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Development and Validation of RP-HPLC Method for Simultaneous Estimation of Atorvastatin and Ezetimibe in Combined Formulation

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Abstract
A simple, selective, robust and sensitive reversed phase high performance liquid chromatography method has been developed and validated for the simultaneous estimation of Atorvastatin and Ezetimibe in bulk drug and pharmaceutical formulations. The separation was achieved on a phenomenex C-18 (250 × 4.6 mm, packed with 5 µ) column by using an isocratic mobile phase mixture composed of Acetonitrile: ammonium acetate buffer pH 3.0 (50:50, v/v) with 1.1 mL/min as flow rate and the eluents were monitored at 247 nm. The retention times for Atorvastatin, Ezetimibe were 3.3, 4.5 min respectively, the linearity for both analytes was found to that r^2 = 0.991 and 0.986 for Atorvastatin and Ezetimibe respectively. The method was validated for its system suitability, accuracy, precision and stability. The proposed method was successfully employed for the simultaneous quantification of Atorvastatin and Ezetimibe in their pharmaceutical formulation.

Keywords
Atorvastatin and Ezetimibe

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INTRODUCTION
Atorvastatin (ATR) is a synthetic lipid-lowering agent is chemically [R-(R*, R*)]-2-(4-flurophenyl) b-dihydroxy-5-1-methyl ethyl)-3-phenyl-4-[(phenylamino)carbonyl]-1*H*-pyrrole-1-heptanoic acid. It lowers the cholesterol level by inhibiting the 3-hydroxy-3-methyl-glutaryl reductase (HMG-CoA reductase) coenzyme. HMG-CoA reductase is responsible for the conversion of HMG-CoA to mevalonate, an early and rate limiting step in the synthesis of cholesterol in liver. Inhibition of cholesterol synthesis in the liver leads to an increase in LDL catabolism. This also reduces the LDL-production to some extent which results into inhibition of hepatic synthesis of very low density lipoprotein, the precursor of LDL-cholesterol.[1-2] There are numerous methods for estimation atorvastatin alone HPLC and in combination with other drugs such as ramipril, aspirin, telmisartan, fenofibrate were reported[3-4].

Ezetimibe (EZT) is chemically 1- (4-flurophenyl) -3 (R) [3- (4-flurophenyl)- 3 (S) - hydroxypropyl]-4(S) (4hydroxyphenyl)-2-azetidione] is also a lipid lowering agent. It reduces the blood cholesterol by preventing intestinal absorption of cholesterol without altering absorption of triglycerides, fatty

acids, bile acids and fat-soluble vitamins[5-7]. Reports revealed that Ezetimibe alone can be estimated by HPLC and LC-MS/ MS methods[8]. Methods such as spectrophotometric, HPLC and HPTLC methods were reported for the simultaneous estimation of Atorvastatin and Ezetimibe. Recently, Bhatt *et al*. reported a simultaneous method for the estimation of Atorvastatin and Ezetimibe. The method was sensitive enough in terms of its limit of detection (LOD) and limit of quantification (LOQ). It utilizes a large quantity of methanol as one of its mobile phase solvent (> 60 % in total volume), in general methanol is a most poisonous and high viscous solvent which cause high back pressure in column used in HPLC, which leads to reduced column life. Hence present paper portray a highly sensitive, more reproducible and robust HPLC method for the estimation of Atorvastatin and Ezetimibe using new mobile phase with acetonitrile a high polar, low viscous less hazardous HPLC solvent with ammonium acetate buffer[9-11].

MATERIALS AND METHODS

Pharmaceutical Formulations

Atorvastatin and Ezetimibe manufactured by Entod pharmaceuticals ltd, Mumbai, India. Methanol HPLC grade was obtained from Merck, Germany. Double distilled water (in house) was used throughout the analysis. Mobile phase was filtered using 0.45 µm membrane filter (Millipore – Millipore Pvt Ltd, Bangalore, India). Pure drugs of Atorvastatin and Ezetimibe were collected from Asha Pharmaceuticals, Hyderabad, Telangana, India.

Equipment and Analytical Conditions

Chromatographic separation was achieved on WATERS HPLC (515 pump) system fitted with a C18 phenomenex (250 x 4.5 mm, 5 µ) column and a UV (2489) detector. All the parameters of HPLC were controlled by EMPOWER-2 software. All chromatographic determinations were carried out in isocratic elution mode with a flow rate of 1.1 ml/min. The detector wavelength was set at 247 nm, sample injection volume was 20 µl. The UV spectrophotometric method was performed on ELICO-SL159 UV-Visible spectrophotometer at appropriately selected wavelength for dual wavelength method of analysis. All the parameters of UV were controlled by SPECTRATREATS software. One-centimeter quartz cells were used for measuring absorbance.

Optimized Chromatographic Conditions for the Proposed HPLC Method:

Stationary Phase: phenomenex C-18 (250 × 4.5 mm, 5 µ) column (waters)
Mobile Phase: 50: 50 Acetonitrile and (0.05M) ammonium acetate buffer (pH 3.0 ± 0.05, adjusted by addition of 10 % acetic acid)
Flow rate 1.1 ml/min
Detection wavelength 247 nm
Injection Volume 20 µl
Run time 8 min

1. Preparation of Standard Stock Solution

25 mg each of Ezetimibe and Atorvastatin were taken in 25 ml volumetric flasks separately, dissolved and made up to the mark with Acetonitrile and (0.05M) ammonium acetate buffer (50:50 v/v).

2. Preparation of Working Standard Solutions

Working standard solutions of concentrations 10, 50, 100, 200, up to 1000 µg/ml were prepared by appropriate dilutions of the standard stock solution with the Acetonitrile and (0.05M) ammonium acetate buffer (50:50 v/v).

3. Preparation of Test Solution

5 ml of marketed formulation of Atorvastatin and Ezetimibe transferred into a 25 ml volumetric flask, dissolved and made volume up to the mark with Acetonitrile and (0.05M) ammonium acetate buffer (50:50 v/v). 3 ml of this solution was transferred into 10 ml volumetric flask and
the volume was made up to the mark with Acetonitrile and (0.05M) ammonium acetate buffer (50:50 v/v). The solution was filtered through 0.45 µ filter paper and then degassed by Sonication

HPLC - Method Development and Optimization

To develop a suitable HPLC method for the determination of Atorvastatin and Ezetimibe, trials were performed with different mobile phases, using methanol and buffer at different pH with different compositions of mobile phases like 50:50 (50 : 50 Acetonitrile and (0.05M) ammonium acetate buffer (pH-3), like 80:20 (50 : 50 Acetonitrile and (0.05M) ammonium acetate buffer pH-5), 50:30:20 (Methanol: Acetonitrile: water), 50:30:20(Methanol: Acetonitrile: water), 70:30 (Methanol: Water) and 70:30 (methanol: di potassium hydrogen phosphate). The method was optimized finally using combination of acetonitrile and ammonium acetate buffer in the ratio of 50:50% v/v with a flow rate of 1.1 ml/min. The drugs were eluted at retention time of 3.3 min (Atorvastatin) and 4.5 min (Ezetimibe) at a detection wavelength of 245 nm. The run time was set for 8 minutes.

1. Preparation of the Mobile Phase

The mobile phase was prepared by mixing of acetonitrile and ammonium acetate buffer (pH-3 adjusted with 10% acetic acid) in the ratio of (50:50 v/v). It was filtered through 0.45 µ filter paper then degassed by sonication.

2. Preparation of Mixed Standard Stock Solution

25 mg of each of Atorvastatin and Ezetimibe were accurately weighed and transferred into a 25 ml volumetric flask, dissolved and was made up to the mark with mobile phase and mixed well. This was used as the standard stock solution.

3. Preparation of Working Standard Solutions

Working standard solution of concentrations 10, 50, 100,200, up to 1000 µg/ml were prepared by appropriate dilutions of the standard stock solution with the mobile phase. The solutions thus prepared were filtered through0.45 µ membrane filter and sonicated for 5 min.

4. Preparation of Test Solution

5 ml of marketed formulation of Atorvastatin and Ezetimibe transferred into 25 ml volumetric flask, dissolved and made volume up to the mark with mobile phase. 4 ml of this solution was transferred into 10 ml volumetric flask and the volume was made up to the mark with mobile phase. The solution was filtered through 0.45 µ filter paper and then degassed by Sonication.

Validation of Assay Methods [4-6]

1. Linearity

For HPLC, Standard solutions in the range of 10 µg/ml to 1mg/ml were prepared by appropriate dilutions of the mixed standard stock solution and 20 µl of each of standard solutions were injected at the optimized overlain chromatographic conditions and calibration curves of Atorvastatin and Ezetimibe (Figure 1) were constructed by plotting concentration on X-axis against peak area on Y-axis and regression equations were computed.

2. Assay of Marketed Formulation

For HPLC, the test solutions were prepared from marketed formulation and this prepared sample solutions were injected under identical chromatographic conditions as mentioned earlier and chromatograms were recorded (Figure 4), this was done in triplicate. The concentration of this test sample determined by substituting the peak area in the regression equations of calibration curve to get the concentration.

3. System Suitability

For HPLC System suitability test was performed by injecting standard solution of (200 µg/ml) concentration into the stabilized HPLC system, six times. The system suitability was established by evaluating repeatability, tailing factor (T) and theoretical plates (N), from the standard chromatograms obtained.

5. Accuracy

To study the accuracy of proposed methods, recovery studies were carried out by standard addition method at three different levels. Known amount of the two drugs was added to pre-analyzed eye drop formulation and percentage recoveries were calculated. For HPLC and UV, recovery studies were carried out at three different levels (50%, 100% and 150%) by the addition of standard drug to pre-analyzed sample solution having the concentration of Ezetimibe and Atorvastatin. Triplicate determinations were carried out at each level. Mean percentage recovery values at three different levels of the drug were calculated for both methods.

% Recovery = (Spiked Concentration- Test Concentration) / Standard Concentration X 100

6. Precision

For HPLC, System precision was performed by injecting six replicate injections of standard solution (100 µg/ml) and the chromatograms were reviewed for the % RSD of peakareas. Method precision was demonstrated by preparing six test solutions at 100 µg/ml and 100 µg/ml concentration as per the test procedure and recording the chromatograms.

7. Estimation of Stability of Drug Solutions

Stability was estimated with standard (200 µg/ml) and test solutions at 200 µg/ml (Ator) and 200 µg/ml (Ezet) level by injecting the solutions at intervals of 24 hrs until there was a significant change (due to degradation) in the peak area values.

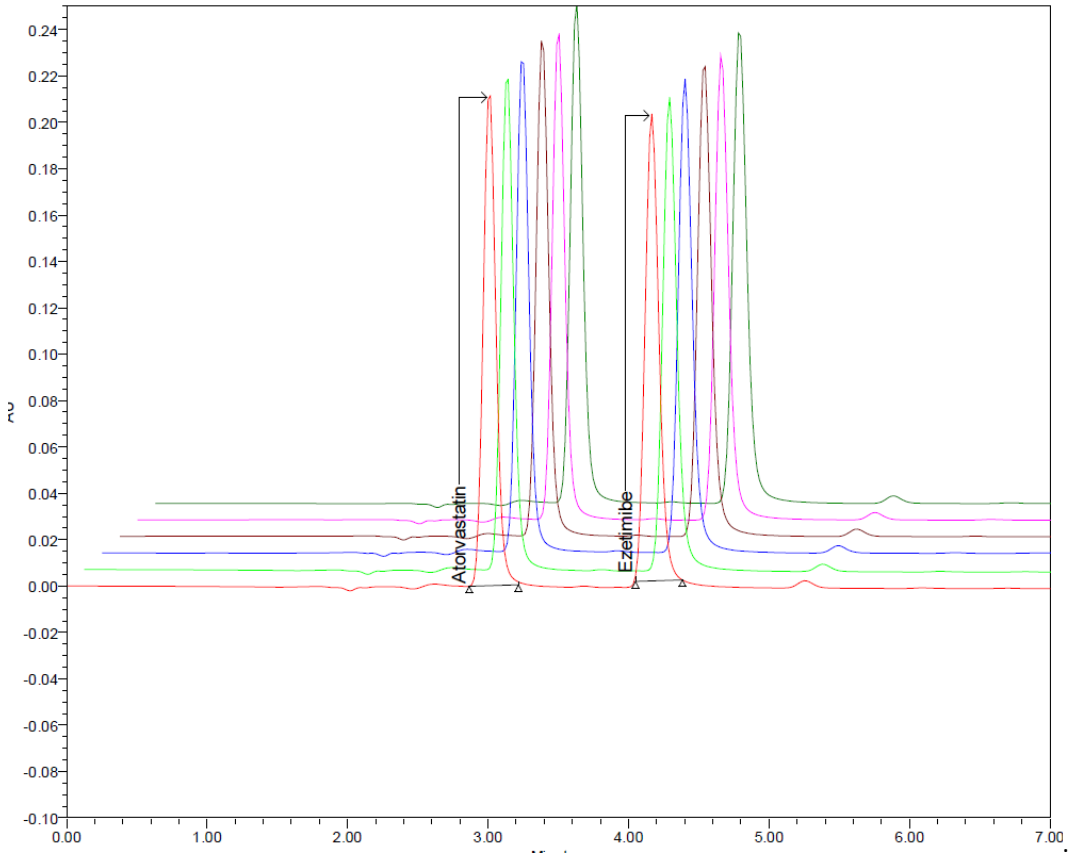

Figure 1: Overlain chromatogram of linearity

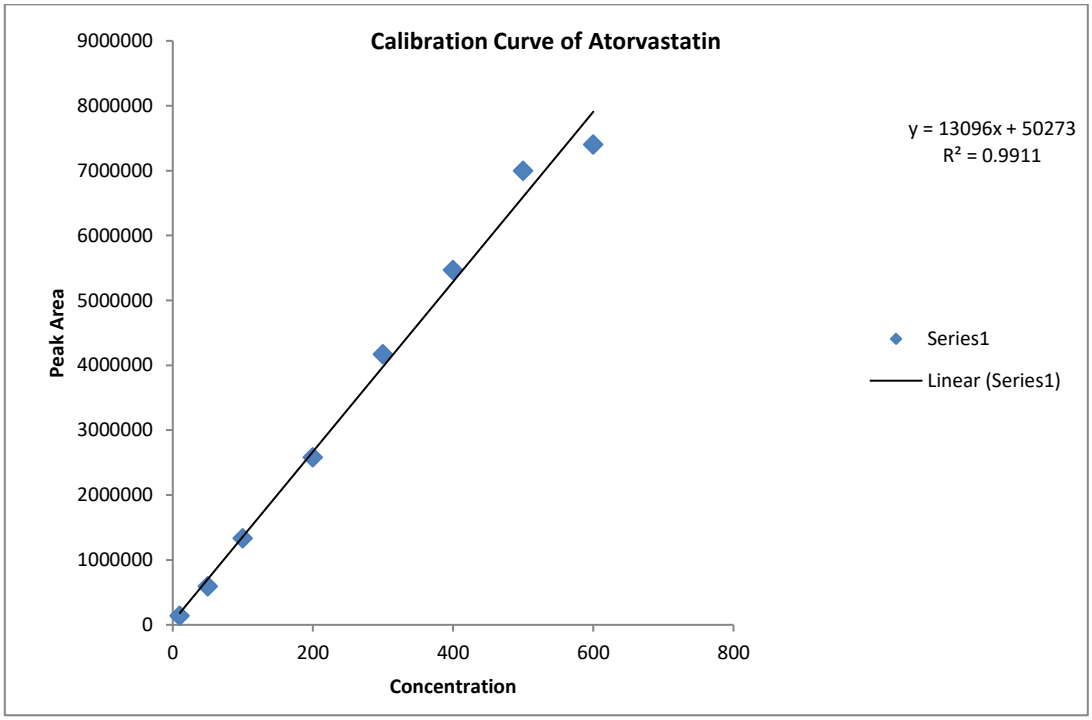

Figure2: Calibration Curve Atorvastatin

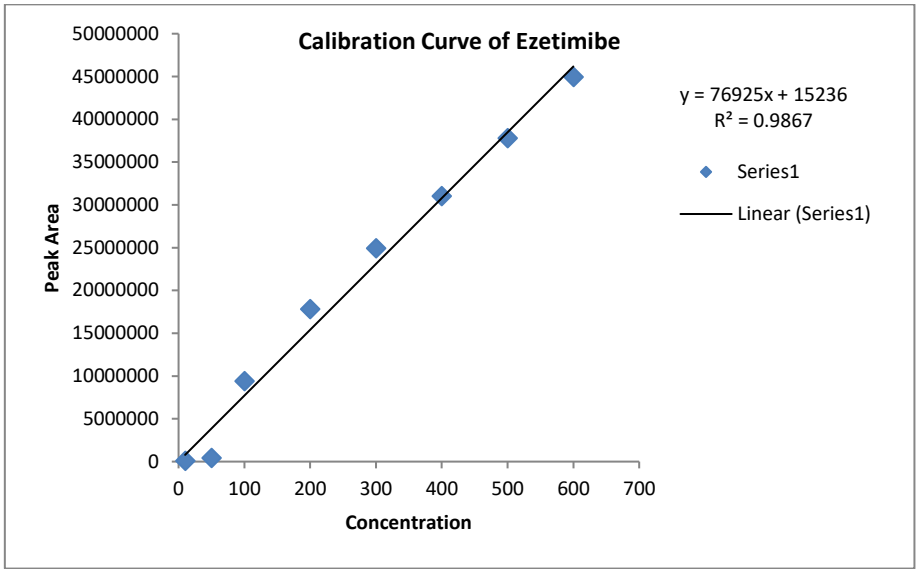

Figure3: Calibration Curve Ezetimibe

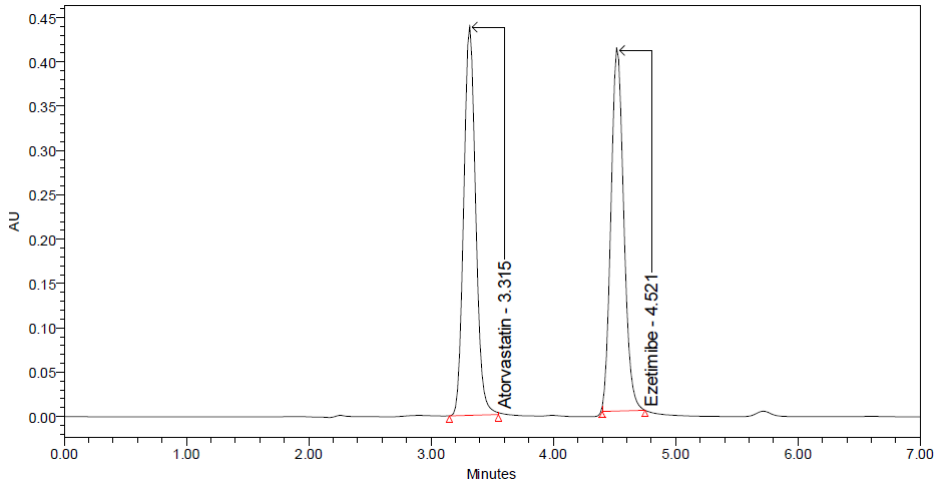

Figure 4: Chromatogram of test sample

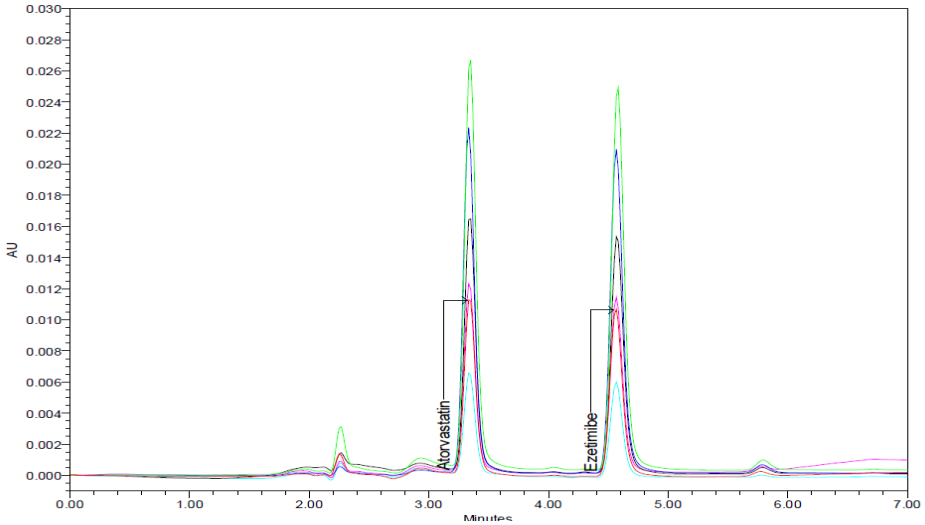

Figure 5: Overlain chromatogram of LOD

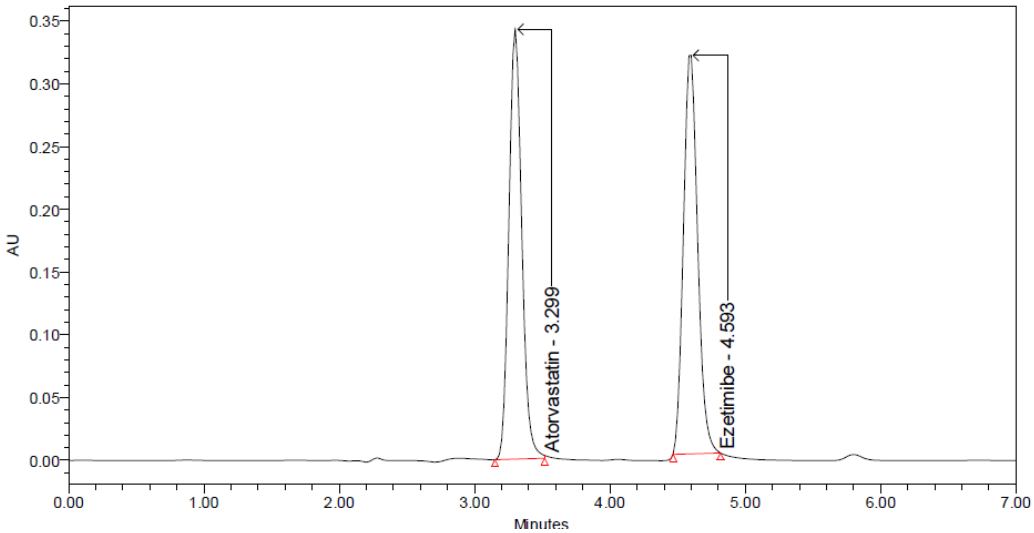

Figure 6: chromatogram of system suitability

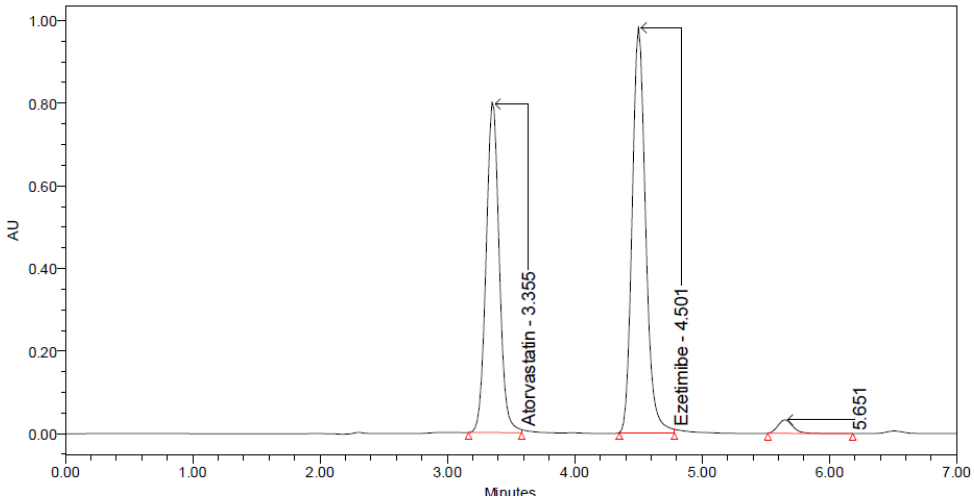

Figure 7: chromatogram of stability

Table 1: Results for Linearity

		PEAK AREA	
S.No	**Conc (µg/mL)**	**Atorvastatin**	**Ezetimibe**
1	10	138817	82878
2	50	592778	4082721
3	100	1333571	9371343
4	200	2583294	17813696
5	300	4172601	24893423
6	400	5470801	31009652
7	500	6996029	37787622
8	600	7400802	44913277
Correlation Coefficient(r^2)		0.991	0.986
Y-Intercept		50273	15236

Table 2: Results of Assay

S. No.	Parameters	Atorva	Ezetim
1	Label claim	10 mg	10 mg
2	Test peak area	47886	41433
3	Amount obtained	9.8mg	9.7mg
4	% Assay	98.00%	97.00%

Table 3: System Suitability

Drug Name	Retention Time	Peak Area	Tailing Factor	Theoretical Plate Count
Atorvastatin	3.299	2261450	1.08	5648
Ezetimibe	4.593	2319442	1.12	6215

Table 4 : Accuracy study data of Atorvastatin

Level of Spiking	Peak Area of Ator	Test Peak Area Conc. (100 µg/ml)	Spiked Peak Area	% Recovery	Mean % Recovery
	2206728	1579115	3776587	99.2	
50%	2197698	1579895	3753425	98.7	99.2
	2207301	1570102	3770605	99.6	
	4806125	1578922	6340399	98.4	
100%	4822099	1579897	6337289	99.1	98.6
	4812899	1570089	6373509	98.3	
	7142458	1579009	8664099	99.2	
150%	7184199	1599098	8684112	99.1	99.2
	7348304	1570089	8893598	99.4	

Table 5: Accuracy study data of Ezetimibe

Level of Spiking	Peak Area of Ezet	Test Peak Area Conc. (100 µg/ml)	Spiked Peak Area	% Recovery	Mean % Recovery
	1281502	180701	1449694	98.2	
50%	1274245	180684	1439105	98.6	98.4
	1296398	180704	1456552	98.4	
	1880884	180705	2032187	98.5	
100%	1871897	180678	2030898	98.9	98.7
	1871125	180712	2033702	99.2	
	2679706	180722	2876358	100.1	
150%	2669987	180754	2876312	99.8	99.8
	2898102	180715	3056655	99.5	

Table 6: Method Precision Data (HPLC)

S. No	Conc (µg/mL)	Peak Area Atorvastatin	% Assay	Conc (µg/mL)	Peak Area Ezetimibe	% Assay
1	100	6457394	98.2	100	5188825	98.7
2	100	6443225	98.4	100	5168578	98.5
3	100	6450158	98.3	100	5169788	98.6
4	100	6453101	98.5	100	5177335	98.4
5	100	6441245	98.6	100	5166012	99.1
6	100	6443584	98.3	100	5158884	98.7
%RSD			0.20	%RSD		0.22

Table 7: Stability Study Data of Atorvastatin

Time (hours)	Std. Ator Peak Area	%Assay	Time (hours)	Test. Ator Peak Area	%Assay
Initial	2446502	99.4	Initial	2447205	99.6
24	2422198	98.7	24	2430000	98.3
48	2420315	97.5	48	2420120	97.6

Table 8: Stability Study Data of Ezetimibe

Time (hours)	Std. Ezeti Peak Area	%Assay	Time (hours)	Test. Ezeti Peak Area	%Assay
Initial	16883546	100	Initial	1382205	99.9
24	16652905	98.7	24	1375258	99.4
48	16625504	97.3	48	1369402	97.5

RESULTS AND DISCUSSION:

Atorvastatin and Ezetimibe are used for the treatment in reduce the cholesterol in blood. The present work focuses on simple, accurate, precise, and economic RP-HPLC method for simultaneous estimation of Atorvastatin and Ezetimibe in pharmaceutical formulation. Method development trials were performed, using several combinations of chromatographic conditions, but the optimum results were achieved with phenomenex C18 (250 x 4.5 mm, 5µ) column, mobile phase mixture constituting 50: 50 Acetonitrile and (0.05M) ammonium acetate buffer (pH 3.0 ± 0.05, adjusted by addition of 10 % acetic acid) at 1.1 ml/min flow rate and a detection wavelength of 247 nm. Atorvastatin and Ezetimibe peaks were achieved with good, resolution, peak shape and symmetry at Rt 3.3 min and 4.5 min respectively. The assay was performed on the marketed formulation and the % drugs content were found to be 98% and 97% respectively (Table 2), which was within the acceptance limits. The linearity of the proposed method for both drugs were accomplished from the correlation coefficient of the standard calibration curves (Table 1), which were constructed at concentration range of 10 µg - 600 µg/ml (Atorvastatin) and 10 µg-600 µg/ml (Ezetimibe) (Figure 2-3). The correlation coefficient was found to be 0.991 and 0.986 for both drugs which was in compliance with the acceptance criteria. The accuracy of the proposed method was evaluated from the recovery studies, by standard addition method which was performed at three levels of 50%, 100% and 150%. The mean percentage recoveries at each level were found to be 98.6–99.8%, (Table 4-5) which indicates good recovery. The precision of the proposed method was established from the % RSDs of the %assays of the both drugs at the levels of method precision The RSDs of peak areas of system precision were found to be 0.20% and 0.22% respectively (Table 6). The system suitability of the proposed method was accomplished from the resolution, theoretical plate count and asymmetric factor at the optimized conditions. The parameters were recorded and tabulated and were found to be in compliance with the acceptance specifications. The solution stability studies were performed, and the standard and test drug solutions were found to be stable for 48 hrs from the time of their preparation (Table 7 and 8). The Percentage recoveries 97.5 – 99.9 %. This indicates that the developed method was found to be accurate. As all the validation parameters studied, complied with the acceptance criteria, the proposed HPLC method was said to be validated accordance with ICH guidelines.

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