Simultaneous RP-HPLC Method Development and Validation of Atorvastatin, Ezetimibe and Fenofibrate

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Keywords: Atorvastatin; Ezetimibe; Fenofibrate; RP-HPLC; Method validation

Abstract

A simple, accurate, precise and robust reverse phase high performance liquid chromatographic method has been developed and subsequently validated for the simultaneous estimation of atorvastatin (AT), ezetimibe (EZ) and fenofibrate (FE) in commercial formulation. The method has shown an adequate separation for AT, EZ and FE. The drugs were resolved on an enable C-18 Column (25 mm x 4.6 mm i.d. 5 µm particle size) using Shimadzu SPD-20A prominence UV-Visible detector with the mobile phase composed of acetonitrile and phosphate buffer (pH 3.3) in the ratio of 90:10% V/V as mobile phase at a flow rate of 1 ml/min and the detection was carried out at 254 nm. The retention time of AT, EZ and FE were found to be 3.155, 5.299 and 6.215 min respectively. The linearity of the proposed method was investigated in the range of 10-100 µg/mL, 10-100 µg/mL, and 160-1600 µg/mL for AT, EZ and FE, respectively. The limit of detection (LOD) was 2.18, 0.87, and 20.9 for AT, EZ and FE, respectively. The limit of quantification (LOQ) was 6.8, 2.6 and 63.6 for AT, EZ and FE, respectively. The % RSD from the precision and accuracy studies was found to be below 2%. The proposed method was statistically evaluated and can be applied in regular quality control of AT, EZ and FE in pharmaceutical dosage forms.

Introduction

Lowering low-density lipoprotein cholesterol (LDL-C) with the use of the most potent statins improves mortality and morbidity related to cardiovascular events in patients with hypercholesterolemia. Statins, which are known as “3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors”, act to block the synthesis of cholesterol in the liver. Statin therapy has been shown to reduce the rate of major vascular events in patients with established vascular disease [1] and is considered the first line therapy for the management of dyslipidemia in such individuals [2]. These medications are generally well tolerated by the vast majority of patients, but a small number experience side effects, most seriously those of myopathies, rhabdomyolysis and elevated liver enzymes - recognition of this fact, that statins are not universally without problems, highlights the need for viable alternatives. EZ is a novel cholesterol absorption inhibitor that prevents cholesterol absorption by inhibiting the transport of cholesterol across the intestinal wall. It is an intestinal cholesterol binder that is known to has a modest effect (approximately 18%) [3] in the lowering of LDL-C. FE is a medication that also works through the liver and has long been used to control blood lipid levels in patients with mixed lipid problems. It is a fibric acid derivative that binds to peroxisome proliferator-activated receptor alpha and alters lipoprotein synthesis [4]. Treatment with FE monotherapy has also been proven to provide modest reductions in LDL-C, and may also be an effective therapeutic option for patients who are intolerant of statins [5].

In contrast to monotherapy where both EZ and FE each provide only modest effect, the combined therapy produces significantly greater reductions in LDL-C. This combination of EZ and FE in most of the cases is effective as AT and may be an effective second-line therapeutic option in patients who are intolerant to statins, but still require medication for elevated cholesterol. Recently many studies have been successfully demonstrated the effectiveness and tolerability of the co-administration of EZ and FE with statin therapy in patients with hypercholesterolemia with complex vascular disease [6]. Hence, a suitable analytical method is highly desirable for simultaneous determination of these drugs in bulk and pharmaceutical formulations.

Figure 1 presents the chemical structures of the three antihyperlipidemic agents used for HPLC study. From the literature search, it was evidenced that few analytical methods such as: RP-HPLC [7-10], High-Performance Thin-Layer Chromatography (HPTLC) [11,12] were reported for simultaneous determination of AT, EZ, and FE. Pathak et al., reported RP-HPLC and chemometric assisted UV-spectrophotometric methods for simultaneous analysis of the three drugs in combined dosage form [13]. A gradient HPLC and a HPTLC method have been demonstrated by Varghese et al., for quantitative simultaneous determination of the said drugs. AT have been determined by multicomponent analysis of HPLC along with EZ [14-17] and FE [18]. Ashutosh et al., reported a stability-indicating RP-HPLC method for simultaneous estimation of AT and EZ in human plasma [19]. Few quantitative methods for determination of AT and...
EZ using HPTLC [20-22], stability-indicating UPLC [23] methods were also reported. A spectrophotometric method for simultaneous estimation of AT and FE in binary mixture is also available. 

Inspite of so many methods dealt with quantification of analytes of interest available, the lack of sensitivity, complex mobile phase and gradient elution mode seem to be responsible for some drawbacks of these analytical tools. Therefore, a sensitive RP-HPLC method has been proposed for multicomponent analysis of AT, EZ, and FE in its bulk and dosage form.

Preparation of buffer solution

Potassium dihydrogen orthophosphate (100 g) was dissolved in 800 mL of HPLC grade water and pH 3.3 was adjusted with Hydrochloric acid and finally makes up to 1000 mL with HPLC grade water.

The mobile phase

A mixture of potassium dihydrogen phosphate buffer pH 3.3 and acetonitrile in the ratio of 90:10 v/v was prepared and used as mobile phase.

The diluent

Acetonitrile was used as diluent.

Method Validation

At the optimal condition, the proposed HPLC method has been validated with respect to the following parameters outlined by ICH [ICH Q2 (R1), 2005]:

Linearity

Suitable dilutions from the working standard solution were prepared to yield a series of solutions in the concentration range of 10-100 μg/mL, 10-100 μg/mL and 160-1280 μg/mL for AT, EZ and FE, respectively. The resultant solutions were chronologically injected in triplicate into the HPLC column. A calibration curve was constructed thereby plotting the corresponding peak areas against the concentrations to obtain regression equation and correlation coefficient, which is used to indicate the linearity of the method.

Detection and quantitation limits

The limit of detection (LOD) and limit of quantification (LOQ) represent the concentration of the analyte that would yield a signal-to-noise ratio of 3 and 10, respectively.

Precision

Intra-day precision of the method was determined using quality control samples (40, 40 and 640 μg/mL of AT, EZ and FE, respectively)
each six injections on the same day and percentage relative standard deviation (% RSD) were calculated. Furthermore, these experiments were repeated on three consecutive days to assess inter-day precision.

**Accuracy**

Accuracy was determined by analyzing a known concentration of drug, viz., 80%, 100%, and 120% spiked with formulation in triplicate and then determining the percent recovery.

**Robustness**

The robustness study was carried out to assess the influence of minor variations in the optimal chromatographic factors. A deliberate variations in the separation parameters i.e., flow rate (± 0.1 mL/min), percent acetonitrile in mobile phase (± 2%) were experimented. The resultant responses to the variations were statistically compared with the proposed method.

**Assay of commercial formulation**

For assay of formulation (fibator, Sun Pharmaceutical Industries Ltd.) was procured from the local market. For sample preparation, 20 tablets containing AT, EZ and FE from the same batch were weighed and finely powdered. Thereafter, tablet powder equivalent to 5 mg of AT, 5 mg of EZ and 80 mg of FE, was transferred into a 100 mL volumetric flask and dissolved in acetonitrile. After the immediate dissolution, the volume was made up to the mark with same solvent and filtered with 0.45 µM filter to get the stock solution. 5 mL of stock solution was further diluted up to 50 mL and filtered to get the working standard solution. The filtrate was then injected onto the HPLC under the optimal chromatographic condition. The % assay of the formulation by the method was determined by using equation:

\[
\% \text{Assay} = \frac{\text{Sample area} \times \text{Standard weight} \times \text{Average weight of 20 tablets}}{\text{Standard area} \times \text{Sample weight} \times \text{Label claim}}
\]

**Results and Discussion**

**Development and optimization of the method**

Column chemistry, solvent type, solvent strength, detection wavelength and flow rate were varied to determine the chromatographic conditions giving the best separation. Separation with good resolutions was studied on different type’s columns (C8 and C18). The mobile phase conditions were optimized so that the components were not interfered from the solvent and excipients. Several buffer systems at different pH values were trailed in various ratios with MeOH, and MeCN as mobile phase. Mobile phase and flow rate selection was based on peak parameters (height, area, tailing, theoretical plates, capacity factor and resolution) and run time. Decisively after several experimental trials, the best result was obtained by use of 90:10 (v/v) ratios of acetonitrile and buffer with 1.0 mL/min flow rate. Among different buffer pH values, better selectivity and good peak parameters were observed at phosphate buffer pH 3.3. The suitable wavelength considered for monitoring the drugs was 265 nm. Solutions of AT, EZ and FE in diluent were also injected directly for HPLC analysis and the responses (peak area) were recorded. It was observed that there was no interference from the mobile phase or baseline disturbances and all the analytes absorbed well at 265 nm. The obtained chromatograms of the mixture sample indicate that separation of all the analytes was successful. The typical chromatogram at the optimum condition is depicted in Figure 2. Under the optimum chromatographic conditions, there tension time obtained for AT, EZ and FE was 3.155, 5.299 and 6.215 min, respectively. The system suitability parameters (retention time, tailing factor, Number of theoretical plates and resolution) obtained is found to be within the acceptance criteria indicating the fitness of the method for separation and determination of the compounds.

**Validation of the method**

The analytical method was validated with respect to the following parameters:

**Linearity**

The peak areas for drug samples were linear in the concentration range between 10 to100 µg/mL, 10 to100 µg/mL and 160 to1600 µg/mL for AT, EZ and FE, respectively. The data were analyzed with least squares linear regression indicating good linearity for all the analytes (R2=0.999). Typically, the regression equations were: \( y=5016.8 \times + 2534.5 \) (R=0.9998), \( y=5043-118.57 \) (R2=1), \( y=5098x - 572.39 \)(R2=0.9994) for AT, EZ and FE, respectively.

**LOQ and LOD**

The method sensitivity was explained by establishing the LOD and LOQ of 2.186 µg/mL and 6.893 µg/mL for AT, 0.877 µg/mL and 2.65 µg/mL for EZ and 20.99 µg/mL and 63.61 µg/mL for FE, respectively. The obtained LOD and LOQ results demonstrate that the method is highly sensitive as compared to the reported methods.

**Precision**

The data attained from precision study is presented in Table 1 for intra-and inter-day precision experiments. The calculated %R.S.D values for intra-day precision study were <0.27% and for inter-day study were <0.20%, proving that the method was suitably precise. The overlaid chromatograms of the study are depicted in Figure 3.

**Accuracy**

The mean percentage recovery from the accuracy study was calculated for fortified and unfortified solutions. Excellent recoveries were obtained (>98.8%) at each added concentration. The represented data were shown in Table 2.
Figure 3: Overlaid chromatograms of precision study.

Table 1: Intra and Inter-day precision data.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Intra-day (n=6)</th>
<th>Inter-day (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Peak area (mean ± SD; %RSD)</td>
<td>Peak area (mean ± SD; %RSD)</td>
</tr>
<tr>
<td>AT</td>
<td>201932.33 ± 47.42; 0.023</td>
<td>201872.5 ± 43.41; 0.021</td>
</tr>
<tr>
<td>EZ</td>
<td>199084.5 ± 544.20; 0.27</td>
<td>199252 ± 406.47; 0.20</td>
</tr>
<tr>
<td>FE</td>
<td>3224740.3 ± 2658.38; 0.08</td>
<td>3225015.2 ± 2017.5; 0.06</td>
</tr>
</tbody>
</table>

Assay of formulation

The amounts of drugs present in the tablet dosage forms were calculated to be >99.21 % for all the three analytes. The represented chromatogram for formulation was shown in Figure 4.

Table 2: Accuracy study of AT, EZ and FE.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Level</th>
<th>Mean peak area ± S.D; %RSD (n=3)</th>
<th>% Recovery</th>
<th>% Accuracy</th>
</tr>
</thead>
<tbody>
<tr>
<td>AT</td>
<td>80%</td>
<td>161515 ± 69.081; 0.04</td>
<td>163562.32 ± 43.09; 0.02</td>
<td>99.09</td>
</tr>
<tr>
<td></td>
<td>100%</td>
<td>201935 ± 45.791; 0.02</td>
<td>204187.68 ± 2036.70; 0.99</td>
<td>99.35</td>
</tr>
<tr>
<td></td>
<td>120%</td>
<td>242228.6 ± 211.09; 0.08</td>
<td>245539 ± 3095; 1.26</td>
<td>99.5</td>
</tr>
<tr>
<td>EZ</td>
<td>80%</td>
<td>159496.6 ± 79.538; 0.05</td>
<td>161317 ± 1761.82; 1.09</td>
<td>98.9</td>
</tr>
<tr>
<td></td>
<td>100%</td>
<td>199390 ± 45.981; 0.02</td>
<td>201715.66 ± 2698.21; 1.33</td>
<td>98.9</td>
</tr>
<tr>
<td></td>
<td>120%</td>
<td>239347.3 ± 48.80; 0.02</td>
<td>246679.3 ± 2079.10; 0.84</td>
<td>98.9</td>
</tr>
<tr>
<td>FE</td>
<td>80%</td>
<td>2580694.6 ± 351.923; 0.01</td>
<td>2541360.93 ± 20033; 0.788</td>
<td>98.8</td>
</tr>
<tr>
<td></td>
<td>100%</td>
<td>3225502.6 ± 786.7; 0.02</td>
<td>3258835.46 ± 20816.72; 0.63</td>
<td>98.8</td>
</tr>
<tr>
<td></td>
<td>120%</td>
<td>3870750.8 ± 837.23; 0.02</td>
<td>3881417.46 ± 10016.65; 0.25</td>
<td>98.87</td>
</tr>
</tbody>
</table>

Robustness

The method was found to be sufficiently robust under the tested conditions (Table 3). There was no significant change in the total analysis time, resolution and tailing factor of AT, EZ and FE. The resulted overlaid chromatograms from the robustness study are shown in Figure 5.
Flow rate (mL/min)$^a$

<table>
<thead>
<tr>
<th>Flow rate (mL/min)$^a$</th>
<th>AT</th>
<th>EZ</th>
<th>FE</th>
</tr>
</thead>
<tbody>
<tr>
<td>-1 (0.9)</td>
<td>9.054±0.004; 0.052</td>
<td>3.347±0.007; 0.225</td>
<td>1.651±0.121</td>
</tr>
<tr>
<td>+1 (1.1)</td>
<td>8.095±0.074</td>
<td>3.002±0.013; 0.433</td>
<td>1.474±0.011; 0.780</td>
</tr>
<tr>
<td>% Acetonitrile in mobile phase$^a$</td>
<td>2 (08)</td>
<td>7.974±0.011</td>
<td>3.853±0.010; 0.172</td>
</tr>
<tr>
<td>2 (12)</td>
<td>7.622±0.053; 0.706</td>
<td>3.311±0.005; 0.168</td>
<td>1.572±0.010; 0.667</td>
</tr>
</tbody>
</table>

Table 3: Robustness study.

System suitability study

System suitability is an integral part of the method development and is used to ensure adequate performance of chromatographic system. The system suitability test (SST) parameters are presented in Table 4. From the SST results, it has been confirmed that the system was deemed to be suitable as it complies with the limits of peak parameters. Resolution (>3.33), peak asymmetry (<2.0) and total analysis time of 6.215 min confirms the good selectivity of the method.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Values obtained</th>
<th>Preferable values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Retention time (min)</td>
<td>AT</td>
<td>EZ</td>
</tr>
<tr>
<td>Theoretical plates (N)</td>
<td>30628</td>
<td>38459</td>
</tr>
<tr>
<td>Resolution (R)</td>
<td>-</td>
<td>8.988</td>
</tr>
<tr>
<td>Tailing factor</td>
<td>1.565</td>
<td>1.601</td>
</tr>
</tbody>
</table>

Table 4: System suitability parameters.

Conclusion

A simple and efficient reversed-phase HPLC method was developed for simultaneous determination of AT, EZ and FE in bulk and tablet formulation and the method was validated according to ICH guideline requirements. The method was found to be precise, accurate, linear, sensitive & robust during validation. Satisfactory results were obtained from the validation of the method. The method can be recommended for routine analysis.

References

