REVERSE PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC (HPLC) METHOD FOR SIMULTANEOUS DETERMINATION OF ATORVASTATIN, EZETIMIBE AND FENOFIBRATE IN COMMERCIAL TABLETS

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ABSTRACT

A simple, rapid, precise and accurate gradient reverse-phase HPLC method was developed and validated for the simultaneous determination of Atorvastatin (AT), Ezetimibe (EZ) and Fenofibrate (FE) in commercial tablets. The method has shown adequate separation for AT, EZ and FE. Separation was achieved on Purospher® C18, 5µm, 250mm X 4.6mm analytical column using gradient elution with acetonitrile – water system at room temperature and the detection was carried out at 254nm using photodiode array (PDA) detector. The linearity of the proposed method was investigated in the range of 2-10µg/ml (r=0.991), 2-10µg/ml (r=0.990), 32-160µg/ml (r=0.994) for AT, EZ and FE respectively. The limit of detection (LOD) was 0.1577, 0.1266 and 1.9544 for AT, EZ and FE respectively. The limit of quantification (LOQ) was 0.4780, 0.3838 and 3.6225 for AT, EZ and FE respectively. The relative standard deviation (RSD) of six replicates is less than 2%. This HPLC method is applied successfully to the simultaneous quantitative analysis of AT, EZ and FE in commercial tablets.

Keywords: Atorvastatin Calcium; Ezetimibe; Fenofibrate; Gradient; Reverse phase HPLC; Photodiode array (PDA).

INTRODUCTION

Atorvastatin (AT) calcium, chemically [R-(R*,R*)]-2-[(4-fluorophenyl)-β, δ-dihydroxy-5-[1-methylethyl]-3-phenyl-4-[(phenylamino)carbonyl]-1H-pyrole-1-heptanoic acid, calcium salt (2:1) trihydrate, is a synthetic lipid-lowering agent. AT is an inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductases, the enzyme which catalyzes the conversion of HMG-CoA to mevalonate an early and rate-limiting step in cholesterol biosynthesis1,2. AT is indicated to reduce the risk of myocardial infarction, stroke and to reduce the risk for revascularization procedures and angina. AT is also prescribed for the stabilization of plaque3,4. AT is official in Indian Pharmacopoeia5 and British Pharmacopoeia6. Bioanalytical, HPLC, HPTLC, UPLC and FT- Raman Spectroscopy methods are reported for its individual determination and in combination with other drugs7-14. Ezetimibe (EZ), chemically (1-[4-[4-(4-chlorobenzoyl)phenoxy]-2-methyl-propanoic acid 1-methylethyl ester, is a lipid regulating agent. It is a white solid and is insoluble in water. FE is official in United States Pharmacopoeia24 and British Pharmacopoeia25. Stability indicating UPLC in combination with AT13 and HPLC methods for assay and purity and an NMR method for purity25; spectroscopy and LC method for its determination with vinpocetine in formulations26 are reported.

Combination of FE and AT has additive beneficial effect in the treatment of mixed dyslipidemia27. The effects of combined therapy of FE and AT on plasma adiponectin levels and insulin sensitivity were significantly greater than those of AT alone and FE alone27. The co-administration of EZ with FE offers a well tolerated, lipid management strategy for patients with mixed dyslipidemia. The combined use of these agents provides a therapy with complementary effects to improve the atherogenic lipid profile observed for these patients28.

The present manuscript first time describes a reverse phase HPLC method which is simple, rapid, accurate and precise method for the simultaneous determination of AT, EZ and FE in the commercial tablets.
MATERIAL AND METHODS

Chemical and Reagents
AT calcium, EZ and FE were supplied by Torrent Research Centre, Astron Research Centre, Zydus Research Centre, Ahmedabad, India, respectively. TriTonact® (Lot: IL258011, Lupin Ltd., Mumbai) was purchased from Indian market. HPLC grade Acetonitrile (ACN), water, and Methanol were purchased from Ranchem (A division of Ranbaxy) Laboratory Ltd.

HPLC instrumentation & conditions
The HPLC system consisted of a Young Lin 9101 vacuum degasser, a Young Lin 9001 quaternary pump and a Young Lin 9160 PDA detector (Seoul, South Korea). An YL-clarity chromatography data system was used to record and evaluate the data collected during and following chromatographic analysis. The chromatographic separation was achieved on a Purospher® 5µm, 250mm x 4.6mm i.d. column using a mobile phase consisting of ACN-water with gradient elution. The eluent was monitored using PDA detector at a wavelength of 254nm. The column was maintained at room temperature and injection volume of 20µl was used. The mobile phase was filtered through 0.45µm Chrom Tech Nylon-66 filter for use.

Preparation of stock and standard solutions
Stock solution of AT calcium (equivalent to 100µg/ml), EZ (equivalent to 100µg/ml) and FE (equivalent to 1600µg/ml) were prepared in methanol. The stock solution were protected from light using aluminium foil and stored for three weeks at 4°C with no evidence of decomposition. Aliquots of standard stock solution of AT, EZ and FE were transferred using A-grade bulb pipettes into 100ml volumetric flasks and the solution were made up to volume with methanol to yield final concentration of 2, 4, 6, 8 and 10 µg/ml for AT; 2, 4, 6, 8 and 10 µg/ml for EZ and 32, 64, 96, 128 and 160 µg/ml for FE.

RESULT AND DISCUSSION

HPLC method development and optimization
Purospher® RP 5µm, 250mm x 4.6mm i.d. column (Merck, Germany) maintained at ambient temperature (25°C) was used for the separation and the method validated for the determination of AT, EZ and FE in TriTonact® tablets. The composition, pH and flow rate of the mobile phase were changed to optimize the separation conditions using main substances of the three compounds of interest. A mobile phase consisting of ACN-water with gradient elution (Table -1) was selected for use for further studies after several preliminary investigatory chromatographic runs. Under the described experimental conditions, all peaks were well defined and free from tailing.

<table>
<thead>
<tr>
<th>Time [min]</th>
<th>Water %</th>
<th>ACN %</th>
<th>Flow Rate [ml/min]</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>48</td>
<td>52</td>
<td>1</td>
</tr>
<tr>
<td>8.0</td>
<td>40</td>
<td>52</td>
<td>1</td>
</tr>
<tr>
<td>9.0</td>
<td>30</td>
<td>70</td>
<td>2</td>
</tr>
<tr>
<td>22</td>
<td>30</td>
<td>70</td>
<td>2</td>
</tr>
<tr>
<td>23</td>
<td>48</td>
<td>52</td>
<td>1</td>
</tr>
</tbody>
</table>

Fig. 2: Representative chromatogram obtained for standard solution corresponding 4, 4 and 64 µg/ml of AT, EZ and FE, respectively

Validation of the method
The analytical method was validated with respect to parameters such as linearity, limit of detection (LOD), precision, accuracy, selectivity, and recovery29.

Linearity
Linearity was established by least squares linear regression analysis of the calibration curve 29. The constructed calibration curves were linear over the concentration range of 2-10µg/ml, 2-10µg/ml and 32-160µg/ml for AT, EZ and FE, respectively. Peak areas of AT or EZ or FE were plotted versus their respective concentrations and linear regression analysis was performed on the resultant curves. Typically, the regression equations were: y = 80.25x - 155.6 (R = 0.9915), y = 109.3x - 213.2 (R = 0.9903), y = 53.97x - 1395.9 (R = 0.9942) for AT, EZ and FE, respectively.

LOQ and LOD
LOD and LOQ were performed on samples containing concentrations of analytes, based on calibration curve method. Standard Solution of AT, EZ and FE were injected in six replicate. Average peak area of six
analytes was plotted against concentration. LOD and LOQ were calculated by using following equations

\[
\text{LOD} = \frac{(3.3 \times \sigma)}{S} \quad \text{LOQ} = \frac{(10.0 \times \sigma)}{S}
\]

Where \(\sigma\) is the standard deviation of y-intercepts of regression lines of the calibration curve, \(S\) is the slope of the calibration curve.

The LOD and LOQ value were found to be 0.1577\(\mu\)g/ml, 0.1266\(\mu\)g/ml, 1.9544\(\mu\)g/ml and 0.4780\(\mu\)g/ml, 0.3830\(\mu\)g/ml, 3.6225\(\mu\)g/ml, for AT, EZ and FE, respectively (Table-2).

**Precission**

The intra and inter-day variability or precision data are summarized in table-3. They were assessed by using standard solutions prepared to produce solutions of three different concentrations of each drug. AT, EZ and FE were used in the same solution for the purpose of these studies. Intra-day precision were investigated by injecting three replicate samples of each of the samples of three different concentrations. Inter-day precision were assessed by injecting the same three samples over three consecutive days.

Repeatability was investigated by injecting six replicate samples of the same three samples over three consecutive days.

Precision Ranges (CV)

<table>
<thead>
<tr>
<th>Parameters</th>
<th>AT</th>
<th>EZ</th>
<th>FE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linear Range ((\mu)g/ml)</td>
<td>2.10 mcg/ml</td>
<td>2.10 mcg/ml</td>
<td>32-160 mcg</td>
</tr>
<tr>
<td>Slope</td>
<td>80.25117</td>
<td>109.2833</td>
<td>53.9716</td>
</tr>
<tr>
<td>Intercept</td>
<td>155.667</td>
<td>213.167</td>
<td>1395.667</td>
</tr>
<tr>
<td>Standard deviation of slope</td>
<td>0.7</td>
<td>1.0778</td>
<td>0.2979</td>
</tr>
<tr>
<td>Limit of Detection ((\mu)g/ml)</td>
<td>0.07</td>
<td>0.1266</td>
<td>1.944</td>
</tr>
<tr>
<td>Limit of Quantification ((\mu)g/ml)</td>
<td>0.4780</td>
<td>0.3838</td>
<td>3.6225</td>
</tr>
<tr>
<td>Linear equation</td>
<td>Y = 80.25x - 155.6</td>
<td>Y = 109.3x - 213.2</td>
<td>Y = 53.97x - 1395.9</td>
</tr>
<tr>
<td>R² value</td>
<td>0.9915</td>
<td>0.9903</td>
<td>0.9942</td>
</tr>
</tbody>
</table>

**Conclusion**

A simple, rapid, accurate and precise HPLC analytical method has been developed and validated for the routine analysis of AT, EZ and FE in API and tablet dosage forms. The proposed method has the ability to separate these drugs from excipients found in tablet dosage forms.

**ACKNOWLEDGEMENT**

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