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RESEARCH ARTICLE

VALIDATED RP - HPLC METHOD FOR THE SIMULTANEOUS ESTIMATION OF ATORVASTATIN AND EZETIMIBE IN PURE AND COMBINED PHARMACEUTICAL DOSAGE FORMS

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Abstract

A simple, specific, precise, and efficient method for the simultaneous estimation of Atorvastatin and Ezetimibe in pure and pharmaceutical dosage forms by a Reverse Phase - High Performance Liquid Chromatography method is developed and validated. Selected mobile phase was in a combination of Methanol and Phosphate buffer (pH - 3.8) (40:60 % v/v). Optimized column is a Phenomenex Gemini C18 (4.6 mm × 250 mm) 5 µm particle size and at a flow rate of 1.0 ml/min with detection wavelength at 251 nm for Atorvastatin and Ezetimibe. In our study, the validation of analytical method for determination of Atorvastatin and Ezetimibe in pure and pharmaceutical dosage forms was performed in accordance with the parameters including - system suitability, specificity, linearity of response, accuracy, precision (reproducibility & repeatability), robustness (change of wave length ± 2 nm). The method is validated according to ICH guidelines. The results obtained by RP - HPLC method are rapid, accurate and precise. Therefore, proposed method can be used for routine analysis of Atorvastatin and Ezetimibe in the pure form as well as in combined pharmaceutical dosage form.

Introduction:-

Atorvastatin (ATV)[1] is chemically [R-(R*, R*)]-2-(4-flurophenyl)-β, δ-dihydroxy-5- (1- methylethyl)-3-phenyl-4-[(phenyl amino) carbonyl]-1H-pyrrole-1-heptanoic acid, calcium salt trihydrate. Drug profile of Atorvastatin is given in Table 1 and its structure is shown in Figure 1. 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. Ezetimibe (EZE)[2,3] is [(3R, 4S)-1-(4- fluorophenyl)-3-[(3S)-3-(4-fluorophenyl)-3-hydroxypropyl]-4-(4-hydroxyphenyl)-2-azetidinone. Drug profile of Ezetimibe is given in Table 2 and its structure is shown in Figure 2. 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. 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. A literature survey regarding quantitative analysis of these drugs revealed that attempts were made to develop analytical methods for atorvastatin using extractive spectrophotometry, HPLC, HPTLC, UPLC.[4-12] A liquid chromatography/ mass spectrometry method for the simultaneous quantitation of Atorvastatin and Ezetimibe in

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human plasma was reported.[13] LC and UPLC MS-MS simultaneous determination of atorvastatin and ezetimibe in human plasma were also reported.[14,15]

Hence, the objective of this work was to develop a simple, precise, reliable and rapid high performance liquid chromatographic analytical method for simultaneous estimation of atorvastatin and ezetimibe in plasma, to validate the method in accordance with ICH guidelines[16-22]. Analytical chemistry derives its principles from various branches of science like chemistry, physics, microbiology, nuclear science and electronics. This method provides information about the relative amount of one or more of these components.[23]

Drug Profile:

Table 1:- Drug profile of Atorvastatin.

DRUG	Atorvastatin
Synonym	Atorvastatin, Lipitor, Atorvaliq
Category	Antihyperlipidemic
IUPAC	(3R,5R)-7-[2-(4-Fluorophenyl)-3-phenyl-4-(phenylcarbamoyl)-5- propan-2-ylpyrrol-1-yl]-3,5-dihydroxyheptanoic acid
Molecular formula	$C_{33}H_{35}FN_2O_5$
Melting point	176°C
pK_a	4.46
Log p	6.98

Fig. 1:- Structure of Atorvastatin.

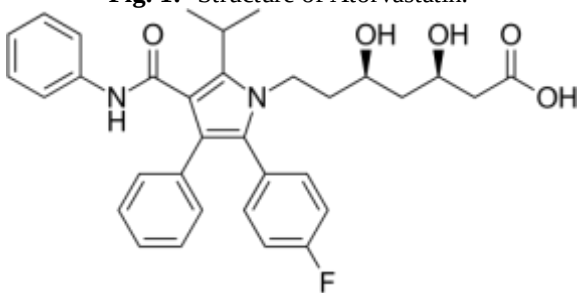
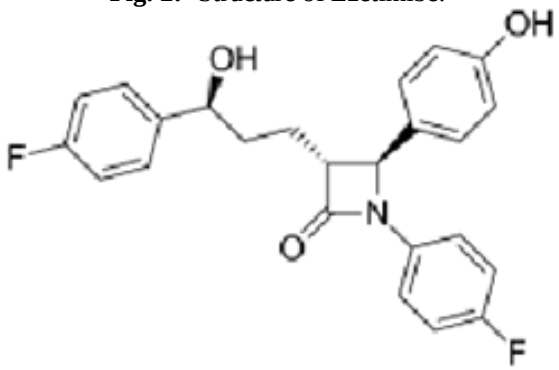


Table 2:- Drug profile of Ezetimibe.

Drug	Ezetimibe
Synonym	Ezetimibe, Ezedoc, Zetia
Category	Antihyperlipidemic
IUPAC	(3R,4S)-1-(4-fluorophenyl)-3-[(3S)-3-(4-fluorophenyl)-3- hydroxypropyl]-4-(4-hydroxyphenyl)azetidin-2-one
Molecular formula	$C_{24}H_{21}F_2NO_3$
Melting point	163°C
pK_a	9.72
Log p	4.14

Fig. 2:- Structure of Ezetimibe.



Introduction to HPLC

HPLC is also called as high-pressure liquid chromatography since high pressure is used to increase the flow rate and efficient separation by forcing the mobile phase through at much higher rate. The pressure is applied using a pumping system. The development of HPLC from classical column chromatography can be attributed to the development of smaller particle sizes. Smaller particle size is important since they offer more surface area over the conventional large particle sizes. The HPLC is the method of choice in the field of analytical chemistry, since this method is specific, robust, linear, precise and accurate and the limit of detection is low and also it offers the following advantages.

1. Improved resolution of separated substances
2. Column packing with very small (3, 5 and 10 µm) particles
3. Faster separation times (minutes)
4. Sensitivity
5. Reproducibility
6. Continuous flow detectors capable of handling small flow rates
7. Easy sample recovery, handling and maintenance. [24-26]

Methods And Materials:-

Table 3:- Instruments used.

S. No.	Instruments and Glass ware	Name of the manufacturer
1	HPLC	WATERS Alliance 2695 separation module, Software: Empower 2, 996 PDA detector
2	pH meter	Lab India
3	Weighing machine	Sartorius
4	Volumetric flasks	Borosil
5	Pipettes and burettes	Borosil
6	Beakers	Borosil
7	Digital ultra sonicator	Lab Man

Table 4:- Chemicals used.

S. No.	Chemicals	Manufacturer/ Supplier
1	Atorvastatin	Sura labs
2	Ezetimibe	Sura labs
3	Water and methanol for HPLC	LICHROSOL V (MERCK)
4	Acetonitrile for HPLC	Merck

HPLC Method Development:

Trails

Preparation of standard solution:

Accurately weigh and transfer 10 mg of Atorvastatin and Ezetimibe working standard into a 10 ml of clean dry volumetric flasks add about 7 ml of Methanol and sonicate to dissolve and removal of air completely and make

volume up to the mark with the same Methanol. Further pipette 0.2 ml of Atorvastatin and 0.6 ml of Ezetimibe from the above stock solutions into a 10 ml volumetric flask and dilute up to the mark with Methanol.

Procedure:
Inject the samples by changing the chromatographic conditions and record the chromatograms, note the conditions of proper peak elution for performing validation parameters as per ICH guidelines.

Mobile Phase Optimization:
Initially the mobile phase tried was Methanol: Water and ACN: Water with varying proportions. Finally, the mobile phase was optimized to Methanol and Phosphate buffer in proportion 40:60 v/v respectively.

Optimization of Column:
The method was performed with various C18 columns like Symmetry, X terra and ODS column. Phenomenex Gemini C18 (4.6 × 250mm) 5 µm was found to be ideal as it gave good peak shape and resolution at 1 ml/min flow.

Validation
Preparation of Mobile Phase:
Accurately measured 400 ml of Methanol (40%) and 600 ml of Phosphate buffer (pH - 3.8) (60%) were mixed and degassed in a digital ultrasonicater for 10 minutes and then filtered through 0.45 µ filter under vacuum filtration.

Diluent Preparation:
The Mobile phase was used as the diluent.

Validation Parameters System Suitability:
Accurately weigh and transfer 10 mg of Atorvastatin and 10 mg of Ezetimibe working standard into a 10 ml of clean dry volumetric flasks, add about 7 ml of diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 0.1 ml of the above Atorvastatin and 0.3 ml of the Ezetimibe stock solutions into a 10 ml volumetric flask and dilute up to the mark with diluent.

Procedure:
The standard solution was injected for five times and measured the area for all five injections in HPLC. The %RSD for the area of five replicate injections was found to be within the specified limits.

Specificity Study Of Drug:
Preparation of Standard Solution:
Accurately weigh and transfer 10 mg of Atorvastatin and 10 mg of Ezetimibe working standard into a 10 ml of clean dry volumetric flasks add about 7 ml of diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)**.** Further pipette 0.1 ml of the above Atorvastatin and 0.3 ml of the Ezetimibe stock solutions into a 10 ml volumetric flask and dilute up to the mark with diluent.

Preparation of Sample Solution:
Weighing the contents of ten tablets allowed us to determine their average weights, which were subsequently triturated into a fine powder. A 10 mg equivalent weight of Atorvastatin and Ezetimibe was measured and transferred into a 10 ml volumetric flask that was cleaned and dried, add about 7 ml of diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. Further pipette 0.1 ml of the above Atorvastatin and 0.3 ml of the Ezetimibe stock solutions into a 10 ml volumetric flask and dilute up to the mark with diluent.

Preparation Of Drug Solutions For Linearity:
Accurately weigh and transfer 10 mg of Atorvastatin and 10 mg of Ezetimibe working standard into a 10 ml of clean dry volumetric flasks add about 7 ml of diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution).

Preparation of Level – I (6 ppm of Atorvastatin & 18 ppm of Ezetimibe):
Pipette out 0.06 ml of Atorvastatin and 0.18 ml of Ezetimibe stock solutions, take in a 10 ml of volumetric flask dilute up to the mark with diluent.

Preparation of Level – II (8 ppm of Atorvastatin & 24 ppm of Ezetimibe):
Pipette out 0.08 ml of Atorvastatin and 0.24 ml of Ezetimibe stock solutions, take in a 10 ml of volumetric flask dilute up to the mark with diluent.

Preparation of Level – III (10 ppm of Atorvastatin & 30 ppm of Ezetimibe):
Pipette out 0.1 ml of Atorvastatin and 0.3 ml of Ezetimibe stock solutions, take in a 10 ml of volumetric flask dilute up to the mark with diluent.

Preparation of Level – IV (12 ppm of Atorvastatin & 36 ppm of Ezetimibe):
Pipette out 0.12 ml of Atorvastatin and 0.36 ml of Ezetimibe stock solutions, take in a 10 ml of volumetric flask dilute up to the mark with diluent.

Preparation of Level – V (14 ppm of Atorvastatin & 42 ppm of Ezetimibe):
Pipette out 0.14 ml of Atorvastatin and 0.42 ml of Ezetimibe stock solutions, take in a 10 ml of volumetric flask dilute up to the mark with diluent.

Procedure:
Inject each level into the chromatographic system and measure the peak area. Plot a graph of peak area versus concentration (on X-axis concentration and on Y-axis Peak area) and calculate the correlation coefficient.

Precision
Preparation of Atorvastatin and Ezetimibe Product Solution for Precision:
Accurately weigh and transfer 10 mg of Atorvastatin and 10 mg of Ezetimibe working standard into a 10 ml of clean dry volumetric flasks add about 7 ml of diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 0.1 ml of the above Atorvastatin and 0.3 ml of the Ezetimibe stock solutions into a 10 ml volumetric flask and dilute up to the mark with diluent.

The standard solution was injected for five times and measured the area for all five injections in HPLC. The %RSD for the area of five replicate injections was found to be within the specified limits.

Intermediate Precision:
To evaluate the intermediate precision (also known as Ruggedness) of the method, Precision was performed on different days by maintaining same conditions.

Procedure:
DAY 1: The standard solution was injected for six times and measured the area for all six injections in HPLC. The %RSD for the area of six replicate injections was found to be within the specified limits.
DAY 2: The standard solution was injected for six times and measured the area for all six injections in HPLC. The %RSD for the area of six replicate injections was found to be within the specified limits.

Accuracy:
For preparation of 50% Standard stock solution:
Accurately weigh and transfer 10 mg of Atorvastatin and 10 mg of Ezetimibe working standard into a 10 ml of clean dry volumetric flasks add about 7 ml of diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution). Further pipette 0.05 ml of the above Atorvastatin and 0.15 ml of the Ezetimibe stock solutions into a 10 ml volumetric flask and dilute up to the mark with diluent.

For preparation of 100% Standard stock solution:
Accurately weigh and transfer 10 mg of Atorvastatin and 10 mg of Ezetimibe working standard into a 10 ml of clean dry volumetric flasks add about 7 ml of diluents and sonicate to dissolve it completely and make volume up to the

mark with the same solvent. (Stock solution). Further pipette 0.1 ml of the above Atorvastatin and 0.3 ml of the Ezetimibe stock solutions into a 10 ml volumetric flask and dilute up to the mark with diluent.

For preparation of 150% Standard stock solution:
Accurately weigh and transfer 10 mg of Atorvastatin and 10 mg of Ezetimibe working standard into a 10 ml of clean dry volumetric flasks, add about 7 ml of diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution). Further pipette 0.15 ml of Atorvastatin and 0.45 ml of Ezetimibe from the above stock solutions into a 10 ml volumetric flask and dilute up to the mark with diluents.

Procedure:
Inject the three replicate injections of individual concentrations (50%, 100%, 150%) which were made under the optimized conditions. Recorded the chromatograms and measured the peak responses. Calculate the Amount found and Amount added for Atorvastatin and Ezetimibe and calculate the individual recovery and mean recovery values.

Robustness:
The analysis was performed in different conditions to find the variability of test results. The following conditions are checked for variation of results.

For preparation of Standard solution:
Accurately weigh and transfer 10 mg of Atorvastatin and 10 mg of Ezetimibe working standard into a 10 ml of clean dry volumetric flasks add about 7 ml of diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution). Further pipette 0.1 ml of the above Atorvastatin and 0.3 ml of the Ezetimibe stock solutions into a 10 ml volumetric flask and dilute up to the mark with diluent.

Effect of variation of flow conditions:
The sample was analyzed at 0.9 ml/min and 1.1 ml/min instead of 1 ml/min, remaining conditions are same. 20 µl of the above sample was injected and chromatograms were recorded.

Effect of variation of mobile phase organic composition:
The sample was analyzed by variation of mobile phase i.e. Acetonitrile: Phosphate Buffer was taken in the ratio 50:50 and 45:55 instead 40:60, remaining conditions are same. 10 µl of the above sample was injected and chromatograms were recorded.

Results and Discussion of System Suitability:-

Chromatographic conditions:
The method was performed with various C18 columns like Symmetry, X terra and ODS column. Phenomenex Gemini C18 (4.6 × 250 mm) 5 µm was found to be ideal as it gave good peak shape and resolution at 1 ml/min flow, equilibrated with Methanol and Phosphate buffer (pH - 3.8) (40:60% v/v) as a mobile phase. Run time was 6 min and here the peaks were seperated and showed better resolution. Conditions of optimized chromatography are shown in table No. 5.

Table No. 5:- Optimized Chromatographic Conditions.

Mobile phase	Methanol and Phosphate buffer (pH - 3.8) (40:60% v/v)
Wavelength	251 nm
Flow rate	1 ml/min
Run time	6 min
Temperature of the column	35ºC
Injection volume	20 µl
Column	Phenomenex Gemini C18 (4.6 × 250 mm) 5 µm particle size

Specificity: There was no other components present at the elution time for Atorvastatin and Ezetimibe. As seen in the figure - 4 , the blank chromatogram is present.

Fig. No. 3:- Optimized chromatogram of Atorvastatin (RT = 2.121 min) & Ezetimibe (RT = 3.643 min).

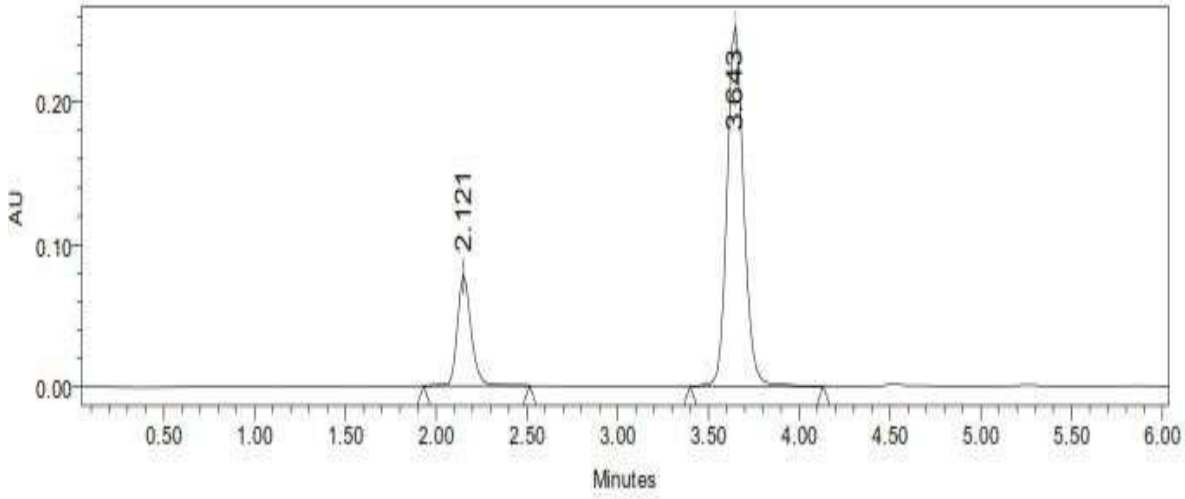
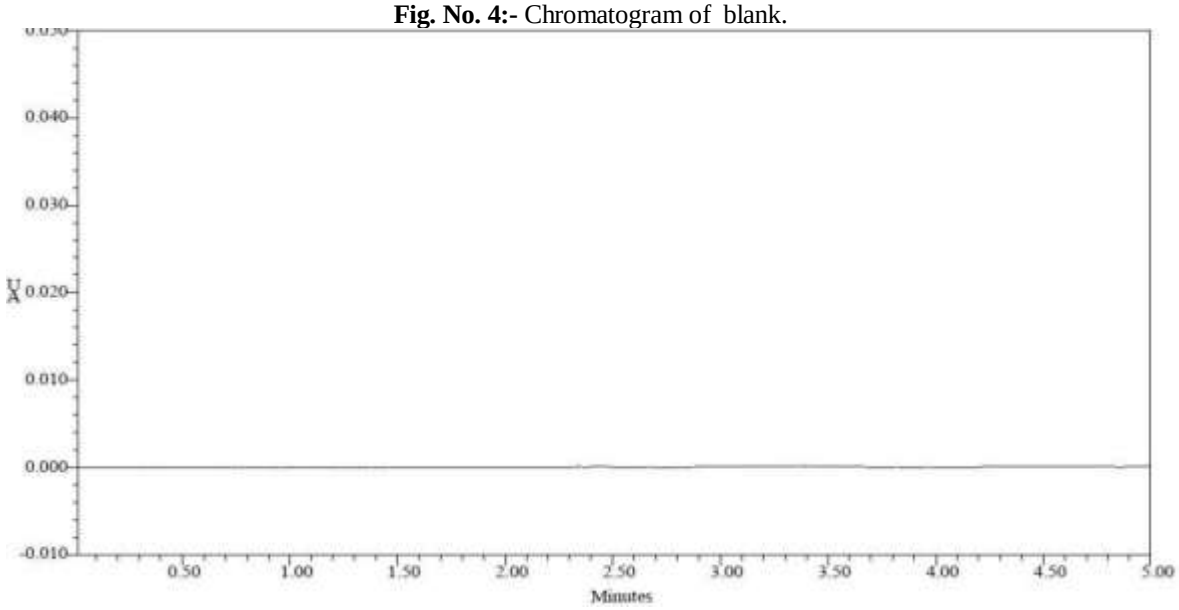


Fig. No. 4:- Chromatogram of blank.



Linearity: The linearity range was found to be 10-30 µg/ml of Atorvastatin, 30-90 µg/ml of Ezetimibe and chromatograms are shown in table no - 6.

Table No. 6:- Linearity Data of Atorvastatin and Ezetimibe.

S. No.	Atorvastatin		Ezetimibe	
	Working conc. (µg/ml)	Peak Area	Working conc. (µg/ml)	Peak Area
1	10	245899	30	863094
2	15	365687	45	1249397
3	20	481526	60	1678592
4	25	589854	75	2050412
5	30	705882	90	2468444
Correlation coefficient (r)		0.999		0.999
Slope		23457		27290
Intercept (c)		7184		20465

Fig. No. 5:- Calibration plot of Atorvastatin.

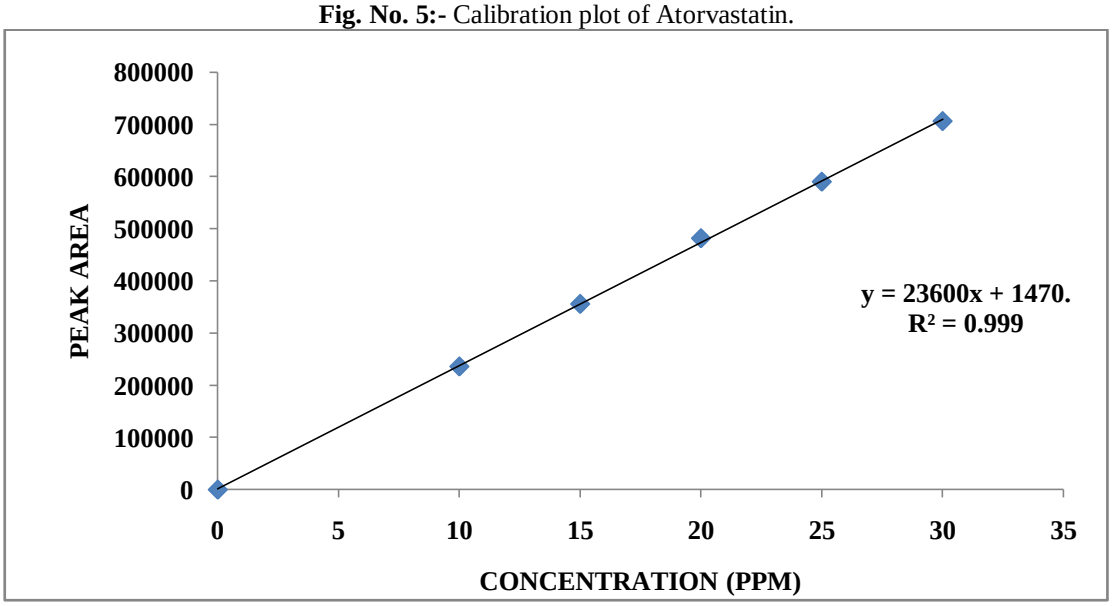
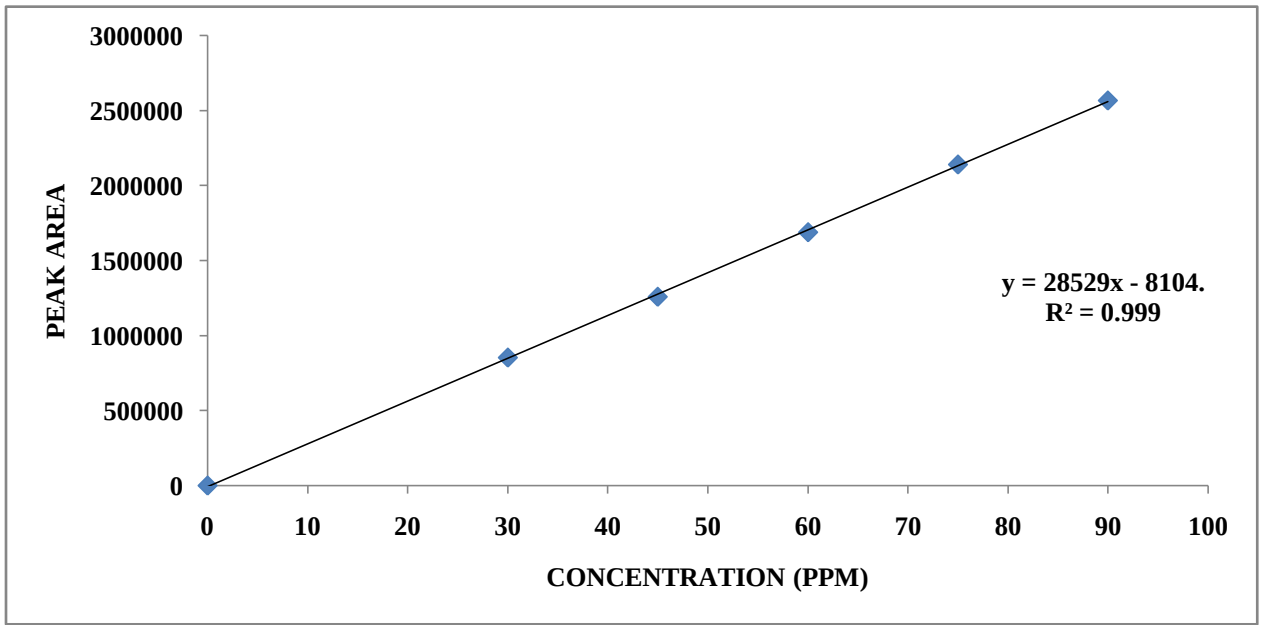


Fig. No. 6:- Calibration plot of Ezetimibe.



Precision: Precision of the method was carried out for both sample and standard solutions as described under experimental work. The corresponding chromatogram and results are shown in table no – 7.

Table No. 7:- Results of method precision.

S. No.	Atorvastatin		Ezetimibe	
	Retention time (min)	Peak Area	Retention time (min)	Peak Area
1	2.198	514658	3.623	1645875
2	2.196	514354	3.611	1658554
3	2.160	513985	3.696	1649854
4	2.160	514875	3.696	1659842
5	2.160	514658	3.696	1645985

6	2.186	516452	3.642	1659852
Mean		5148303		1653327
Std. Dev.		852.3705		6838.733
%RSD		0.165563		0.413635

Table No. 8:- Results of intermediate precision.

S. No.	Atorvastatin		Ezetimibe	
	Retention time (min)	Peak Area	Retention time (min)	Peak Area
1	2.198	514658	3.611	1638732
2	2.196	514895	3.623	1637438
3	2.178	514658	3.684	1638474
4	2.142	514784	3.697	1634273
5	2.177	515268	3.684	1636372
6	2.177	514598	3.684	1639283
Mean		5148102		1637429
Std. Dev.		248.5224		1860366
%RSD		0.048275		0.113615

Accuracy:
Sample solutions at different concentrations (50%, 100%, and 150%) were prepared and the % recovery was calculated.

Table 9:- The Accuracy results for Atorvastatin.

Accuracy level	Atorvastatin			Ezetimibe		
	Amount added (µg/ml)	Amount found (µg/ml)	% Recovery	Amount added (µg/ml)	Amount found (µg/ml)	% Recovery
50%	10	10.179	101.79%	30	30.114	100.38%
100%	20	20.316	101.58%	60	60.068	100.113%
150%	30	30	100.72%	90	90.268	100.297%
Mean % Recovery	101.36%			100.26%		

Limit of detection and Limit of quantification (LOD & LOQ):
The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value. The quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined.

Table No. 10:- LOD & LOQ data for Atorvastatin and Ezetimibe.

Drug	**LOD (µg/ml)**	**LOQ (µg/ml)**
Atorvastatin	1.0 µg/ml.	11.0 µg/ml.
Ezetimibe	3.1 µg/ml.	3.2 µg/ml.

Robustness
The robustness was performed for the flow rate variations from 0.9 ml/min to 1.1 ml/min and mobile phase ratio variation from more organic phase to less organic phase ratio for Atorvastatin and Ezetimibe . The method is robust only in less flow condition and the method is robust even by change in the Mobile phase ± 5 %.

Table No. 11:- Robustness data for Atorvastatin and Ezetimibe.

Parameter used for sample analysis	Atorvastatin		Ezetimibe	
	Retention Time	Tailing factor	Retention time	Tailing factor
Actual Flow rate of 1.0 ml/min	2.179	1.2	3.610	1.1
Less Flow rate of 0.9 ml/min	2.210	0.9	4.498	0.9
More Flow rate of 1.1 ml/min	2.184	1.0	3.505	0.8
Less organic phase	2.200	0.9	4.504	0.9
More Organic phase	2.172	0.8	3.512	0.9

Conclusion:-

The study is focused to develop and validate RP - HPLC method for estimation of Atorvastatin and Ezetimibe in bulk and tablet dosage form. For routine analytical purpose it is desirable to establish methods capable of analysing huge number of samples in a short time period with good robustness, accuracy and precision without any prior separation steps. HPLC method generates large amount of quality data, which serve as highly powerful and convenient analytical tool. The method shows good reproducibility and good recovery. From the specificity studies, it was found that the developed methods were specific for Atorvastatin and Ezetimibe.

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