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Der Pharmacia Lettre, 2010: 2 (1) 427-436
(http://scholarsresearchlibrary.com/archive.html)

ISSN 0975-5071
USA CODEN: DPLEB4

Simultaneous Estimation of Atorvastatin and Ezetimibe in Pharmaceutical Formulations by RP-HPLC Method

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Abstract

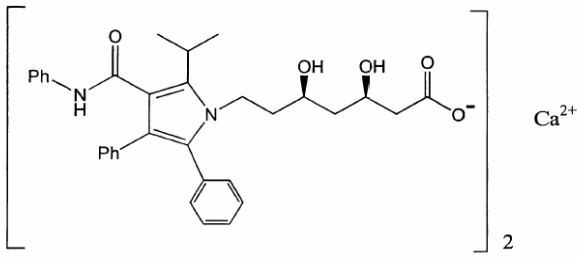
Atorvastatin and Ezetimibe are used to treat hyperlipidemia. A simple, precise cost effective and stability indicating RP-HPLC method has been developed and validated for the simultaneous determination of Atorvastatin and Ezetimibe in pharmaceutical formulations. Separation of both Atorvastatin and Ezetimibe was achieved with in 5 minutes with required resolution, accuracy and precision thus enabling the utility of the method for routine analysis. Chromatographic separation was achieved on a Waters Symmetry C18 3.5µm, 150 mm x 3.0 mm using a mobile phase consisting of 0.05M phosphate buffer pH3.0 and Acetonitrile in the ratio of 45:55 at a flow rate of 0.8ml per minute. The detection was made at 236nm. The retention time of Atorvastatin and Ezetimibe were2.5 and 2.0 minutes respectively. The method was found linear over the range of 5-15 µg per ml for Ezetimibe and 40-120 µg per ml for Atorvastatin. The proposed method was validated as per the ICH and USP guidelines.

Key words: Atorvastatin and Ezetimibe, HPLC and validation

Introduction

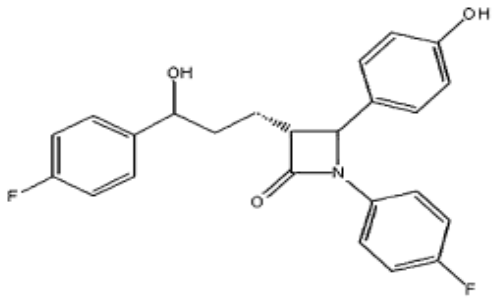
Atorvastatin calcium(Figure 1), is [R-(R*,R*)]-2-(4-fluorophenyl)-β,δ-dihydroxy-5-(1-methyl ethyl)-3-phenyl-4-[(phenylamino)carbonyl]-1Hpyrrole-1-heptanoic acid, calcium salt (2:1) trihydrate. The empirical formula of atorvastatin calcium is $(C_{33}H_{34}\ FN_2O_5)2Ca{\bullet}3H_2O$ and its molecular weight is 1209.42. Atorvastatin is an inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase. This enzyme catalyzes the conversion of HMG-CoA to mevalonate, an early and rate-limiting step in cholesterol biosynthesis [1-2]. The typical dose of Atorvastatin calcium is 10-80 mg per day and it reduces 40-60% LDL [3].

Figure 1: Chemical Structure of Atorvastatin Calcium



Ezetimibe (Figure 2), (1-(4-flurophenyl)-3(R)-[3(S)-(4-flurophenyl) –3-hydroxy propyl] –4(S) (4 –hydroxyphenyl) azetidin- 2- one), which belongs to a group of selective and very effective 2-azetidione cholesterol absorption inhibitors acts at the level of cholesterol entry into enterocytes [4]. It prevents transport of cholesterol through the intestinal wall by selectively blocking the absorption of cholesterol from dietary and billiary sources. This reduces the overall delivery of cholesterol to the liver, thereby promoting the synthesis of LDL receptors and a subsequent reduction in serum LDL-C [5-6].

Figure 2: Chemical Structure of Ezetimibe



Clinical studies have shown that co-administration of ezetimibe with statins could provide an additional reduction in LDL cholesterol as well as total cholesterol [7].

A few methods based on HPLC [8-9], GC-MS [10] LC-MS [11] HPLC – Electrospray tendem mass spectrometry [12] and HPTLC [13] were reported earlier for the determination of Atorvastatin individually and in combination with other drugs.

A few analytical procedures were also proposed for the determination of ezetimibe in dosage forms [14] in human serum, urine and feces [15].

Although the combinational use of Atorvastatin and Ezetimibe is continuously increasing, simultaneous analysis of these two components in their pharmaceutical preparation is not official in Indian Pharmacopoeia, British Pharmacopoeia, United states and European Pharmacopoeia. There is an urgent need to develop and validated analytical methods for the simultaneous analysis of Atorvastatin and Ezetimibe in pharmaceutical dosage forms. We describe herein a simple, sensitive and validated stability indicating HPLC method utilizing isocratic mobile phase with short retention time for the simultaneous determination of these two components in

pharmaceutical formulations like tablets. The developed method can be successfully applied to quality control and other analytical purposes.

Materials and Methods

I. Chemicals and Reagents

Atorvastatin and Ezetimibe working standards were procured from Cipla Labs, and the tested pharmaceutical formulations (Atorvastatin (80mg) and Ezetimibe (10mg) tablets) were procured from commercial pharmacy. Potassium dihydrogen phosphate, acetonitrile, methanol, ortho phosphoric acid were of suitable analytical grade.

II. Apparatus and Chromatographic Conditions

HPLC analysis was performed on Waters HPLC system equipped with a 2696 separation module and 2996 Photo Diode Array Detector. Separations were carried on a Waters Symmetry C18 3.5µm, 150 mm x 3.0 mm using isocratic elution. The flow rate was 1.0 mL min-1. UV detection was performed at 236 nm. Injection volume was 10 µL. Peak identity was confirmed by retention time comparison and the HPLC was operated at room temperature.

III. Preparation of Mobile Phase

The mobile phase is composed of a mixture of 0.05M-Potassium dihydrogen phosphate buffer (pH 3.0) and acetonitrile in the ratio of 45:55 (v/v)., filtered through a 0.45 µm nylon filter (Millipore, USA) and degassed by sonication prior to use.

IV. Preparation of Standard Solution

The standard stock solution of Atorvastatin (0.8 mg/mL) and Ezetimibe (0.125 mg/ mL) was prepared in methanol since both drugs are soluble in this solvent. The working standard solution Atorvastatin (80 µg/mL) and ezetimibe (10 µg/mL) was prepared by diluting the stock solution in mobile phase solution.

V. Preparation of Sample Solution

Twenty tablets were weighed to get the average weight and then ground. An amount of powder equivalent to 80 mg of Atorvastatin and 10mg of ezetimibe was transferred to a 100 mL volumetric flask, added 70 mL of methanol and sonicated for 30 min with intermediate shaking. Followed by making up to volume with methanol to obtain a solution containing 0.8 mg/ mL Atorvastatin and 0.1 mg/ mL Ezetimibe. An aliquot was then removed and centrifuged at 5000rpm for 10min. The solution was filtered using 0.45µm membrane filter paper. After filtration, the solutions were diluted with mobile phase to give a final concentration of Atorvastatin (80 µg/mL) and Ezetimibe (10 µg/mL).

Results and Discussion

Method Development

Drug quality control, stability, metabolism, pharmacokinetics, and toxicity studies all necessitate the determination of drugs in pharmaceutical formulations and biological samples. Correspondingly, efficient and validated analytical methods are very critical requirements for all these investigations. Chromatographic parameters were preliminary optimized to develop a LC

method for simultaneous determination of Ezetimibe and Atorvastatin with short analyses time (<4 min), and acceptable resolution (Rs > 2). The polarity of Ezetimibe and Atorvastatin differ greatly as Atorvastatin is more lipophilic than Ezetimibe.

The sample retention increases with increased column length so a shorter column (150 x 3.0 mm i.d.) was selected to have a shortest possible runtime not compromising on the resolution.
Lower particle size (3.5µm) column was chosen to increase the resolution between the Ezetimibe and Atorvastatin.

In order to identify a suitable organic modifier, various compositions of acetonitrile and methanol were tested. Methanol produced a high retention time for Atorvastatin and high column pressures due to the high viscosity. Acetonitrile was found to display advantageous separations. Change of percentage of acetonitrile in the mobile phase brought about a great influence on retention time of the two drugs. When the acetonitrile content was lower than 50%, retention time of Atorvastatin increased rapidly and when the acetonitrile content was higher than 60%, Resolution between Ezetimibe and Atorvastatin dropped below the value of 3.0.

Effects of the mobile phase pH on retention of the both drugs were investigated at pH values of 3, 4, 5, 6, and 7, respectively. It was found that the mobile phase pH had no effect on the retention of Atorvastatin and Ezetimibe.

Finally separation for simultaneous determination of Ezetimibe and Atorvastatin was carried out by isocratic elution using 55% acetonitrile with a flow rate of 0.8 mL per min. Under this separation condition, retention time was 2.01 for Ezetimibe and 2.55 for Atorvastatin.
The above method is suitable routine pharmaceutical applications involving the analysis of Atorvastatin and Ezetimibe. As shown in Table 1 retention time of each analyte was very reproducible with relative standard deviations between 0.03 and 0.04% (n = 6) for Ezetimibe and Atorvastatin respectively. The peak area responses were also reproducible with relative standard deviations between 0.6 and 0.5% (n = 6) for Ezetimibe and Atorvastatin respectively.

Table: 1

Parameter	Result		Acceptance Criteria
	Ezetimibe	Atorvastatin	
Tailing Factor	1.2	1.3	NMT 2.0
USP Resolution	NA	3.8	NLT 2.0
%RSD of Peak Area	0.6	0.5	NMT 2.0%
%RSD of retention time	0.03	0.04	NMT 2.0%

Method Validation
The above method was validated according to ICH and USP guidelines to establish the performance characteristics of a method (expressed in terms of analytical parameters) to meet the requirements for the intended application of the method.

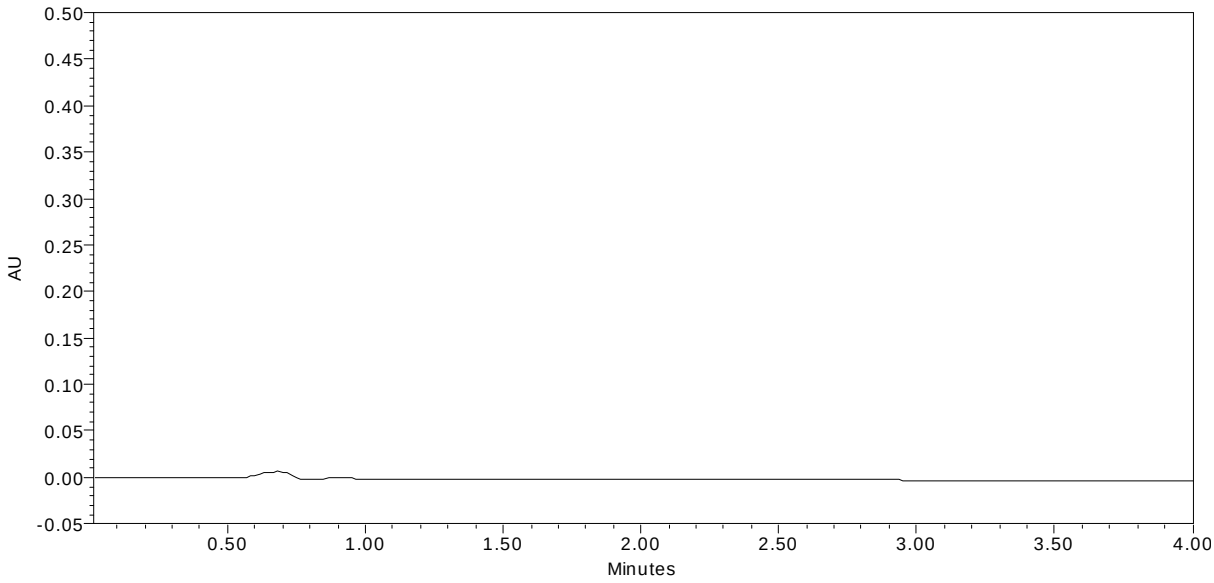
System Suitability

In order to determine the adequate resolution and reproducibility of the proposed methodology, suitability parameters including retention time, resolution, Tailing factor, %RSD of retention time and peak areas were investigated. The results are summarized in Table 1.

Specificity

The specificity of an analytical method may be defined as the ability to unequivocally determine the analyte in the presence of additional components such as impurities, degradation products and matrix. Specificity was evaluated by preparing the analytical placebo and it was confirmed that the signal measured was caused only by the analytes.

Figure 3: Chromatogram of Placebo



A solution of analytical placebo (containing all the tablet excipients except Ezetimibe and Atorvastatin was prepared according to the sample preparation procedure and injected. To identify the interference by these excipients, a mixture of inactive ingredients (placebo), standard solutions, and the commercial pharmaceutical preparations including Ezetimibe and Atorvastatin were analyzed by the developed method The representative chromatograms did not show any other peaks, which confirmed the specificity of the method. (Fig. 3 and 4).

Linearity:

The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample. Linearity of detector response for Atorvastatin/Ezetimibe was established by analyzing serial dilutions of a stock solution of the working standard. Ten concentrations ranging from 50% to 150% of the test concentration were prepared and analyzed. The final concentration of each solution in µg per mL was plotted against peak area response. Slope, correlation coefficient (R) and intercept were found to be 24103.12, 0.99996and 2224 for Ezetimibe, 224050.06, 0.99998 and 7630 for Atorvastatin respectively. The linear graphs were shown in figure 5 and 6.

Figure 4: Chromatogram of Standard

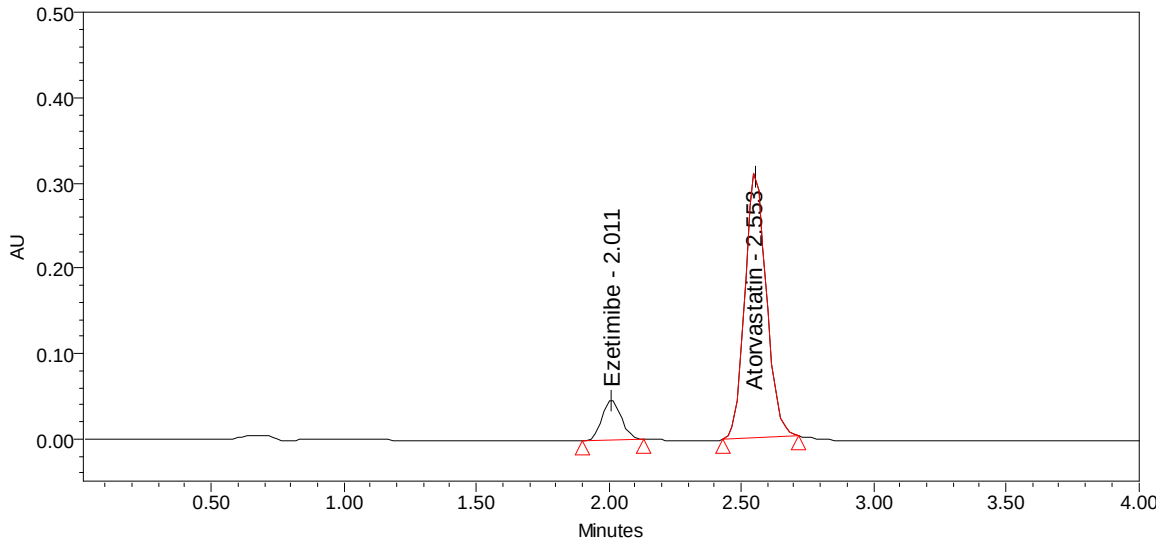


Figure 5. Linearity graph for Ezetimibe

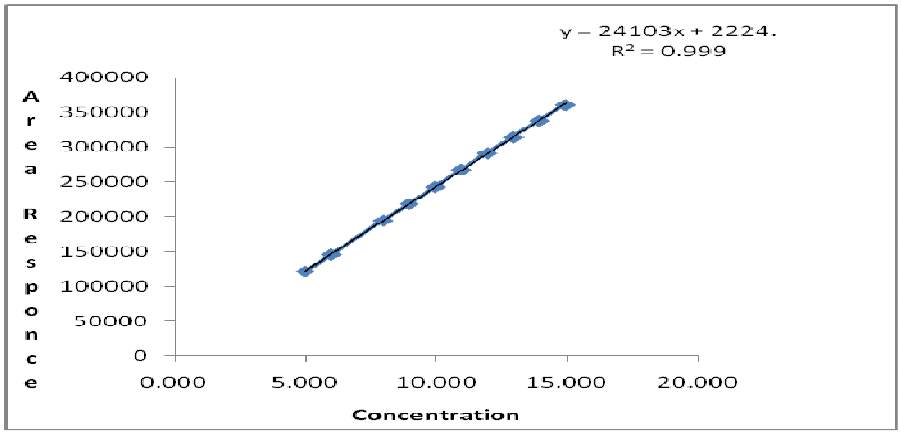


Figure 6 Linearity graph for Atorvastatin

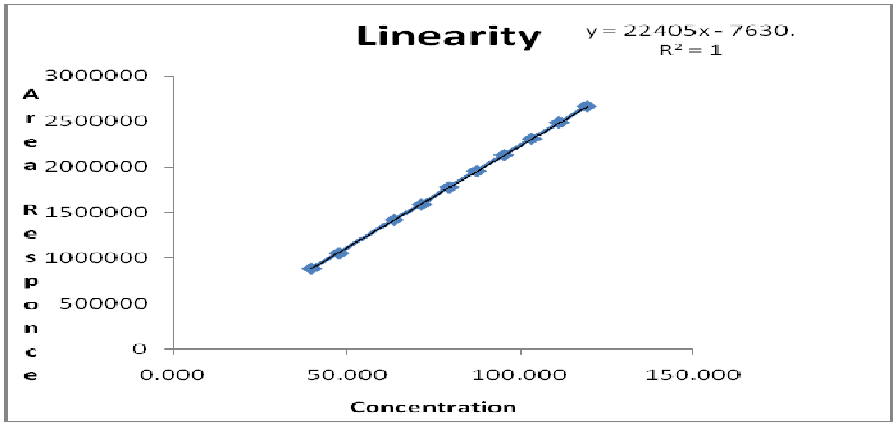


Table. 2

Sample No.	Assay	
	Ezetime	**Atorvastatin**
1	99.2	101.9
2	99.5	101.1
3	99.8	101.7
4	100.1	100.8
5	100.3	101.3
6	100.2	100.6
Mean ($\overline{X}$)	99.9	101.2
s	0.4324	0.5046
%RSD	0.4	0.5
Lower 95% CI	99.6	100.8
Upper 95% CI	100.2	101.6

Table 3 . Recovery-Ezetimibe

Sample No.	Spike Level	Amount Spiked, mg	Amount Recovered, mg	Percent Recovery	Mean Percent Recovery
1	50%	5.15	5.25	101.9	100.1
2		5.26	5.32	101.1	
3		5.22	5.29	101.3	
4		5.35	5.27	98.5	
5		5.26	5.22	99.2	
6		5.34	5.26	98.5	
1	75%	7.28	7.19	98.8	99.2
2		7.88	7.82	99.2	
3		7.92	7.89	99.6	
1	100%	10.52	10.48	99.6	99.5
2		10.26	10.21	99.5	
3		10.58	10.51	99.3	
1	125%	12.67	12.57	99.2	98.8
2		12.68	12.45	98.2	
3		12.92	12.81	99.1	
1	150%	15.32	15.16	99.0	99.0
2		15.26	15.11	99.0	
3		15.28	15.18	99.3	
4		15.55	15.18	97.6	
5		15.27	15.09	98.8	
6		15.26	15.29	100.2	

Precision:

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous

sample under the prescribed conditions. Precision may be considered at three levels: repeatability, intermediate precision and reproducibility.

Six replicate samples were prepared and analyzed as per the sample preparation procedure. Assay of each replicate, the average of 6 replicates, its standard deviation, %RSD and the 95% confidence interval were calculated. The results are shown in Table 2.

Accuracy:
The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found.

Recovery study was performed at 50%, 75%, 100%, 125% and 150% of the target concentration by spiking placebo blend with the drug substance. Six replicates each were spiked at 50% & 150% levels, and 3 replicates each at 75%, 100% and 125% levels. Spiked samples were extracted and analyzed. The amount spiked, amount recovered, percent recovery and its mean were calculated. The results are shown in Table 3 and 4.

Table 4 . Recovery-Atorvastatin

Sample No.	Spike Level	Amount Spiked, mg	Amount Recovered, mg	Percent Recovery	Mean Percent Recovery
1	50%	40.25	40.18	99.8	99.5
2		40.52	40.45	99.8	
3		40.62	40.28	99.2	
4		40.98	40.75	99.4	
5		40.26	40.02	99.4	
6		40.52	40.35	99.6	
1	75%	61.52	61.22	99.5	99.4
2		60.95	60.26	98.9	
3		60.58	60.42	99.7	
1	100%	80.45	80.25	99.8	99.7
2		80.62	80.29	99.6	
3		80.15	80.05	99.9	
1	125%	101.52	100.85	99.3	99.3
2		100.25	99.79	99.5	
3		102.52	101.56	99.1	
1	150%	120.58	119.56	99.2	99.3
2		121.35	120.35	99.2	
3		122.35	121.92	99.6	
4		122.58	122.06	99.6	
5		120.95	120.25	99.4	
6		122.12	120.95	99.0	

Range:
The range of an analytical procedure is the interval between the upper and lower concentration (amounts) of analyte in the sample (including these concentrations) for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity. The results are shown in Table 5.

Table 5 Range

Parameter	Acceptance Criteria	Result	
		Ezetimibe	Atorvastatin
Linearity	R ≥ 0.999	0.99991	0.99998
Precision	%RSD of 6 Replicates NMT 2.0%	0.4% to 0.5%	0.3% to 0.4%
Accuracy	Recovery 97.0% to 103.0%	98.8%-100.1%	99.3%-99.7%

Robustness:
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. The variations like flow rate of mobile phase, column temperature, ratio of organic content in the mobile phase etc. does not have any significant effect on the method performance.

Conclusion

A simple, rapid, cost effective and accurate RP-HPLC method was developed for the simultaneous determination of Atorvastatin and Ezetimibe in pharmaceutical formulations by isocratic mode elution. The analytical conditions and the solvent system developed provided good resolution for Atorvastatin and Ezetimibe within a short run time. The HPLC method was validated and demonstrated good linearity, precision, accuracy and specificity. Thus, the developed HPLC method can be utilized for routine analysis during the analysis of Atorvastatin and/or Ezetimibe.

Acknowledgment

The authors are thankful to Cipla labs for providing the working standards of atorvastatin and Ezetimibe.

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