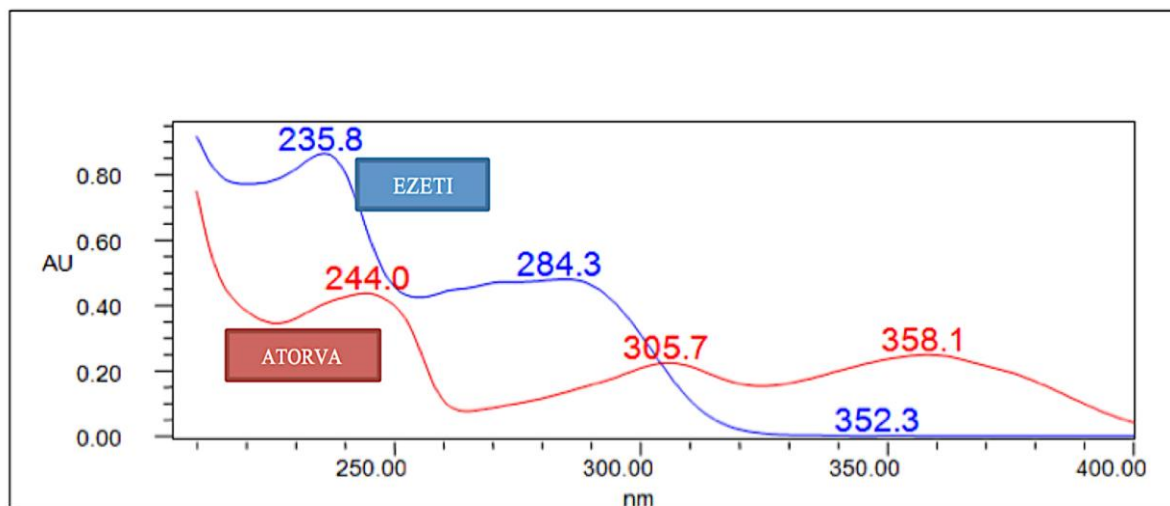


Fig. No. 5 Analytical wavelength Selection for analytes

METHOD OPTIMIZATION:

A well-defined symmetrical peak was obtained upon measuring the response of eluent under the optimized conditions after thorough experimental trials that can be summarized. Two columns were used for performance investigations, including Inertsil C18 (4.6 mm x 25 cm, 5.0 μ) and Hypersil C18 (250 mm x 4.6 mm, 5.0 μ), the First column was the most suitable one since it produced symmetrical peaks with high resolution. The UV detector response of was studied and the best wavelength was found to be 244 nm showing highest sensitivity.

Mobile phase composition:

Several modifications in the mobile phase composition were performed in order to study the possibilities of changing the selectivity of the chromatographic system. These modifications included the change of the type and ratio of the organic modifier, the pH, the flow rate, the temperature, the concentration of Ammonium acetate Buffer with Acetonitrile and Tetrahydrofuran (THF) etc.

Standard Solution Preparation:

5.0 mg of Ezetimibe was accurately weighed and transferred to 100 ml volumetric flask, and then 50 ml of diluent was added. The dilution was kept for sonication for 10.0 minutes and volume was made up with the diluent. Further 1ml of this solution was withdrawn and volume make up to 10 ml was done with Water and Acetonitrile.

Placebo Preparation:

Placebo equivalent to 20 mg of Atorvastatin was weighed and transferred to a 20 ml volumetric flask. 10 ml of diluent was added and it was kept in Ultra-sonicator for 10.0 minutes. The solution was cooled to room temperature and diluted with the diluent up to the mark. Further, it was filtered through Whatman GF/C filter paper. First few ml of the solution was discarded and further injected into the system.

Sample Preparation:

20 tablets were powdered and weighed equivalent to 20 mg of Atorvastatin. Then it was transferred to 20 ml volumetric flask. 10 ml of diluent was added & it was kept for sonication for 10 minutes, then it was cooled to room temperature & further it was diluted with diluent up to the mark. Then the solution was filtered through Whatman GF/C filter. First few ml of the solution was discarded and then solution injected into the HPLC system. The Diluent and, System suitability solution and five replicate standard solutions were injected separately. The chromatogram of system suitability was recorded & then observed the resolution between Ezetimibe and Atorvastatin is not less than 3.0 and, the % RSD for five replicate injections in standard solution is not more than 5.0

Type of organic modifier:

Initially, the methanol was used as an organic modifier, which gives the poor baseline with baseline drift. Hence the response for drug compounds was reduced to improve the peaks shape and peak response, Acetonitrile was tried as an organic modifier. The baseline was found good and peak response was improved. The peak shape and peak symmetry was also improved and hence Acetonitrile was selected as organic modifier.

Ratio of organic modifier:

The effect of changing the ratio of organic modifier on the selectivity and retention times of the test solutes was investigated by using mobile phases containing concentrations of 70-50% Acetonitrile. Table 1 showed that 70% Acetonitrile was the best one giving well symmetrical peak and higher number of theoretical plates. Ratios less than 70% resulted in peak with very long unacceptable retention times, whereas ratios higher than 70% with decreased peak purity angle.

Effect of pH:

The effect of changing the pH of the mobile phase on the selectivity and retention times of the test solutes was investigated using mobile phases of pH ranging from 2.0-6.0. The pH 4.0 with Ammonium acetate in presence of glacial acetic acid was the most appropriate one giving well-resolved peaks and highest number of theoretical plates. At pH values > 4.0 produced peak broadening in the mobile phase.

Effect of Flow rate:

The effect of flow rate on the formation and separation of peaks was studied by varying the flow rate from 0.8- 2.0 a flow rate of 1.5 ml/min was optional for good shape and symmetry of peaks in a reasonable time.

Effect of Temperature:

The effect of temperature on the formation, separation and resolution was studied by varying the temperature from 10 - 40°C; we found that at higher Temperatures the peaks are not well resolved, whereas at temperature 10°C the peaks show good symmetry and purity.

Table No. 3 System suitability parameters with peak purity data as per USP; NF*

System Suitability Parameter	ATORVASTATIN	EZETIMIBE
Retention Time	18	13
Theoretical plates ^a (T.P.)	5142	5764
Peak Tailing ^a	NMT 1.6	1.12
K prime	1.23	2.49
% R.S.D. (T.P.)	0.75	0.78
PA and PT	0.117(0.739)	0.117(0.748)

Validation of Established method:

Parameters studied:

The established method was validated in terms of system suitability, specificity, linearity and range, precision, accuracy, limit of detection, limit of quantification, solution stability and robustness as per USP and ICH guidelines.

System suitability

The system suitability test was performed to ensure that the complete testing system was suitable for the intended application and it was performed by injecting the five replicate injections of standard preparation (20 µg mL⁻¹). Thus, 10.4 mg of Atorvastatin and 5.0 mg Ezetimibe were weighed accurately and transferred to 10 ml volumetric flask, further 5 ml of diluents was added. Then dilution was kept in ultra-sonicator for 5.0 minutes and further volume was made up with diluent i.e. water and Acetonitrile (60:40). The parameters measured were retention time, resolution, peak area and peak threshold of Atorvastatin and Ezetimibe.



Linearity:

The stock solution was prepared by dissolving accurately weighed 10.4mg of Atorvastatin and 5.0 mg of Ezetimibe in 100 ml of mobile phase to obtain a final concentration of 1.0 mg/ml of ATV and 0.50mg/ml of EZT respectively. From this stock solution, Standards within concentration range 1-15 µg/ml for ATV and 0.50-7.50 µg/ml for EZT were freshly prepared in mobile Phase prior to analysis.

a) Working Standard and Test Sample Details:

Analyte:	Atorvastatin
Working Standard No.:	1
Purity (As determined):	95.00%
Average Wt./ Net Fill Content:	0.10 mg
Label Claim:	1.00 mg

Molecular Weight of Base: 1115.34

Molecular Weight of Salt: 1155.42

Multiplication Factor: 0.965

b) Working Standard and Test Sample Details:

Analyte: Ezetimibe

Working Standard No.: 1

Purity (As determined): 99.28%

Average Wt./ Net Fill Content: 0.10 mg

Label Claim: 1.00 mg

Molecular Weight of Base: 1.00

Molecular Weight of Salt: 1.00

Multiplication Factor: 1.00



Eight replicates (n=8) per concentration were injected and Chromatograms were recorded. Respective calibration curves were plotted of Area against concentration of each drug. The following table was followed for preparing stock solution in order to determine Linearity.

Table No. 4 Area observed for different ranges of Concentrations of ATV

Levels	Linearity Stock Soln. (ml)	Placebo (mg)	Diluted to (Volume make up ml)	Conc. of ATV (ppm)	Conc. of EZT (ppm)
10 %	1	161.5	100	1.0	0.50
15 %	1.5	160.9	100	1.5	0.75
20 %	2	159.9	100	2.0	1.00
30 %	3	160.8	100	3.0	1.50
40 %	4	160.5	100	4.0	2.00
50 %	5	160.3	100	5.0	2.50
100 %	10	160.1	100	10.0	5.00
150 %	15	160.5	100	15.0	7.50

a) Evaluation of Linearity Data: For Atorvastatin:

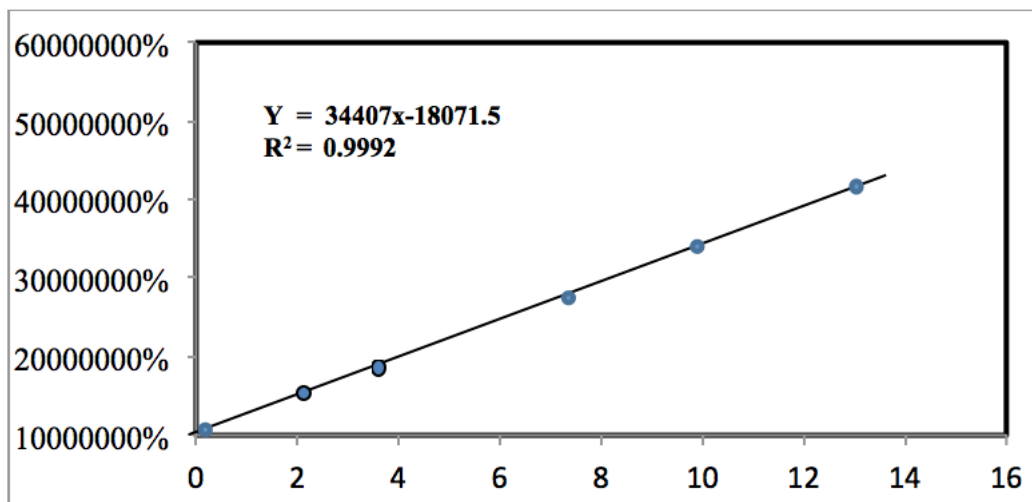


Fig. No. 6 Calibration Graph for Atorvastatin

Table No. 5 Observation by Calibration graph of ATV

Correlation Coefficient (R^2)	0.9992
Slope	34407.6569
Intercept	-18071.0505

b) Evaluation of Linearity Data: For Ezetimibe

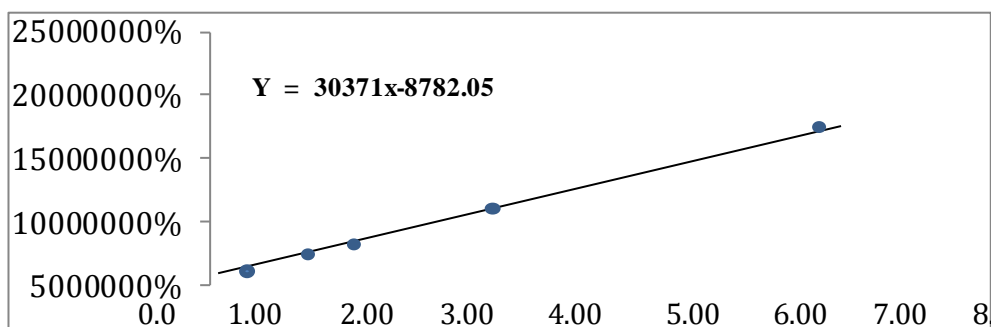


Fig. No.7 Calibration Graph for Ezetimibe

Table No. 6 Observation by Calibration graph of EZT

Correlation Coefficient (R^2)	0.9994
Slope	22253.6019
Intercept	-8782.0528

Hence, the regression analysis equation was found to be ($Y=34407x -18071.05$) and ($Y=30371x - 8782.05$) and correlation coefficient (r) was 0.9992 and 0.9994 for Atorvastatin and Ezetimibe respectively showing good linearity. The results confirmed the linearity of the standard curves over the range studied and the excellent reproducibility of the assay method.

5.3.3. Formulation Analysis

The assay for the marketed tablets was established with present chromatographic condition developed. The average drug content was found to be 99.42 % for ATV and 99.60 % for EZT of the labeled claim. No interfering peaks were found in chromatogram, as indicated by Peak Purity test. The results are given in Table 7.

Table No.7 Results of Tablet analysis

Sr. No.	Label Claim (mg/tab)		Amount found (mg/tab)		% of Label claim determine	
	ATV	EZT	ATV	EZT	ATV	EZT
1	20	10	19.25	10.14	99.07	100.02
2	20	10	20.16	9.51	100.2	99.89
3	20	10	20.09	9.09	98.76	98.81
4	20	10	19.34	10.11	99.16	99.99
5	20	10	19.61	9.08	99.15	98.98
6	20	10	20.61	10.04	100.20	99.94
Mean			19.8433	9.66167	99.42333	99.605
SD			0.53081	0.50221	0.61901	0.52704
% RSD			2.6754	5.19886	0.622621	0.529164

Accuracy

Accuracy of the method was calculated by recovery studies at three levels by standard addition method. The proposed method when used for extraction and subsequent estimation of ATV and EZT from pharmaceutical dosage form after spiking with additional drug afforded recovery of 98–102%. Hence, the accuracy of the assay method was evaluated with the recovery of the standards from excipients. The mean percentage recoveries obtained for ATV and EZT were 99.73% and 99.56%, respectively, reported in Table 8.

Table No.8 Accuracy (recovery) of ATV and EZT

Compound	Recovery Level (%)	Qty. spiked (µg/ml)	Qty. recovered (µg/ml)	Recovery (%)	R.S.D (%)
ATV	50	5.0	5.03	99.35	1.07
	100	10.0	9.01	100.01	0.54
	150	15.0	15.01	99.83	1.03
EZT	50	2.50	1.03	99.55	0.60
	100	5.00	4.05	99.67	0.89
	150	7.50	6.99	99.48	0.56

Precision

The precision of the method was done by replicate (n=3) analysis of tablet preparations. The intra-day and inter-day variation were calculated in terms of percentage relative standard deviation and the results show that the mean assay value and %RSD are well within the acceptance criteria for the precision study. The results of precision and intermediate precision are shown in Table 9 and 10.



Table No. 9 Intraday and Inter-day precision of ATV (n=3).

ATV	Measured concentration (µg/ml), % R.S.D	
Conc. (µg/ml)	Intra -day	Inter-day
5	5.03, (1.38)	4.03, (1.35)
10	9.01, (0.75)	10.01, (0.70)
15	15.01, (0.57)	14.02, (0.29)

Table No. 10 Intraday and Inter-day precision of EZT (n=3).

EZT	Measured concentration (µg/ml), % R.S.D	
Conc. (µg/ml)	Intra day	Inter day
2.5	1.03, (0.93)	1.04, (1.76)
5.0	4.05, (0.69)	5.02, (0.75)
7.5	6.99, (0.76)	6.55, (0.65)

Method Precision:

The Method precision is a measure of the method variability that can be expected for a given analyst performing the analysis, which analyses of the same working standard solution of Ezetimibe. Hence, the developed method was found to be precise in order to determine unknown impurities present in the sample by taking 6 sample replicates and the mean, SD, % RSD was calculated from the retention time of the API and unknown impurities present in the sample.

Table No. 11 Retention Time and Area of Impurities Observed In Test Samples

Test spl.	RT	RRT	Spl-1	Spl-2	Spl-3	Spl-4	Spl-5	Spl-6
Unk imp.	10.911	0.74	63248	65571	62074	61705	62213	61004
Unk imp.	12.645	0.85	28220	31896	25824	25812	25221	24954
Unk imp.	16.583	1.12	54055	56171	57972	57005	54075	48521
Unk imp.	21.240	1.43	64888	59162	64067	66349	56633	67709
Unk imp.	30.125	2.03	23735	22156	22820	24238	22498	22949
Unk imp.	31.910	2.15	20884	16130	17673	17526	15922	16788
Unk imp.	35.890	2.42	61121	57367	56134	56369	56774	51633
Unk imp.	38.565	2.60	19366	12631	13811	11213	16716	12477
Unk imp.	43.092	2.91	37055	24488	24710	34132	17907	25805

Table No. 12 % Impurities observed by Method Precision

Test Spls.	Spl -1	Spl-2	Spl-3	Spl-4	Spl-5	Spl-6	Mean	SD	%RSD
Unk imp.	0.38	0.39	0.37	0.37	0.37	0.36	0.37	0.01	2.58
Unk imp.	0.17	0.19	0.15	0.15	0.15	0.15	0.16	0.02	9.89
Unk imp.	0.32	0.33	0.34	0.34	0.32	0.29	0.32	0.02	6.19
Unk imp.	0.39	0.35	0.38	0.39	0.34	0.40	0.38	0.03	6.84
Unk imp.	0.14	0.13	0.14	0.14	0.13	0.14	0.14	0.00	3.38
Unk imp.	0.12	0.10	0.11	0.10	0.09	0.10	0.10	0.01	10.34
Unk imp.	0.36	0.34	0.33	0.34	0.34	0.31	0.34	0.02	5.36
Unk imp.	0.12	0.08	0.08	0.07	0.10	0.07	0.09	0.02	21.42
Unk imp.	0.22	0.15	0.15	0.20	0.11	0.15	0.16	0.04	25.69
Unk Max. Imp.	0.39	0.39	0.38	0.39	0.37	0.40	0.39	0.01	2.92
Total imp.	2.21	2.05	2.05	2.11	1.95	1.97	2.06	0.10	4.66

Specificity:

The specificity defined as the ability of method to measure the analytes accurately and specifically in the presence of components present in the sample matrix was determined by analysis of chromatograms of drug-free and drug-added placebo formulation. In peak purity analysis with UV detector, purity angle should be less than purity threshold for both the analytes. Hence, the specificity chromatogram was observed for ATV (20 µg/ml) and EZT (10 µg/ml) as per Fig. No.8.

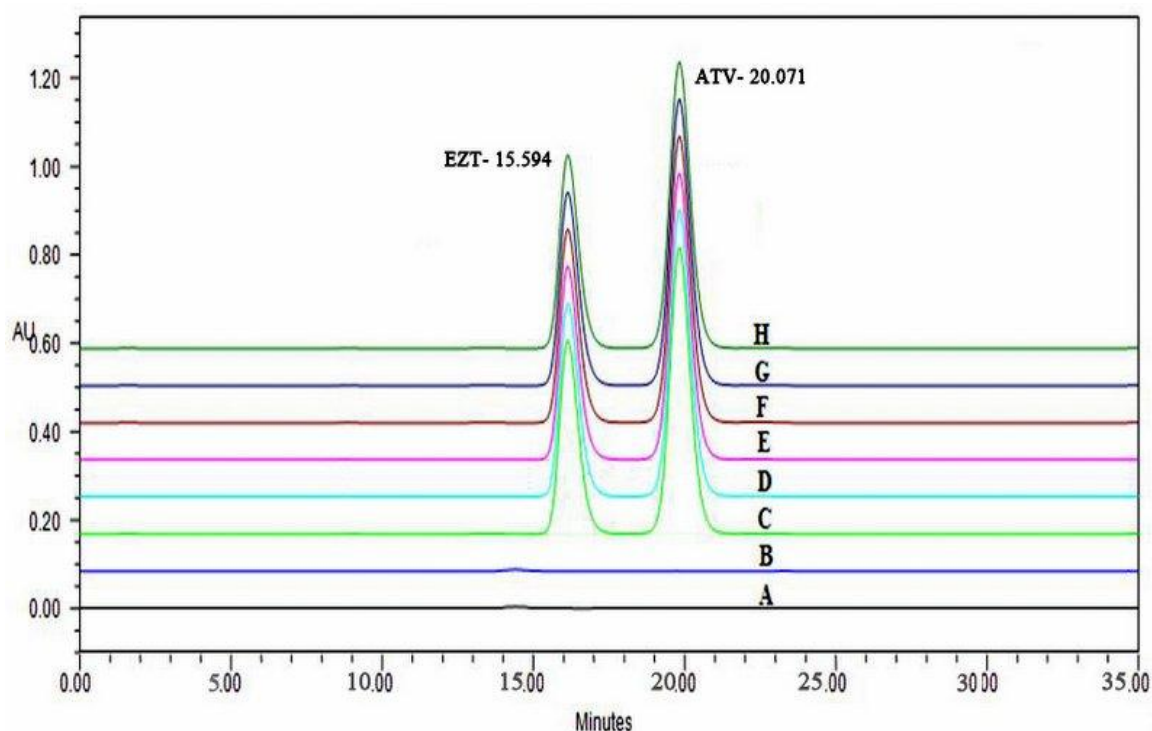


Fig. No. 8 Specificity Chromatogram consists of (A) Mobile Phase, (B) Placebo, (C) Formulation, (D-H) System suitability Standards of ATV (20 µg/ml) and EZT (10 µg/ml)

Limit of Detection and Limit of Quantification:

LOD is the lowest concentration of an analyte that can reliably be differentiated from background levels. LOQ of an individual analytical procedure is the lowest amount of analyte that can be quantitatively determined with suitable precision and accuracy. LOD and LOQ were calculated from standard deviation of the response and the slope of the three linearity curves using the formula $3.3 \sigma/S$ for LOD and $10 \sigma/S$ for LOQ where σ is standard deviation of response and S is mean of slope of three Calibration curves. The LOQ was verified by

injecting six replicates at its concentration at the LOQ level of ATV and EZT. Hence, the LOD and LOQ values were found to be 0.5µg/ml, 0.15µg/ml and 0.07 µg/ ml, 0.22µg/ml for ATV and EZT, respectively.

Robustness of the method:

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage. Hence, robustness of the method was determined by making slight changes in the chromatographic conditions. It was observed that there were no marked changes in the chromatograms, which demonstrated that the RP-HPLC method developed, and System suitability parameters were found to be within acceptable limits. Results were shown in Table No. 13 indicating that the test method was robust for all variable conditions.

Table No. 13 Result of robustness study

Parameter (Limit)	Level	Analyte Name	System Suitability Parameters (SD) n=3				% Assay, % RSD, n=3
			t _R	N	Rs	K	
Flow rate ml/min (± 0.1 mL)	1.4 (-)	ATV	20.46	28977	4.40	4.46	99.59, 0.46
	1.6(+)	EZT	15.24	13796	---	5.24	100.01, 0.57
% of Organic (± 2%)	49 (-)	ATV	20.23	28914	4.16	4.42	99.66, 0.52
	53(+)	EZT	15.14	13682	---	5.21	99.88, 0.60
pH of Mobile Phase (±2)	3.8(-)	ATV	20.12	28826	4.21	4.47	99.48, 0.78
	4.2(+)	EZT	15.26	13702	---	5.32	100.02, 0.55
Separation column	Column I ^a	ATV	20.18	28897	4.26	4.48	99.65, 0.77
	Column II ^b	EZT	15.19	13697	---	5.15	99.74, 0.58
Measurement Wavelength (± 0.2 nm)	243.8(-)	ATV	20.48	28993	4.18	4.36	99.59, 0.65
	244.2(+)	EZT	15.45	13845	---	5.17	99.83, 0.95
Buffer strength (± 5millimoles)	40(-)	ATV	20.11	28615	4.27	4.49	98.88, 0.87
	50(+)	EZT	15.23	13789	---	5.27	99.86, 0.92
Column Temp. (± 3°C)	47(-)	ATV	20.10	28812	4.12	4.45	100.01, 0.76
	53(+)	EZT	15.22	13791	---	5.19	99.58, 0.98

Range:

The calibration range was established through consideration of the practical range necessary, according to each compound concentration present in the pharmaceutical product, to give accurate, precise and linear results.

Forced degradation studies

For preparing solutions for forced degradation studies, following table was followed.

Table No. 14 Preparation of Solutions for Forced Degradation Studies (Available in Literature for Degradation studies)

Sr. No.	Degrading agents	Condition	Sample Preparations
1	Acid degradation	0.1N HCl	50.0 mg of sample was weighed and transferred into three 50 ml volumetric flasks, 5 ml of 0.1N HCl (methanolic) added to each flask. These solutions kept at room temperature for 0 hour, 12 hours and 24 hours. These solutions neutralized with 0.1N NaOH (methanolic) and 25-30 ml of diluent was added and sonicated for complete solubility. Then these solutions were made up to the mark.
2	Base degradation	0.1 N NaOH	50 mg of sample weighed and transferred it into three 50 ml volumetric flasks, 5 ml of 0.1N NaOH (methanolic) added to each flask. These solutions kept at room temperature for 0 hour, 12 hours and 24 hours. These solutions neutralized with 0.1N HCl (methanolic) and 25-30 ml of diluent was added and sonicated for complete solubility. Then these solutions were made up to the mark.
3	Peroxide degradation	3% H ₂ O ₂	50 mg of sample weighed and transferred it into three 50 ml volumetric flasks, 5 ml of 3% H ₂ O ₂ added to each flask. These solutions kept at room temperature for 0 hour, 12 hours and 24 hours and 25-30 ml of diluent was added and sonicated for complete solubility. Made up to the mark with diluent. Blank solution was also prepared

Sr. No.	Degrading agents	Condition	Sample Preparations
			in similar way.
4	Heat degradation	105°C for 24 hours	500 mg of Atorvastatin API and Ezetimibe API kept at 105°C and withdrawn after 24 hours, from that 50 mg of sample weighed and transferred it into a 50 ml volumetric flask to that 25-30 ml of diluent was added and sonicated for complete solubility. This solution was then made up to the mark with diluents
5	Humidity degradation	75% R.H. for 24 hours	500 mg of Atorvastatin API and Ezetimibe API kept in an open atmosphere at 75%RH and withdrawn after 24 hours, from that 50 mg of sample weighed and transferred it into a 50 ml volumetric flask, to that 25-30 ml of diluent was added and sonicated for complete solubility. The volume was then up to the mark with diluent.
6	Photolytic degradation	Light	500 mg of Atorvastatin API and Ezetimibe API was kept in the light for a period equivalent to about 1.20 million-lux hours, from that 50 mg of sample weighed and transferred it into a 50 ml volumetric flask, 25-30 ml of diluent was added to the flask and sonicated for complete solubility. The solution was then made up to the mark with diluent.


Control sample of Atorvastatin API and Ezetimibe was prepared and injected along with the forcefully degraded samples. Blank was also prepared in same way as that of the sample (as specified in Table No.14) except for heat, humidity and photolytic treatment. The above analysis was performed on HPLC equipped with photodiode array detector. The system suitability was checked with the standard solution before proceeding with the experiment. The samples were then injected after every 12 and 24 hours during the sequence.


Data Evaluation

The data was processed for peak purity of the Atorvastatin and Ezetimibe and all other impurities were present in the sample upon degradation. Also, all the injections were

processed at a wavelength provided in the method and their selectivity was demonstrated with respect to non-interference peak obtained for the diluent, other impurities, Atorvastatin and Ezetimibe in the sample preparation. The peak purity data was compared with that obtained from a Control sample. According to following table as per IP and USP guidelines for forced degradation studies.

Table No. 15 Observation table for forced degradation (Available in literature for Degradation studies) (PA-purity angle, PT-purity threshold)**

Sr. No.	Chromatogram Name.	Appearance of Extra Peak	PA	PT	Remarks
1.	Acid degradation	--			No degradation Observed
	Sample-0.1N HCl (0.0) hr		0.059 0.125	0.247 0.240	
	Sample-0.1N H (12.0 hr)	-- 	0.057 0.119	0.249 0.238	No degradation Observed
2.	Base degradation				
	Sample-0.1N NaOH (0.0) hr	15.107 min (18.81%)	0.5s64	3.085	19.01% degradation
	Sample-0.1N NaOH (12.0 hr)	20.099 min (0.61%)	3.243	3.691	About 24.5% degradation
	Sample-0.1N NaOH (24.0 hr)	15.115 min (23.89%)	2.648	2.438	About 25.9% degradation
		20.038 min (0.82%)			
		15.199 min (23.94%)			
		20.087 min (1.11%)			

3.	Oxidative degradation Sample-3% H_2O_2 (0.0) hr Sample-3% H_2O_2 (12.0) hr Sample-3% H_2O_2 (24.0)	-- --	0.062 0.137	0.244 0.236	No degradation No degradation No degradation
3.	Oxidative degradation Sample-3% H_2O_2 (0.0) hr Sample-3% H_2O_2 (12.0) hr Sample-3% H_2O_2 (24.0)	-- -- 	0.062 0.137	0.244 0.236	No degradation No degradation No degradation
4.	Sample-Heat	--	0.062 0.137	0.244 0.236	No degradation
5.	Sample-Humidity	--	0.057 0.102	0.249 0.228	Minor degradation
6.	Sample-Photo	15.106 min (18.81%) 20.107 min (0.61%)	0.056 0.119	0.247 0.238	About 19.42% degradation observed

The resulting chromatograms obtained are shown below:

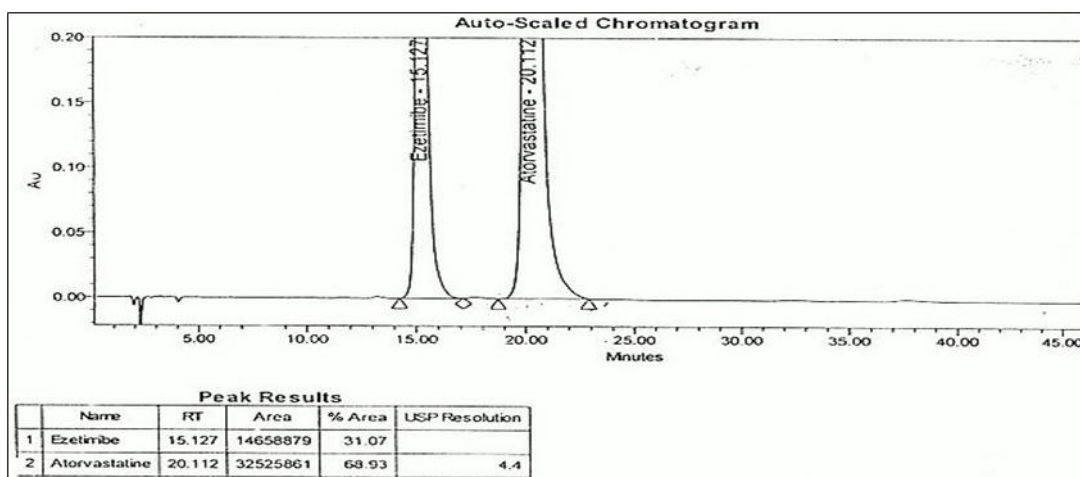
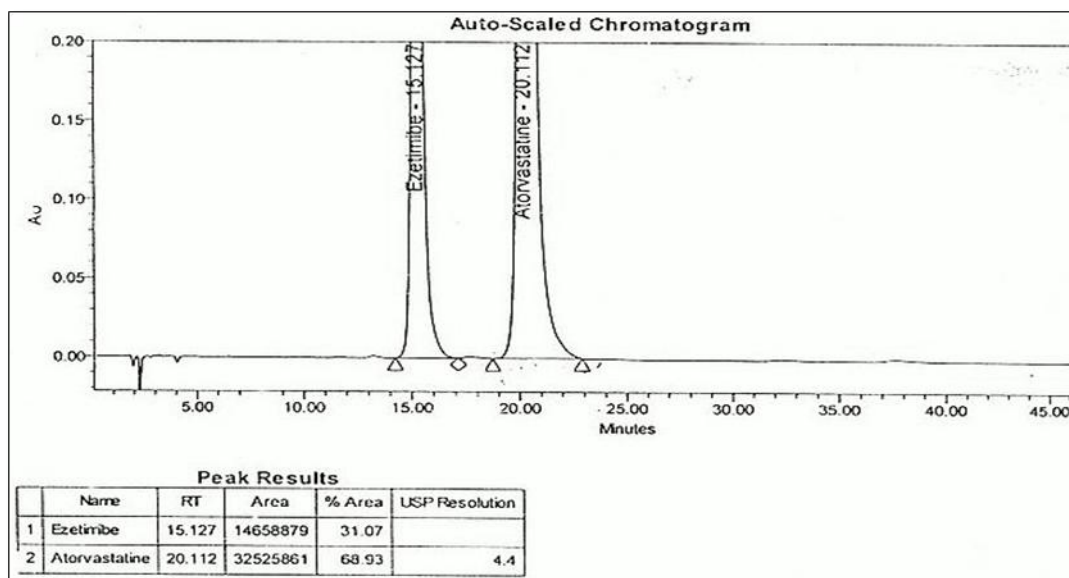


Fig. No. 9: Chromatogram of acid degradation (a,b) : (0.1N HCl for 12, 24 hr).

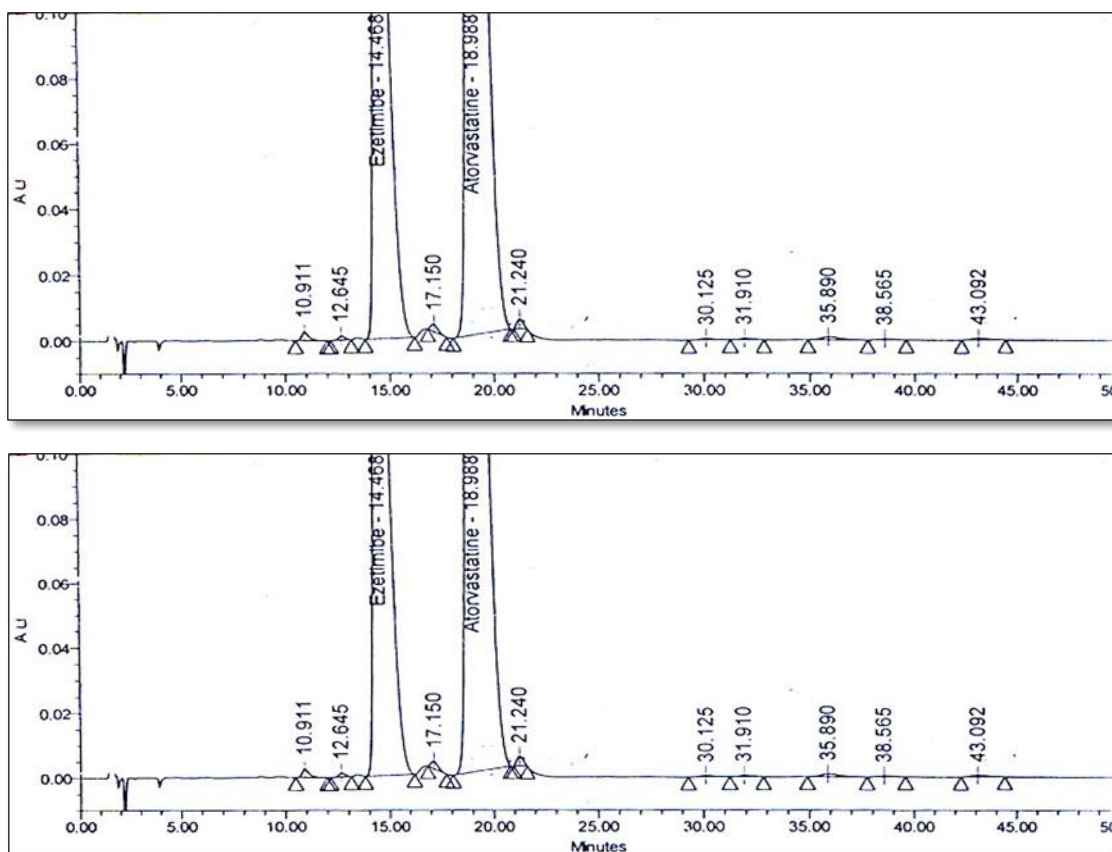


Fig. No. 10: Chromatogram of base degradation (a,b) : (0.1N NaoH for 12,24 hr).

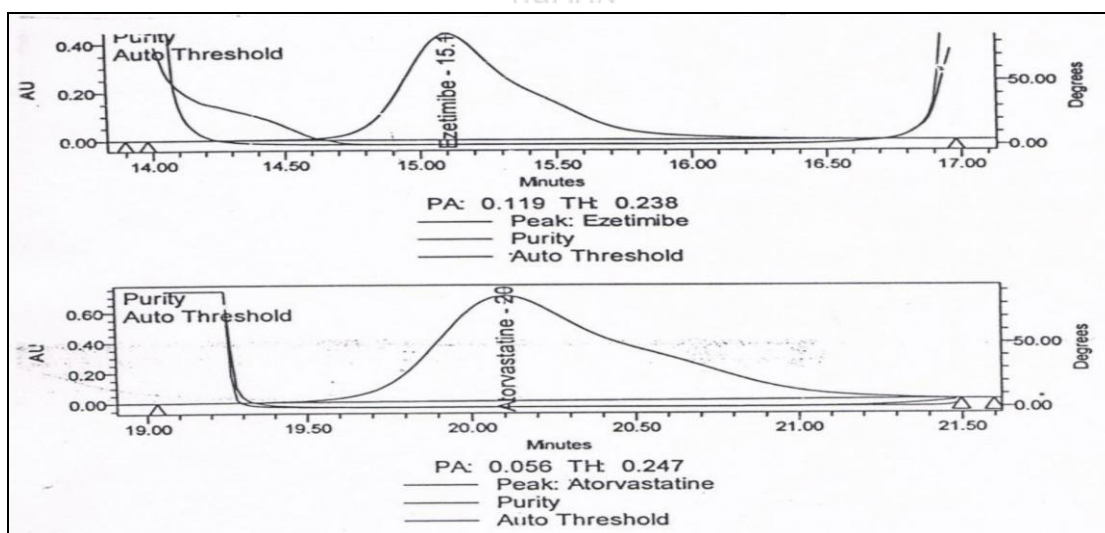
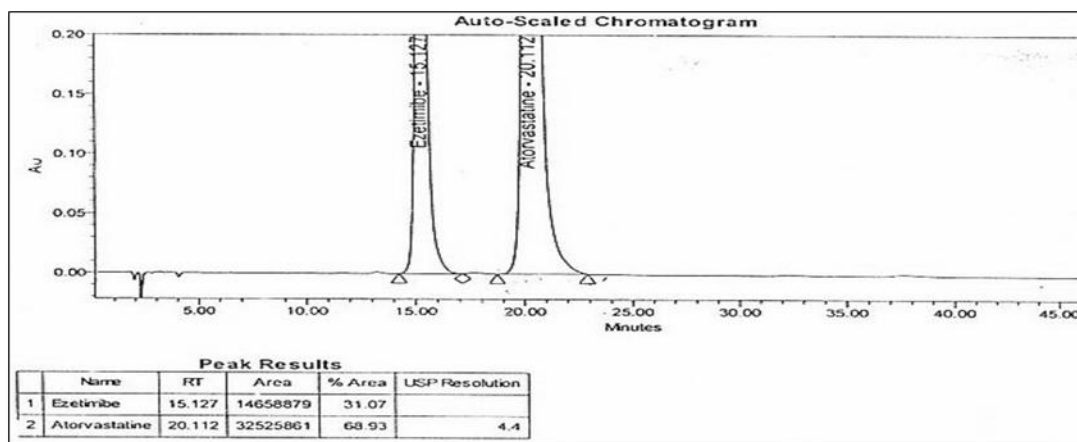
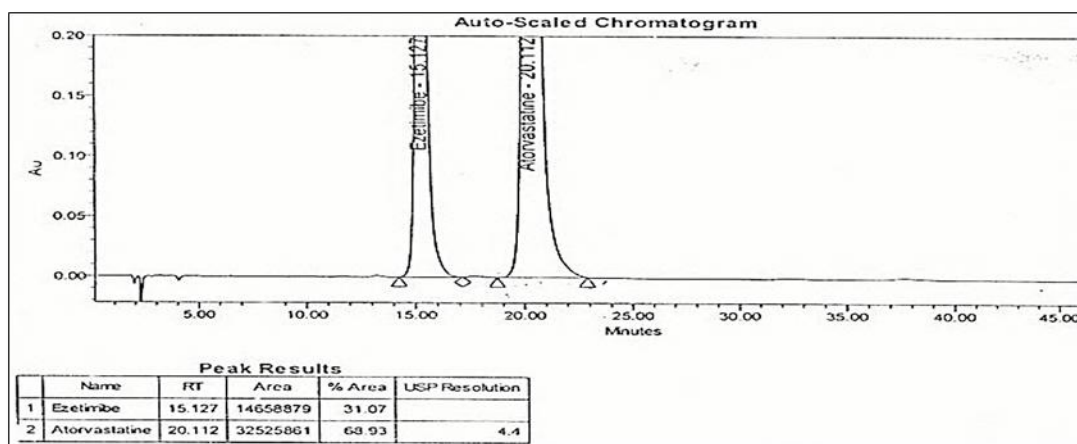


Fig. No. 11: PA & PT Observed for base degradation



HUMAN

Fig. No.12: Chromatogram of Oxidative degradation (a,b): (3% H_2O_2 for 12, 24 hr.)

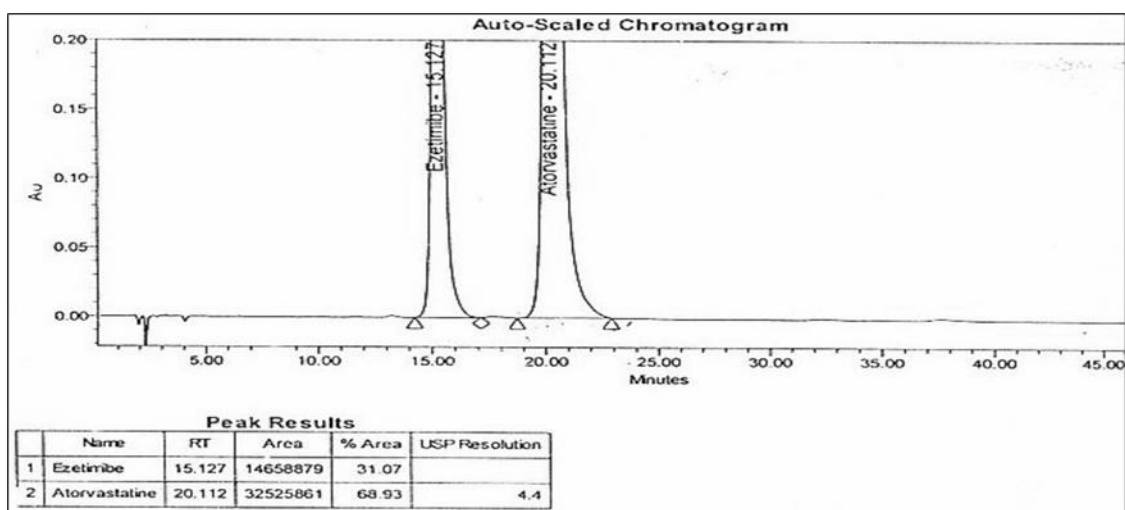


Fig. No. 13: Chromatogram of Heat degradation (105°C for 24 hr.)

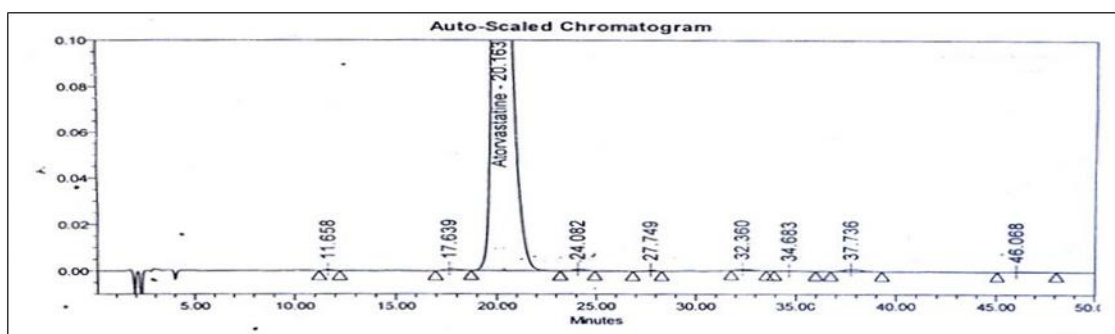


Fig. No.14: Chromatogram of Photo-degradation (1.20 million-Lux). Ezetimibe API

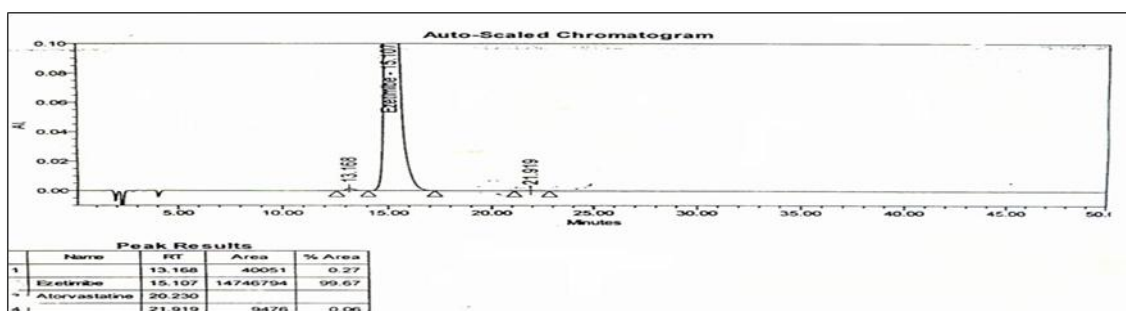


Fig. No. 15: Chromatogram of Photo-degradation (1.20 million-Lux). Sample (ATV+EZT)

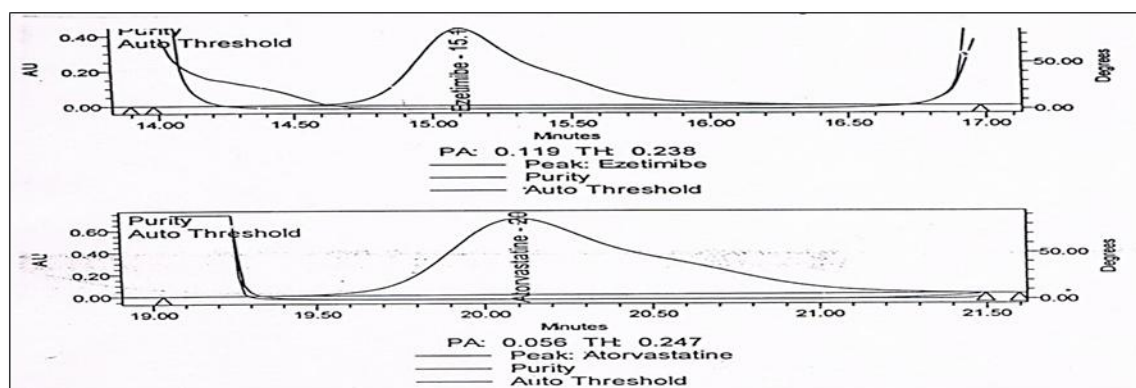
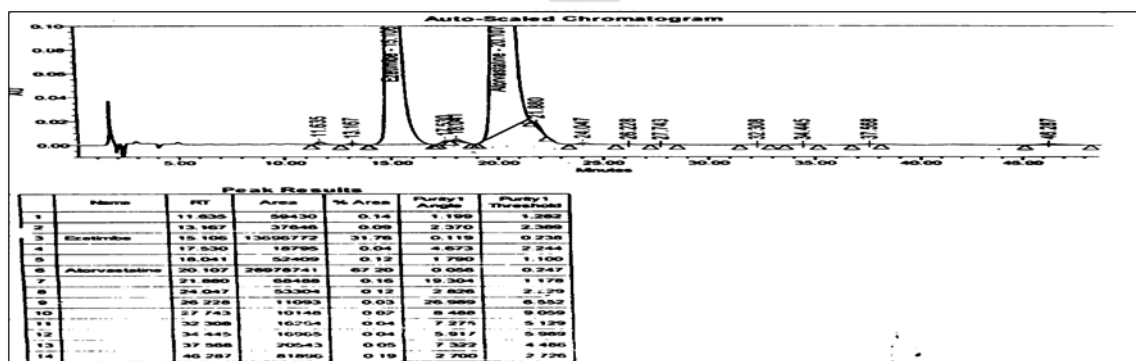


Fig. No. 16: PA & PT (Purity Auto Threshold) Observed for Photolytic degradation

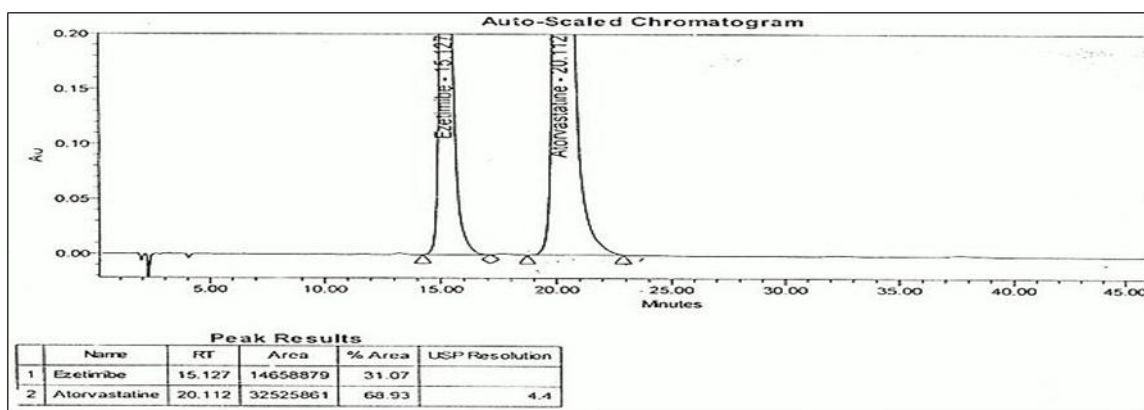


Fig. No. 17: Chromatogram of Humidity degradation (75%R.H. for 24 hours).

Observation by Stress studies

Table No. 16: Photolytic Degradation of ATV & EZT (API & Sample)

Test spls.	Atorvastatin API	Ezetimibe API	Sample API	Atorvastatin Photolytic	Ezetimibe Photolytic	Sample Photolytic
Unk Imp.	14450	-	60731	16562	0	59430
Unk Imp.	0	38538	37754	0	40051	37646
Unk Imp.	0	0	13237	42457	0	18795
Unk Imp.	0	0	60782	0	0	52409
Unk Imp.	14169	4672	60923	0	9476	68488
Unk Imp.	0	0	0	34612	0	53304
Unk Imp.	0	0	0	17164	0	11093
Unk Imp.	0	0	0	0	0	10148
Unk Imp.	44157	0	30207	36436	0	16264
Unk Imp.	11010	0	33610	10520	0	16965
Unk Imp.	65789	0	61619	64237	0	20543
Unk Imp.	18073	0	16164	22521	0	81896
Unk Imp.	18073	0	16164	22521	0	81896

Table No. 17 Observed unknown impurities by photolytic and alkaline degradation

Test Spls.	Atorvastatin API	Ezetimibe API	Sample API	Atorvastatin Photolytic	Ezetimibe Photolytic	Sample Photolytic
Unk Imp.	0.09	0.00	0.39	0.11	0.00	0.38
Unk Imp.	0.00	0.24	0.24	0.00	0.25	0.24
Unk Imp.	0.00	0.00	0.08	0.27	0.00	0.12
Unk Imp.	0.00	0.00	0.39	0.00	0.00	0.33
Unk Imp.	0.00	0.03	0.39	0.00	0.06	0.43
Unk Imp.	0.09	0.00	0.00	0.22	0.00	0.00
Unk Imp.	0.00	0.00	0.00	0.11	0.00	0.01
Unk Imp.	0.00	0.00	0.00	0.00	0.00	0.00
Unk Imp.	0.28	0.00	0.19	0.23	0.00	0.10
Unk Imp.	0.07	0.00	0.21	0.07	0.00	0.11
Unk Imp.	0.42	0.00	0.39	0.41	0.00	0.13
Unk Imp.	0.11	0.00	0.10	0.14	0.00	0.52
Unk Imp.	0.11	0.00	0.10	0.14	0.00	0.22
Single Max. Unk. Imp.	0.42	0.24	0.39	0.41	0.25	0.52
Total impurities	1.17	0.27	2.48	1.70	0.31	2.59

CALCULATION:



Calculation for Estimation of Unknown Impurities Present In Sample:

1) Atorvastatin (API)

$$\begin{aligned}
 \% \text{Unknown impurities} &= \frac{\text{Area of unknown impurity (max)} \times 1}{\text{Area of Atorvastatin in standard solution}} \\
 &= \frac{65789 \times 1}{156640} \\
 &= 0.42 \%
 \end{aligned}$$

2) Ezetimibe (API)

$$\begin{aligned}
 \% \text{Unknown impurities} &= \frac{\text{Area of unknown impurity (max)} \times 1}{\text{Area of Ezetimibe in standard solution}}
 \end{aligned}$$

$$= \frac{38538 \times 1}{160575}$$

$$= 0.24 \%$$

Sample (ATV+EZT)

$$\% \text{Unknown impurities} = \frac{\text{Area of unknown impurity (max)} \times 1}{\text{Area of (ATV + EZT) in standard solution}}$$

$$= \frac{61619 \times 1}{157997}$$

$$= 0.39 \%$$

Calculation of total impurities:

Total Impurities = Sum of all impurities present in the sample

1. Atorvastatin (API):

$$\text{Total Impurities} = 0.09 + 0.09 + 0.28 + 0.07 + 0.42 + 0.11 + 0.11 = \mathbf{1.17}$$

2. Ezetimibe (API):

$$\text{Total Impurities} = 0.24 + 0.03 = \mathbf{0.27}$$

3. Sample (Marketed Formulation in combination) (ATV+ EZT):

$$\begin{aligned} \text{Total Impurities} &= 0.39 + 0.24 + 0.08 + 0.39 + 0.39 + 0.19 + 0.21 + 0.39 + 0.10 + 0.10 \\ &= \mathbf{2.48} \end{aligned}$$

Table No. 18 % Degradation observed by Photolysis

Name	Unknown Max. Imp	Total Impurity (%)	% Degradation
Atorvastatin (API)	0.42	1.17	-
Ezetimibe (API)	0.24	0.27	-
Sample (API)	0.39	2.48	-
ATV (Photolytic)	0.41	1.70	0.53 %
EZT (Photolytic)	0.25	0.31	0.04%
Sample (Photolytic)	0.52	2.59	0.11%
Sample (Photolytic)	0.52	2.59	0.11%

IMPURITY PROFILING:

Atorvastatin & Ezetimibe and their Impurities:

The synthesis of Atorvastatin, as well as Ezetimibe, is multi step process and hence there is a need to eliminate toxic and unstable intermediates from these synthetic steps. The process and degradation impurities Complete forced degradation study was performed on Atorvastatin and Ezetimibe taking into consideration the above mentioned aspects. Atorvastatin and Ezetimibe were subjected to thermolytic, photolytic, hydrolytic (acidic and alkaline) and oxidative stress conditions. The stressed and unstressed samples of Atorvastatin and Ezetimibe were injected into a developed and validated High Performance Liquid Chromatography (HPLC) method. In this method, a Hypersil C18 column (150 mm x 4.6 mm i.d., 5µm particle size) kept at a temperature of 40°C was used. Chromatographic separation was achieved under isocratic elution using a mobile phase consisting of a mixture of KH₂PO₄ buffer (pH 2.5; 0.05 M) – Methanol (45:55, v/v). UV detector was set at 242 nm. Flow rate was adjusted to 1.0 mL/min. An injection volume of standard and test solutions was kept as 10 µL. Water-methanol mixture (50:50, v/v) was used as the diluent.

Atorvastatin and Ezetimibe were found to be stable in acidic, oxidative, thermal stress conditions. Extensive degradation of Atorvastatin and Ezetimibe occurred in Photolytic degradation and alkaline hydrolytic conditions respectively.

Photolytic degradation of Atorvastatin and Ezetimibe was performed by keeping the samples in the light for a period equivalent to about 1.20 million-lux hours, from that 50 mg of sample

weighed and transferred it into a 50 ml volumetric flask, 25-30 ml of diluent was added to the flask and sonicated for complete solubility. The solution was then made up to the mark with diluent.

Alkaline hydrolysis of Atorvastatin and Ezetimibe was performed by heating with 0.01 M aqueous sodium hydroxide solution at 60°C for 10 min. Major degradation product of photolytic degradation of Atorvastatin was found at RT of 21.880. Hence, during forced degradation studies it was observed that one major impurity was formed during photolytic degradation in case of Atorvastatin. While in case of Ezetimibe, major degradation product of alkali hydrolysis was found at RT of 17.639. Hence, during forced degradation studies, it was observed that one major impurity was formed during alkaline degradation of Ezetimibe. The objective was now to identify these degradants and check their purity. This degradant was synthesized and further confirmed by isolation using preparative HPLC. In the synthetic reaction, 20 mg of Atorvastatin drug substance was subjected to light for 48 hours. Around 10 mg of Atorvastatin was taken in an evaporating dish and again subjected to 1.20 million lux hours of natural sunlight. Further, it was taken into a 50 ml round bottom flask and diluted with 50 ml methanol and refluxed at 75°C on a constant temperature water bath for 20 min. The solution was allowed to cool to room temperature and neutralized with 1M methanol. The photolytic degradation solution of was then concentrated on Rotavapor with water bath under high vacuum to around 25 mL of Methanol. The solution was allowed to cool and around 50 ml of ice cold water was added. Yellowish white solids were precipitated out, were washed and dried in freeze dryer. Further confirmation of the degradant of Atorvastatin was done by isolation using preparative HPLC.

Ezetimibe was reacted with 0.1 M methanolic sodium hydroxide solution. Around 0.5 g of Ezetimibe drug substance was taken in 100 ml round bottom flask. About 50 ml of 0.1 M methanolic sodium hydroxide solution was added and the solution refluxed at 80°C on a constant temperature water bath for 15 min. The solution was allowed to cool to room temperature and neutralized with 1 M methanolic hydrochloric acid solution. The alkaline degradation solution of Ezetimibe was then concentrated on Rotavapor with water bath under high vacuum to around 50 mL of Methanol. The solution was allowed to cool and around 100 mL of ice cold water was added. White solids precipitated out, were washed and dried in freeze dryer. Further confirmation of the alkaline degradant was done by isolation using preparative HPLC. Waters preparative HPLC (Delta Prep) 4000 system (Waters Corporation,

Milford, USA) with high pressure unit of 4000 psi, operated through Empower software equipped with fraction collector was used. Waters Symmetry C18 (100 mm x 30 mm.)

Preparative column packed with 8 μ m particle size was employed. Mobile phase consisted of ammonium acetate buffer (pH 4.5, 50 mM) - acetonitrile (50:50, v/v).

Flow rate was kept at 30 mL/min and UV detection was at 244 nm. Methanolic alkaline degradation solution of Ezetimibe was concentrated on rotavapor R-124 and loaded on the preparative column for the isolation of alkaline degradant. The isolated fractions were collected and methanol was then evaporated using rotavapor with water bath to get the solids, which were then dried using freeze dryer.

For the identification of the degradant by MS detector, an LC-MS compatible HPLC method was developed. The solids were then checked for purity, which was found to be more than 99.0%. HPLC method involved an isocratic elution on a Waters Symmetry C18 250 x 4.6 mm, 5 m column using ammonium acetate buffer (pH 4.5, 50 mM) – acetonitrile (60:40,v/v) as the mobile phase at the flow rate of 1.0mL/min and UV detection at 244nm. Solids obtained by the two methods were established to be the same, looking at the RT of 21.880 for Atorvastatin and 17.639 for Ezetimibe.



The structure of this alkaline degradant was elucidated using spectroscopic techniques like MASS and IR spectroscopy. The degradants were identified as (3R,5R)-7-[2-(4-fluorophenyl)-3-phenyl-4-(phenylcarbamoyl)-5-propan-2-ylpyrrol-yl]-3-one,5-hydroxyheptanoate and 4-(5-desfluorophenyl)-[(1,4-fluoro-phenylamino)-2-hydroxy-methyl-1-pent(4-enyl)]-phenol for Atorvastatin and Ezetimibe respectively.

Sr. No.	Name of Impurity	Structure	IUPAC Name
1	Benzyl Ezetimibe Impurity		(3R,4S)-1-(4-Fluorophenyl)-3-[(3S)-3-(4-fluorophenyl)-3-hydroxyphenyl]-4-(4-benzyloxyphenyl)-2-azetidinone
2	Benzyl Ezetimibe Diol Impurity		4-[4-benzyloxyphenyl]-[4-Fluorophenylamino]-methyl]-1-(4-fluorophenyl)-pentane-1,5-diol
3	Lactam cleaved alcohol Impurity		4-(5-Fluorophenyl)-1-[(4-fluorophenylamino)-2-hydroxymethyl-pent-4-enyl]-phenol
4	Ezetimibe Diol Impurity		(3R,4S)-1-(4-Fluorophenyl)-(4-hydroxymethyl)-5-(4-hydroxyphenyl)-5-N-(4-fluorophenylamino)-pentanol
5	Lactam cleaved acid Impurity		5-(4-fluorophenyl)-2-[(4-fluorophenylamino)-(4-hydroxyphenyl)methyl]-pent-4-enoic acid
6	Hydroxyl related desfluoro Impurity		1-(4-fluorophenyl)-4(S)-(4-hydroxyphenyl)-3(R)-(3-hydroxy-3-phenyl-propyl)-2-azetidinone
7	Lactam related desfluoro Impurity		3-[3-(4-fluorophenyl)-3-hydroxypropyl]-4-(4-hydroxyphenyl)-1-phenyl-2-azetidinone

Fig. No. 18: Known Impurities of EZT from available literature survey

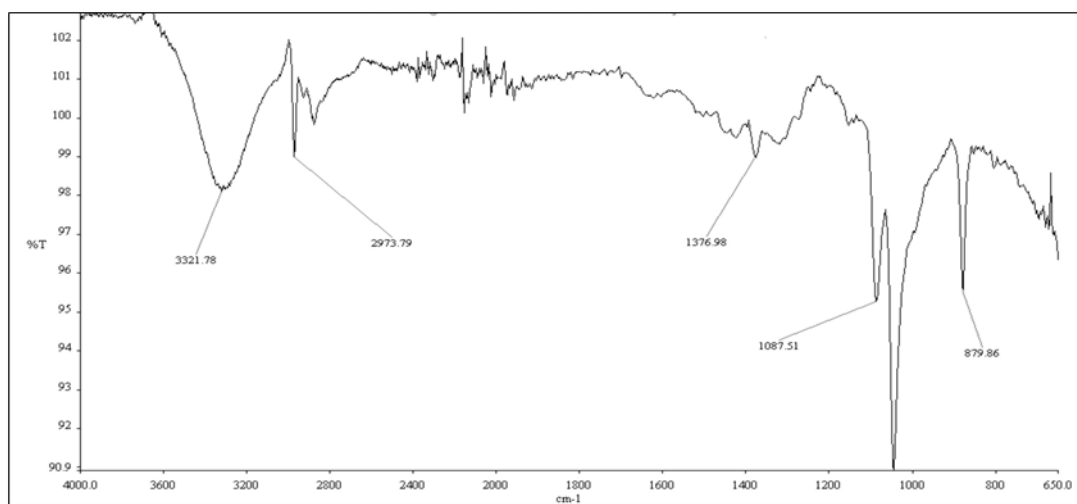


Fig. No. 19: Fourier Transform Infra-Red Spectrum of Atorvastatin Impurity

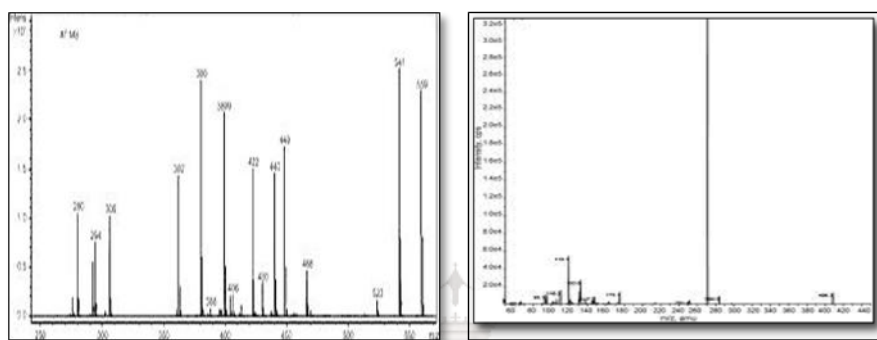


Fig. No. 20: Mass Spectrum of Atorvastatin Impurity & Ezetimibe Impurity

Structural representation of observed major unknown impurities:

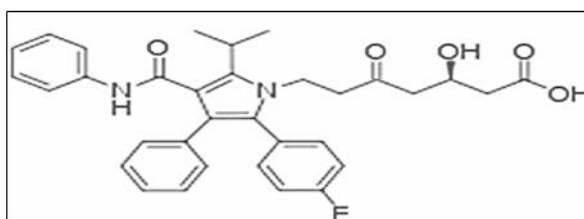


Fig. No. 21: Chemical structure of proposed Atorvastatin Impurity estimated

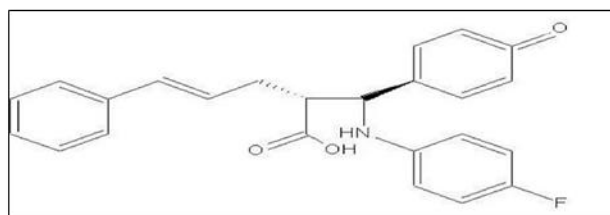


Fig. No. 22: Chemical structure of proposed Ezetimibe impurity estimated

Dissolution studies:

The dissolution study was carried out for the above combination and was validated. A calibrated dissolution apparatus (USP II) paddle 60 rpm & bath temp at $37 \pm 1^\circ\text{C}$. Nine hundred milliliter freshly prepared and degassed 0.1N HCl solution was used as the dissolution medium. Six tablets were evaluated and dissolution sample was collected at 5, 10, 15, 20, 25, 30, 35, 40 min interval. At each time point, a 5mL sample was removed from each vessel sample, filtered through a nylon filter ($0.45\mu\text{m}$, 25 mm), 1.8 mL of filtrate was diluted to 10 ml with distilled water and analyzed by HPLC method. Percentage release of ATV and EZT was calculated by using equations 3 and 4, respectively.

$$\text{ATV \% release} = (C_{\text{ATV}} \times 900 \times 10 \times 100) / (1000 \times 20) \dots (3)$$

$$\text{EZT \% release} = (C_{\text{EZT}} \times 900 \times 10 \times 100) / (1000 \times 10) \dots (4)$$

We found that there is a release of 93.88% & 96.89% for ATV and EZT. The results of Dissolution studies were shown in Fig. No.23

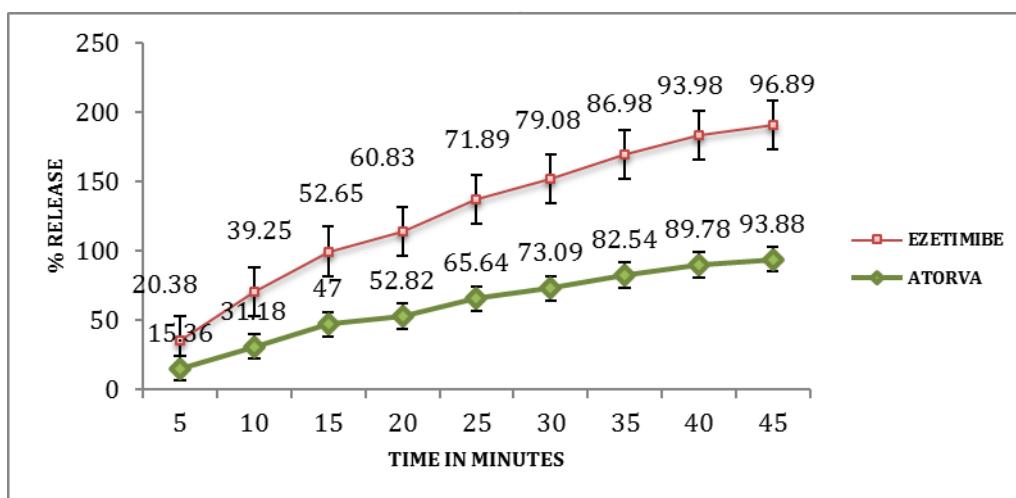


Fig. No. 23 Dissolution profile of ATV and EZT.

Analytical Solution Stability:

Stability, as described in method development under experimental section, was studied. Result of short-term, long-term and the autosampler stability of the ATV and EZT solutions were calculated from nominal concentrations and found concentration. Results of the stability studies were within the acceptable limit (98–102%) and the retention time, peak area of ATV and EZT remained almost unchanged (% R.S.D less than 2.0) indicating that no significant

degradation within the indicated period, which was sufficient to complete the whole analytical process. Hence, to demonstrate the stability of standard working solutions and of tablet sample solutions during analysis, both solutions were analyzed over a period of 12 h while being stored at room temperature and for 24 h when refrigerated at 4°C.

X-ray Diffraction study:

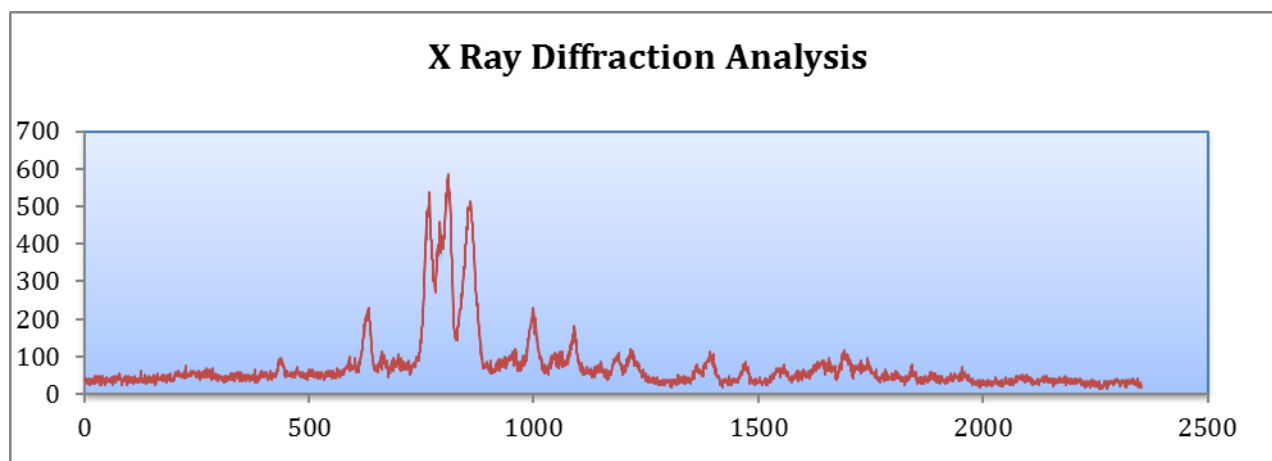


Fig. No. 24: X-ray Diffraction of Atorvastatin Ezetimibe Marketed Tablet Formulation

Thus X-Ray Diffraction studies exhibit that triturated powder of compact intact tablet (a marketed formulation of Graslip Forte has high degree of crystalline form. Thus highest crystallinity in the tablet formulation correlates with solubility of the drug. Hence when analyzed with USP II calibrated dissolution apparatus, the solubility parameter is elongated when compared to powder drug samples (alone and in physical mixture 60:40).

CONCLUSION:

In proposed study, sensitive isocratic RP-HPLC method has been developed for estimation of unknown impurities in Atorvastatin and Ezetimibe tablets including impurity profiling. The developed method was validated and was found to be simple, sensitive, accurate and precise. The method was successfully used for determination unknown impurities with structural elucidation in Atorvastatin and Ezetimibe in their pharmaceutical tablet formulations. Also, X-Ray diffraction is one of the essential analytical technique and in this study, we determined nature of the formulation which is used for correlation analysis of crystallinity and dissolution solubility parameters. As the method separates the drug from its degradation products as well as all the degradation products from each other therefore the method can be

used for routine quality control analysis of Atorvastatin and Ezetimibe in industries for batch release. Also, presence of unknown impurities and related substances could be one of the reasons that are attributed to complete dissolution time. This study can be further extended to bioanalytical experiments.

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