CHAPTER VII
High-performance liquid chromatographic determination of ezetimibe in human plasma and pharmaceutical formulations

Abstract

A simple and sensitive RP-HPLC method for the determination of ezetimibe (EZT) in human plasma and pharmaceutical formulations has been developed and validated. The analysis of EZT was carried out on a CLC C18 (5μ, 25 cm x 4.6 mm i.d) column using UV detector at 200 nm with the mobile phase consisting of acetonitrile-water (90:10 v/v). The linear range of detection was noticed to be 0.6-20 μg/ml (R^2 = 0.9979). Intra- and inter-day assay relative standard deviation values were observed to be less than 1.0%. The applicability of the method was demonstrated by analyzing the spiked human plasma and pharmaceutical preparations containing EZT. The results were subjected to statistical analysis.

The results of this chapter have been communicated for publication.
Ezetimibe (EZT)

Chemical name: 
\[(3R, 4S)-1-(4\text{-fluorophenyl})-3-[(3S)-3-(4\text{-fluorophenyl})-3\text{-hydroxypropyl}]-4-(4\text{-hydroxyphenyl})\text{-2-azetidinone}

Molecular formula: \(C_{24}H_{21}F_2NO_3\)

Molecular weight: 409.43

Description: White, crystalline powder

Solubility: Freely to very soluble in ethanol, methanol and acetone and practically insoluble in water

Category: Anticholesteremic agent
**INTRODUCTION**

Ezetimibe, (3R,4S)-1-(4-fluorophenyl)-3-[(3S)-3-(4-fluorophenyl)-3-hydroxypropyl]-4-(4-hydroxyphenyl)-2-azetidinone, is a selective cholesterol absorption inhibitor, which inhibits the absorption of biliary and dietary cholesterol [1] from the small intestine without affecting the absorption of fat soluble vitamins triglyceride or bile acids [2]. It reduces the small intestinal enterocyte uptake and absorption of cholesterol that keeps the cholesterol in the intestinal lumen for excretion [3]. Since, EZT does not affect the activities of CYP 450 enzymes, significant pharmacokinetic interactions with other medications viz., statins, fibrates, digoxin and warfarin have not been reported. It complements the lipid lowering effects of other therapies, such as statins. Clinical studies have shown that EZT exhibited significant reductions in both the low density lipoproteins (LDL) and the total cholesterol, with slight increases in the high density lipoproteins (HDL)[4-7] when administered with statins besides reducing the risk of coronary heart disease (CHD) events in patients with hypercholesterolemia [8].

In view of its importance, a few analytical methods have been reported for the determination of EZT alone or in combination with other drugs. These methods include UV-Visible spectrophotometry [9-12], LC-MS [13], LC-tandem MS [14] and HPTLC [15-17].

Seshachalam and Chandrasekhar [18] have developed a method for quantitative determination of atorvastatin and ezetimibe in pharmaceutical formulations. The system comprised 0.01 M ammonium acetate buffer.
(pH: 3.0) and acetonitrile mixture (50:50 v/v) as mobile phase. The detection was monitored at 254 nm. These drugs obeyed Beer’ law in the range of 4-400 μg/ml for atrovastatin and 5-500 μg/ml for EZT, respectively. The limit of detection for EZT was found to be 1.48 μg/ml. This method worked out at higher concentration of the drug and found to be less sensitive.

Doshi et al [19] have described a validated stability indicating method for the assay of EZT in tablets. The system employed phosphoric acid (0.1%, v/v)-acetonitrile mixture [50:50 (v/v)] as mobile phase. Beer’ law was obeyed in the range of 20–80 μg/ml. The drug decomposed into numerous products under different stress conditions. In order to overcome these limitations, the investigator has developed a simple and reliable RP-HPLC (coupled with UV detection) method for the determination of EZT in pharmaceutical formulations and spiked human plasma samples.

EXPERIMENTAL

Stock solutions
1) Standard solution: Pure EZT solution (500 μg/ml) was prepared in acetonitrile.
2) Internal standard solution: A stock solution of PRX (500 μg/ml) prepared in acetonitrile was used in the study.

The stock solutions of EZT and PRX were diluted as and when necessary

Standard working solutions

Working solutions of each of 100 μg/ml pure EZT and PRX were prepared separately in the mobile phase from the respective stock solution after
suitable dilution. The working solutions were observed to be stable even after storing for 4 days at 4 °C.

**Sample preparation**

**a) Blood Plasma**

Human blood samples from different healthy volunteers were collected in dry and evacuated tubes containing saline and sodium citrate solution. The samples were handled at 28 °C and were centrifuged for 12 min at 1500 rpm for the separation of plasma within 60 min of collection. The samples were then transferred to polypropylene tubes and stored at -20 °C until analysis.

**b) Tablets**

Twenty tablets of EZT were finely powdered. An amount equivalent to 25 mg of the drug was weighed accurately and transferred into a 100 ml beaker containing mobile phase. Using a mechanical stirrer, the powder was completely disintegrated in mobile phase and then filtered through 0.45 μ Millipore membrane filter and the filtrate was made up to 100 ml in a volumetric flask with the same mobile phase. It was diluted as and when required.
Operating conditions

Column: CLC C_{18} column (5 \mu, 25 \text{ cm} \times 4.6 \text{ mm i.d.) with CLC ODS (4 \text{ cm} \times 4.6 \text{ mm, i.d.}) as a guard column to protect analytical column.}

Mobile phase: acetonitrile : water (90:10 v/v)
Flow rate: 1.0 ml/min
Internal standard: PRX
Temperature: Ambient
Mode: Reverse phase
Membrane: 0.45 \mu Millipore
Run time: 5 min
Wavelength: 200 nm

Procedures

Establishment of calibration curve

Working solutions containing pure EZT (0.6-20 \mu g/ml) and internal standard (6 \mu g/ml) were prepared in the mobile phase. With the above chromatographic conditions, triplicate 20 \mu l solutions were injected for each working solution and the chromatograms were recorded. A typical chromatogram obtained is shown in Fig. 1. The values of ratio of peak area of standard to internal standard were plotted against the concentration of EZT to obtain the calibration graph (Fig. 2). The results were subjected to regression analysis to get the calibration equation and correlation coefficient.
Throughout the study, the suitability of the chromatographic system was monitored by calculating the capacity factor ($k'$), resolution (R), selectivity ($\alpha$) and peak asymmetry ($A_S$).

**Analysis of plasma samples**

The plasma samples obtained as described in plasma sample preparation were allowed to thaw at 28 °C before processing. The plasma samples were spiked with EZT and a fixed amount of PRX (6 μg/ml) and the tube was briefly shaken. Then the mixture was vortex mixed with ether. The ethereal layer was evaporated to dryness on a water bath under gentle stream of nitrogen gas at 40 °C. The residue was dissolved in suitable amount of mobile phase and triplicates of 20 μl solutions were injected on to the column for analysis and the chromatogram was recorded (Fig. 3).

**Analysis of pharmaceutical formulations**

Suitable amounts of the aliquot of the formulation containing EZT were taken and analyzed using the above mentioned chromatographic conditions.

**RESULTS AND DISCUSSION**

**Method development**

**Mobile phase**

For reverse phases, the retention of organic samples is always high with water as eluent. Increasing the concentration of organic solvent in water may accelerate the elution of organic compounds. The mobile phase was chosen...
after several trials with methanol, acetonitrile, isopropyl alcohol, triethylamine, water and various buffer solutions of different pH in various proportions. Acetonitrile was selected along with water as the mobile phase due to its high transparency in UV region. Moreover, satisfactory results were observed with acetonitrile-water combination. Hence, different proportions of acetonitrile and water were examined to select the suitable ratio for quantification. It was noticed that at higher concentration of water in the mobile phase, the retention times of EZT and PRX were observed to be very high and EZT was eluted poorly off the column. The best separation of EZT and PRX was achieved with acetonitrile and water combination in the ratio of 90:10.

Flow rate

Flow rate of the mobile phase plays a crucial role in chromatographic determination of pharmaceutical drugs. The effect of flow rate was investigated by varying the flow rate of the mobile phase from 0.25 to 2.5 ml/min. Lower flow rates led to increase in resolution time while higher flow rates led to considerable increase in pressure. However, a flow rate of 1.0 ml/min gave an optimal signal to noise ratio with satisfactory separation time and hence permitted ideal conditions for quantification of the drug.

Internal standard

Various compounds viz., ceterizine hydrochloride, nimesulide, amoxycillin, ibuprofen, cefadroxil, methdilazine hydrochloride and PRX were examined as internal standards. It was observed that the proper resolution and
less time for analysis was achieved with PRX and hence, PRX was employed as an internal standard throughout the work.

*Order of elution*

Under the experimental conditions, it was noticed that the PRX was eluted first followed by EZT.

*Retention times*

The reproducibility of the retention times of EZT and PRX was checked by analyzing nine replicates. The retention times of EZT and PRX were observed to be 1.78 min and 0.76 min, respectively.

*Wavelength selection*

The chromatographic assays of a drug do depend on the proper selection of the wavelength. The effect of wavelength on the response factor and on the peak resolution was studied over the wavelength range of 180-240 nm. Ideal chromatographic conditions were achieved at 200 nm.

*Linearity*

Linearity and range of the method were determined by injecting 20 μl of different solutions containing 0.1-22 μg/ml of EZT and fixed amount of internal standard (6 μg/ml) under the chromatographic conditions mentioned above (n = 9). Chromatograms were recorded. The plot of peak area ratio of EZT to PRX *versus* concentration of EZT yielded the linearity range (Fig. 2). Table 1 gives the regression line, correlation coefficient, slope, intercept and % RSD. Excellent linearity was noticed in the range of 0.6-20 μg/ml of EZT with $R^2 = 0.9979$. 

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Limits of detection and quantification

Limit of detection (LOD) was established at a signal-to-noise ratio (S/N) of 3 while limit of quantification (LOQ) was calculated at a S/N value of 10. The LOD and LOQ values were calculated to be 0.186 μg/ml and 0.62 μg/ml, respectively (Table 1).

Suitability of the method

System performance parameters viz., resolution, selectivity and peak asymmetry are important in deciding the suitability of a chromatographic method. The suitability of the method was examined by evaluating the above system performance parameters and the corresponding results are shown in Table 2. The observed values of resolution (more than 1), selectivity (more than 1) and peak asymmetry (less than 2) revealed ideal chromatographic conditions for the assay of EZT.

Precision

Method repeatability (intra-day precision) was evaluated by assaying 9 replicates of the drug (12 μg/ml). The % RSD values were found to be less than 1%. The intermediate precision (inter-day precision) was performed by assaying 9 replicates of the drug on different days. The low values of RSD revealed that the results are reproducible on different days, well within experimental errors. The corresponding results are shown in Table 3.

Accuracy

A standard working solution containing 12 μg/ml of EZT and 6 μg/ml PRX, was prepared. 20 μl of the mixture was injected (n = 9) and
chromatogram was recorded each time. From the respective area counts, the concentrations of the EZT were determined using the calibration graph. The accuracy, defined in terms of % bias is listed in Table 3.

**Recovery studies**

In order to examine the accuracy and reproducibility of the proposed method, recovery experiments were carried out [20]. The recovery of the added standard was studied at five different levels. Each level was repeated six times. To aliquots of the previously analyzed preparations, a known concentration of standard solution of EZT was added. The content of EZT was once again determined by the proposed method. From the amount of drug present, percentage recovery was calculated using the following formula:

\[
\text{% Recovery} = \frac{N (\sum xy) - (\sum y)(\sum x)}{N (\sum x^2)(\sum x)^2}
\]

Where  
\( x = \) Amount of standard drug added  
\( y = \) Amount of drug found by proposed method  
\( N = \) Number of observations

The results were found to be in the range of 98.2-101.88%. High recovery values indicated the accuracy of the proposed method.

**Ruggedness**

Chromatographic parameters were not affected significantly with the slight changes in the chromatographic conditions like the composition of the mobile phase and flow rate. Assay procedure described in the proposed method was repeated with different C18 columns and no significant change in

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chromatographic parameters was observed. Also, three different persons (analysts) carried out the analyses and no considerable changes were noticed in the chromatographic parameters. All these revealed that the proposed chromatographic method has reasonable ruggedness.

Applications

Analysis of plasma samples

The proposed method was applied to the determination of EZT in spiked plasma samples. The results obtained for precision and accuracy at three different concentrations in plasma are recorded in Table 4. Low values of relative standard deviation indicate high precision of the proposed method. The proposed method was found to be accurate as evident from low % Bias values.

Analysis of pharmaceutical preparation

The proposed method was successfully applied to the analysis of EZT in tablet and the results of analysis are shown in Table 5. The low value of relative standard deviation indicated good reproducibility of the result.

CONCLUSIONS

The proposed HPLC method showed acceptable linearity, precision and accuracy over the concentration range mentioned. The total chromatographic run time was about 5 min. The chromatographic method could be used to analyze several plasma samples each day in clinical laboratories and

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pharmaceutical formulations in quality control laboratories. Hence, the proposed method could be readily employed for the assay of EZT in plasma samples and pharmaceutical preparations.
REFERENCES


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Fig. 1. A typical chromatogram showing the separation of PRX and EZT in pure form.
Fig. 2. Calibration graph for EZT.
Fig. 3. A typical chromatogram showing the separation of PRX and EZT in plasma sample.
Table 1. Linearity results, limit of detection and limit of quantification.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>EZT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linear dynamic range (μg/ml)</td>
<td>0.6-20</td>
</tr>
<tr>
<td>Regression equation (Y=a)</td>
<td></td>
</tr>
<tr>
<td>Slope (b)</td>
<td>0.198</td>
</tr>
<tr>
<td>Intercept (c)</td>
<td>0.1024</td>
</tr>
<tr>
<td>Correlation coefficient (R²)</td>
<td>0.9979</td>
</tr>
<tr>
<td>LOD (μg/ml)</td>
<td>0.186</td>
</tr>
<tr>
<td>LOQ (μg/ml)</td>
<td>0.62</td>
</tr>
<tr>
<td>% RSD</td>
<td>1.84</td>
</tr>
</tbody>
</table>

*Y = bX + c, where X is concentration of drug in μg/ml.
Table 2. System performance parameters of PRX (6 μg/ml) and EZT (12 μg/ml).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Pure sample</th>
<th>Plasma sample</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PRX</td>
<td>EZT</td>
</tr>
<tr>
<td>Number of theoretical plates</td>
<td>68.91</td>
<td>122.08</td>
</tr>
<tr>
<td>Retention time (t&lt;sub&gt;R&lt;/sub&gt;) in min</td>
<td>0.76</td>
<td>1.78</td>
</tr>
<tr>
<td>Capacity factor (k')</td>
<td>5.16</td>
<td>13.75</td>
</tr>
<tr>
<td>Selectivity factor (α)</td>
<td>2.66</td>
<td></td>
</tr>
<tr>
<td>Resolution (R)</td>
<td></td>
<td>1.449</td>
</tr>
<tr>
<td>Peak asymmetry (A&lt;sub&gt;S&lt;/sub&gt;)</td>
<td>1.20</td>
<td>1.71</td>
</tr>
<tr>
<td>Height equivalent to theoretical plate (H)</td>
<td>0.20</td>
<td>0.12</td>
</tr>
</tbody>
</table>

Table 3. Intra-day (1 representative day) and inter-day precision and accuracy for the determination of EZT.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Concentration added, μg/ml</th>
<th>Intra-day (n = 9) Concentration measured</th>
<th>% RSD</th>
<th>% Bias</th>
<th>Inter-day (n = 9) Concentration measured</th>
<th>% RSD</th>
<th>% Bias</th>
</tr>
</thead>
<tbody>
<tr>
<td>EZT</td>
<td>5</td>
<td>4.91</td>
<td>0.85</td>
<td>-1.8</td>
<td>4.91</td>
<td>0.97</td>
<td>-1.8</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>9.83</td>
<td>0.94</td>
<td>-1.7</td>
<td>9.81</td>
<td>0.88</td>
<td>-1.9</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>14.81</td>
<td>0.72</td>
<td>-1.26</td>
<td>14.73</td>
<td>0.82</td>
<td>-1.8</td>
</tr>
</tbody>
</table>
Table 4. Analysis of EZT in human plasma samples.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Concentration added µg/ml</th>
<th>Measured concentration*, µg/ml</th>
<th>% RSD</th>
<th>% Bias</th>
</tr>
</thead>
<tbody>
<tr>
<td>EZT</td>
<td>5</td>
<td>4.90</td>
<td>1.85</td>
<td>-2.0</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>9.82</td>
<td>1.97</td>
<td>-1.8</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>14.71</td>
<td>1.68</td>
<td>-1.9</td>
</tr>
</tbody>
</table>

*Average of five determinations.

Table 5. Analysis of EZT in pharmaceutical formulations.

<table>
<thead>
<tr>
<th>Tablet*</th>
<th>Labeled, mg/ml</th>
<th>Found*, mg/ml</th>
<th>% RSD</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zetistat</td>
<td>10</td>
<td>10.18</td>
<td>1.67</td>
<td>101.8</td>
</tr>
</tbody>
</table>

* Average of five determinations.
* Marketed by Torrent pharmaceuticals Ltd.