A NOVEL REVERSE PHASE LIQUID CHROMATOGRAPHIC METHOD DEVELOPMENT AND VALIDATION FOR THE SIMULTANEOUS ESTIMATION OF ATORVASTATIN, EZETIMIBE AND FENOFIBRATE IN BULK AND TABLET DOSAGE FORM

B. Praveen Kumar *1, S. Vidyadhara 1, T.E.G.K. Murthy 2, B. Venkateswara Rao 1 and V. Nikhila 1

Department of Pharmaceutical Analysis 1, Chebrolu Hanumaiah Institute of Pharmaceutical Sciences, Chandramoulipuram, Chowdavaram, Guntur, Andhra Pradesh, India.

Department of Pharmaceutics 2, Bapatla College of Pharmacy, Bapatla, Guntur, Andhra Pradesh, India.

ABSTRACT: A novel reverse phase liquid chromatographic method has been developed for the simultaneous estimation of Atorvastatin Calcium, Ezetimibe and Fenofibrate in bulk and Pharmaceutical formulations by using reverse phase Agilent 100-5 C18 column [250mm x 4.6mm]. The mobile phase (Methanol: water) in the ratio of 70:30% v/v was pumped at a flow rate of 1ml/min and the column effluents were monitored at 250nm using Variable Wavelength UV detector. Linearity was obtained in the concentration range of 20-100 µg/ml for Atorvastatin and Ezetimibe and 50-250µg/ml for Fenofibrate. The established method was statistically validated according to the ICH Q2B guidelines and the percentage relative standard deviation for precision, robustness and ruggedness was found to be less than 2% indicating high degree of precision and robustness. The percentage recovery for the accuracy was found to be 100.52%, 100.45% and 101.31% for Atorvastatin, Ezetimibe and Fenofibrate respectively which were within the specified limits of recovery. Assay for the marketed formulation proved that 99.86% of Atorvastatin, 99.61% of Ezetimibe and 99.83% of Fenofibrate. Hence due to its simplicity, rapidity, precision and accuracy the developed HPLC method can be applied for the estimation of Atorvastatin, Ezetimibe and Fenofibrate in pure and marketed formulations by a modern analyst.

INTRODUCTION: Atorvastatin calcium is chemically (3R,5R) – 7 - [2 -(4-Fluorophenyl) - 3-phenyl 4 (phenylcarbamoyl)-5-propan-2-ylpyrrol-1-yl]-3,5-dihydroxyheptanoic acid (Fig.1.a) and is a competitive inhibitor of HMG-CoA reductase. HMC-CoA reductase catalyzes the conversion of HMG-CoA to mevalonate, an early rate-limiting step in cholesterol biosynthesis.

Ezetimibe is (3R,4S)-1-(4-fluorophenyl)-3-[(3S)-3-(4-fluorophenyl)-3-hydroxypropyl]-4-(4 hydroxyphenyl)azetidin-2-one (Fig.1.b). Ezetimibe is a selective cholesterol absorption inhibitio, which potently and selectively prevents absorption of cholesterol from dietary and biliary sources by preventin transport of cholesterol through the intestinal wall. Fenofibrate is propan-2-yl 2-{4-[(4-chlorophenyl) carbonyl] phenoxy}-2- methyl propanoate (Fig.1.c).

The active moiety of Fenofibrate is fenofibric acid. The effects of fenofibrac acid seen in clinical practice have been explained in vivo in transgenic mice and in vitro in human hepatocyte cultures by the activation of peroxisome proliferator activated

Keywords: Atorvastatin Calcium, Ezetimibe, Fenofibrate, RP-HPL and Method validation
A receptor α (PPARα). Through this mechanism, Fenofibrate increases lipolysis and elimination of triglyceride-rich particles from plasma by activating lipoprotein lipase and reducing production of apoprotein C-III (an inhibitor of lipoprotein lipase activity)

Extensive literature search revealed that very few methods were reported for the estimation of combination of Atorvastatin, Ezetimibe and Fenofibrate. The objective of the present work was to design a validation procedure which can determine the three drugs in tablet dosage form with economical and eco-friendly mobile phase, with good resolution and peak symmetry. The present established liquid chromatographic method was validated by following ICH Q2B guidelines.

**MATERIALS AND METHODS:**

**Equipment used:** The chromatographic separation was performed on Agilent 1120 compact liquid chromatographic system integrated with a variable wavelength programmable UV detector and a Rheodyne injector equipped with 20 μl fixed loop. A reverse phase C18 [Agilent 100-5 column, 250mm × 4.5 mm] was used. Lab India 3000”double beam UV visible spectrophotometer and Axis AGN204-PO electronic balance was used for spectrophotometric determinations and weighing purposes respectively.

**Reagents and chemicals:** Pharmaceutical grade pure Atorvastatin from Mylan Laboratories Hyderabad, Ezetimibe and Fenofibrate gift samples were procured from Yarrow chem products, Mumbai. Marketed formulation Tablets with dose of 10mg of Atorvastatin, 10mg of Ezetimibe and 160mg of Fenofibrate were procured from local market. (Mfd. by Sun Pharma). HPLC grade Acetonitrile and Water were procured from Merck specialties private limited, Mumbai.

**Chromatographic conditions:** Agilent 100-5C18 column [250mm × 4.6mm] was used for the chromatographic separation at a detection wavelength of 250 nm. Mobile phase of composition Methanol: water in a ratio of 70:30 v/v was selected for elution and same mixture was used in the preparation of standard and sample solutions. Flow rate was adjusted to 1ml/min and the injection volume was 20 μl.

**Preparation of Mobile Phase:** Mobile phase was prepared by mixing Methanol and Water in the ratio of 70:30 and was initially filtered through 0.45μm Millipore membrane filter and sonicated for 15 min before use.

**Preparation of Standard solutions:** 25mg each of Atorvastatin, Ezetimibe and Fenofibrate were accurately weighed and transferred into three 25ml volumetric flasks, dissolved using mobile phase and the volume was made up with the same solvent to obtain primary stock solutions A (Atorvastatin), B (Ezetimibe) and C(Fenofibrate) of concentration 1000 μg/ml of each drug. From the primary stock solutions, 1ml of each were pipette out from A, B and C respectively, transferred to a 10ml volumetric flask and the volume was made up with the mobile phase to obtain final concentrations of 100 μg/ml of each drug individually and this solution is (working stock solution A).
Preparation of Sample Solution: Twenty tablets of Atorvastatin, Ezetimibe and Fenofibrate were weighed and crushed. Tablet powder equivalent to 10mg of Atorvastatin, 10mg of Ezetimibe and 250mg of Fenofibrate was weighed accurately and transferred to a 10ml volumetric flask. The content was dissolved with 5ml of mobile phase and then sonicated for 15min. The volume was made up with the mobile phase and filtered with 0.45μm membrane filter and sonicated for 20min. 1ml of this solution was pipette out and transferred to a 10ml volumetric flask and the volume was made up with the mobile phase to obtain a concentration of 100 μg/ml of Atorvastatin, 100μg/ml of Ezetimibe and 250μg/ml of Fenofibrate (working stock solution B).

Optimization of RP-HPLC method: The HPLC method was optimized with an aim to develop a simultaneous estimation procedure for the assay of Atorvastatin, Ezetimibe and Fenofibrate. For the method optimization, different mobile phases were tried, but acceptable retention times, theoretical plates and good resolution were observed with Methanol and Water (70:30 v/v) using Agilent 100-5C\textsubscript{18} column [250mm x 4.6mm].

Validation of the RP-HPLC method: Validation of the optimized method was performed according to the ICH Q2 (B) guidelines.

System suitability: System suitability was carried out with five injections of solution of 100% concentration having 100μg/ml of Atorvastatin and Ezetimibe and 250μg/ml of Fenofibrate in to the chromatographic system. Number of theoretical plates (N) obtained and calculated tailing factors (T) were reported in Table 1.

Linearity: For the determination of linearity, appropriate aliquots were pipetted out from working stock solution A to a series of 10ml volumetric flasks and volume was made up with the solvent to obtain concentration ranging from 20-100μg/ml of Atorvastatin, 20-100μg/ml of Ezetimibe and 50-250μg/ml of Fenofibrate. Each solution was injected in triplicate. Calibration curves were plotted with observed peak areas against concentration followed by the determination of regression equations and calculation of the correlation coefficients. The calibration curves for Atorvastatin, Ezetimibe and Fenofibrate were shown in Fig. 3, 4 and 5. Their corresponding linearity parameters were given in Table 2.

Limit of Detection (LOD) and Limit of Quantitation (LOQ): The LOD and LOQ were calculated from the slope(s) of the calibration plot and the standard deviation (SD) of the peak areas using the formulae LOD = 3.3 σ/s and LOQ = 10 σ/s. The results were given in Table 2.

Precision: The repeatability of the method was verified by calculating the %RSD of six replicate injections of 100% concentration (100μg/ml of Atorvastatin and Ezetimibe and 250μg/ml of Fenofibrate) on the same day and for intermediate precision % RSD was calculated from repeated studies on different days. The results were given in Table 3.

Accuracy: To ensure the reliability and accuracy of the method recovery studies were carried out by standard addition method. A known quantity of pure drug was added to pre-analyzed sample and contents were reanalyzed by the proposed method and the percent recovery was reported. The results were given in Table 4.

Specificity: Specificity of a method was determined by testing standard substances against potential interferences. The method was found to be specific when the test solution was injected and no interferences were found because of the presence of excipients. The optimized chromatogram of Atorvastatin, Ezetimibe and Fenofibrate without any interference was shown in Fig. 2.

Robustness: Robustness of the method was verified by altering the chromatographic conditions like mobile phase composition, wave length detection, flow rate, etc. and the % RSD should be reported. Small changes in the operational conditions were allowed and the extent to which the method was robust was determined. A deviation of ±2nm in the detection wave length and ±0.2ml/min in the flow rate, were tried individually. A solution of 100% test concentration with the specified changes in the operational conditions was injected to the instrument in triplicate. %RSD was reported in the Table 5.
Assay of Marketed Formulations: 20µl of sample solution of concentration 100µg/ml of Atorvastatin, 100µg/ml of Ezetimibe, and 250µg/ml of Fenofibrate was injected into chromatographic system and the peak responses were measured. The solution was injected three times in to the column. The amount of drug present and percentage purity was calculated by comparing the peak areas of the standards with that of test samples. A typical chromatogram for assay of marketed formulation was shown in Fig. 6 and the obtained values were reported in the Table 6.

RESULTS AND DISCUSSION: After a number of trials with mobile phases of different composition, Methanol and Water in the ratio 70:30v/v was selected as mobile phase because of better resolution and symmetric peaks. Atorvastatin, Ezetimibe and Fenofibrate were found to show appreciable absorbance at 250nm when determined spectrophotometrically and hence it was selected as the detection wavelength. An optimized chromatogram showing the separation of Atorvastatin, Ezetimibe and Fenofibrate at different Rₜs was shown in Fig. 2.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Retention (min)</th>
<th>Tailing Factor (T)</th>
<th>Theoretical Plates (N)</th>
<th>Resolution (Rₛ)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atorvastatin</td>
<td>2.103</td>
<td>1.3</td>
<td>7251</td>
<td>1.988</td>
</tr>
<tr>
<td>Ezetimibe</td>
<td>3.660</td>
<td>1.1</td>
<td>8652</td>
<td></td>
</tr>
<tr>
<td>Fenofibrate</td>
<td>5.887</td>
<td>1.2</td>
<td>9643</td>
<td></td>
</tr>
</tbody>
</table>

* n= No. of determinants

Concentration range of 20-100µg/ml for Atorvastatin and Ezetimibe and 50-250µg/ml for Hydrochlorothiazide were found to be linear with correlation coefficients 0.999, 0.998 and 0.999 for Atorvastatin, Ezetimibe and Fenofibrate respectively. Respective calibration curves were shown in Fig. 3, 4 and 5. The results were given in Table 2.
The limits of detection for Atorvastatin, Ezetimibe and Fenofibrate were found to be 0.658µg/ml, 0.544µg/ml and 0.548µg/ml respectively and the limits of Quantitation were 2.171µg/ml, 1.795µg/ml and 1.808µg/ml respectively. Values were represented in Table 2.

### Table 2: Results for Linearity (n=3)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Atorvastatin</th>
<th>Ezetimibe</th>
<th>Fenofibrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linearity Range (µg/ml)</td>
<td>20-100</td>
<td>40-120</td>
<td>50-250</td>
</tr>
<tr>
<td>Regression Equation</td>
<td>y = 577005x + 20806</td>
<td>y = 863182x + 30810</td>
<td>y = 605781x + 17306</td>
</tr>
<tr>
<td>Slope (m)</td>
<td>577005</td>
<td>863182</td>
<td>605781</td>
</tr>
<tr>
<td>Intercept (c)</td>
<td>20806</td>
<td>308106</td>
<td>17306</td>
</tr>
<tr>
<td>Regression Coefficient (r²)</td>
<td>0.9992</td>
<td>0.998</td>
<td>0.9992</td>
</tr>
<tr>
<td>Limit of Detection (µg/ml)</td>
<td>0.658</td>
<td>0.544</td>
<td>0.548</td>
</tr>
<tr>
<td>Limit of Quantitation (µg/ml)</td>
<td>2.171</td>
<td>1.795</td>
<td>1.808</td>
</tr>
</tbody>
</table>

*%n= No. of determinants

The proposed method was found to be precise and reproducible with %RSD of 0.58, 0.77 and 0.86 for Atorvastatin, Ezetimibe and Fenofibrate respectively. %RSD was reported in Table 3.

### Table 3: Results of Precision (n=6)

<table>
<thead>
<tr>
<th>Drug</th>
<th>Intraday Precision (% RSD)</th>
<th>Interday Precision (% RSD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atorvastatin</td>
<td>0.58</td>
<td>0.61</td>
</tr>
<tr>
<td>Ezetimibe</td>
<td>0.77</td>
<td>0.79</td>
</tr>
<tr>
<td>Fenofibrate</td>
<td>0.86</td>
<td>0.88</td>
</tr>
</tbody>
</table>

*%n= No. of determinants

Accuracy of the method was verified by performing recovery studies by standard addition method. The percent recovery of the standard added to the pre-analysed sample was calculated and it was found to be 99.78% to 100.52% for Atorvastatin, 99.5 to 99.9% for Ezetimibe and 99.3 to 100.5 % for Fenofibrate. This indicates that the method was accurate. Values obtained were given in Table 4.

### Table 4: Results for Accuracy (n=3)

<table>
<thead>
<tr>
<th>Recovery level</th>
<th>Amount of Standard drug added (µg/ml)</th>
<th>Amount of test added (µg/ml)</th>
<th>Total Amount Recovered (µg/ml)</th>
<th>% Recovery w/w</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ator</td>
<td>Ezet</td>
<td>Fen</td>
<td>Ator</td>
<td>Ezet</td>
</tr>
<tr>
<td>80%</td>
<td>20</td>
<td>20</td>
<td>50</td>
<td>60</td>
</tr>
<tr>
<td>100%</td>
<td>40</td>
<td>40</td>
<td>100</td>
<td>60</td>
</tr>
<tr>
<td>120%</td>
<td>60</td>
<td>60</td>
<td>150</td>
<td>60</td>
</tr>
</tbody>
</table>

*%n= No. of determinant

The method was found to be robust after changing the conditions like detection wavelength (± 2nm) and flow rate (± 0.2 ml). %RSD was calculated for each variation and reported. Values obtained were given in Table 5.
The method was found to be specific for the combination of interest after verifying the chromatograms showing no interference of the excipients present. Hence, the method was well suitable for the estimation of the commercial formulations of the selected combination with a percentage purity of 98.69% for Atorvastatin, 99.61% for Ezetimibe and 99.56% for Fenofibrate. The typical chromatogram for assay of marketed formulations was shown in Fig. 6 and Values obtained were given in Table 6.

**CONCLUSION:** The RP-HPLC method developed and validated allows a simple and fast quantitative determination of Atorvastatin, Ezetimibe and Fenofibrate from their formulations. All the validation parameters were found to be within the limits according to ICH guidelines. The proposed method was found to be specific for the drugs of interest irrespective of the excipients present and the method was found to be simple, accurate, precise, rugged, robust, economical and ecofriendly. So the established method can be employed in the routine analysis of the marketed formulations.

**ACKNOWLEDGMENTS:** The authors are thankful to the Mylan Laboratories, Hyderabad, Yarrow chem. products, Mumbai for providing the gift samples of Atorvastatin, Ezetimibe and Fenofibrate and also to the management of Chebrolu Hanumaiah Institute of Pharmaceutical Sciences, Chowdavaram, Guntur for providing facilities and great support to carry out the research work.

**REFERENCES:**


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