High performance liquid chromatography with PDA detector for combined determination of emtricitabine, tenofovir and efavirenz

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ABSTRACT

A simple, rapid, precise and accurate reverse-phase HPLC method was developed and validated for the simultaneous determination of emtricitabine, tenofovir and efavirenz in commercial tablets. The method has shown adequate separation for emtricitabine, tenofovir and efavirenz. Separation was achieved on Inertsil C18 (250 mm × 4.6mm; 5 µm) column using isocratic method with 0.1% OPA: Methanol (55:45) system at room temperature and the detection was carried out at 260 nm using photodiode array (PDA) detector. The linearity of the proposed method was investigated in the range of 20-60 µg/ml (r²=0.9999), 30-90 µg/ml (r²=0.9999), 60-180 µg/ml (r²=0.9988) for emtricitabine, tenofovir and efavirenz respectively. The limit of detection (LOD) was 0.066, 0.1540 and 0.353 for emtricitabine, tenofovir and efavirenz respectively. The limit of quantification (LOQ) was 0.290, 0.0773 and 1.176 for emtricitabine, tenofovir and efavirenz 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 emtricitabine, tenofovir and efavirenz in commercial tablets.

Key words: Emtricitabine, Tenofovir, Efavirenz, HPLC, Simultaneous Determination.

INTRODUCTION

Emtricitabine [1,2], 4-amino-5-fluoro-1-[(2R,5S)-2-(hydroxymethyl)-1,3-oxathiolan-5-yl]-1,2-dihydropyrimidin-2-one (Figure 1), is a nucleoside reverse transcriptase inhibitor belonging to the class of compounds known as 3'-thia pyrimidine nucleosides. The drug works by inhibiting reverse transcriptase, the enzyme that copies HIV RNA into new viral DNA. By interfering with this process, which is central to the replication of HIV, emtricitabine can help to lower the amount of HIV, or "viral load", in a patient's body and can indirectly increase the number of immune system cells (called T cells or CD4+ T-cells). Both of these changes are associated with healthier immune systems and decreased likelihood of serious illness.

Tenofovir [3,4] chemically known as ([(2R)-1-(6-amino-9H-purin-9-yl)propan-2-yl]oxy)methyl)phosphonic acid (Figure 2). Tenofovir belongs to the class of compounds known as 6-aminopurines. Tenofovir inhibits the activity of
HIV reverse transcriptase by competing with the natural substrate deoxyadenosine 5'-triphosphate and, after incorporation into DNA, by DNA chain termination. Specifically, the drugs are analogues of the naturally occurring deoxynucleotides needed to synthesize the viral DNA and they compete with the natural deoxynucleotides for incorporation into the growing viral DNA chain.

Efavirenz [5,6] is a non-nucleoside reverse transcriptase inhibitor (NNRTI) and is used as part of highly active antiretroviral therapy (HAART) for the treatment of a human immunodeficiency virus (HIV) type 1. Efavirenz belongs to the class of compounds known as benzoxazines. Chemically, it is described as (4S)-6-chloro-4-(2-cyclopropyl ethynyl)-4-(trifluoromethyl)-2,4-dihydro-1H-3,1-benzoxazin-2-one (Figure 3). Efavirenz is also used in combination with other antiretroviral agents as part of an expanded postexposure prophylaxis regimen to prevent HIV transmission for those exposed to materials associated with a high risk for HIV transmission.


The aim of the present investigation is to develop and validate a sensitive, precise and accurate RP-HPLC method for the simultaneous quantification of Emtricitabine, Tenofovir and Efavirenz in bulk and in its combined pharmaceutical formulation.

**MATERIALS AND METHODS**

**Apparatus**
A Waters 2695 alliance with binary HPLC pump equipped with Waters 2998 PDA detector and Waters Empower2 software is used in the present investigation.

**Mobile phase**
The solvents and chemicals used in the preparation of mobile phase were of HPLC grade and analytical grade, respectively. The mobile phase used is 0.1% OPA and methanol in the ratio of 55:45 v/v. The mobile phase is filtered before using, through millipore membrane filter and degassed for 15 min by sonication.

**Chromatographic conditions**
Inertsil C18 (250 x 4.6 mm; 5 μm particle size) analytical column was used for separation and simultaneous analysis of the Emtricitabine, Tenofovir and Efavirenz. The column temperature was maintained at 30 ± 1°C. The separation
was carried out under isocratic elution. The flow rate was maintained 1.0 ml/min. The injection volume was 10 µl. The selected drugs were analyzed using a PDA detector covering the range of 200–400 nm.

**Standard solutions**
The standard stock solution was prepared by dissolving 200 mg of emtricitabine, 300 mg of tenofovir and 600 mg of efavirenz in 100 ml mobile phase. Working standard solutions equivalent to 20-60 µg/ml emtricitabine, 30-90 µg/ml tenofovir and 60-180 µg/ml efavirenz was prepared from stock solution by appropriately diluting the stock standard solution with the mobile phase.

**Sample Solution**
Ten tablets were weighed and crushed to a fine powder. The powder equivalent to dissolving 200 mg of emtricitabine, 300 mg of tenofovir and 600 mg of efavirenz was taken in a 100 ml volumetric flask containing 20 ml of mobile phase, sonicate for 20 min and made up to mark with the same solvent. The resultant mixture was filtered through 0.45 µm filter paper. The resultant solution was diluted appropriately with the mobile phase to get a final concentration of 200 µg/ml emtricitabine, 300 µg/ml tenofovir and 600 µg/ml efavirenz.

**RESULTS AND DISCUSSION**

**HPLC parameters optimization**
Chromatographic parameters such as mobile phase composition, wavelength of detection, column and column temperature were optimized to achieve better efficiency of the chromatographic system. Two HPLC analytical columns, Zorbax C18 (150 mm x 4.6 mm x 5 µm) and Inertsil C18 (250 mm x 4.6 mm x 5 µm) were tested during method development. The system suitability parameters like tailing factor, resolution, and plate count were taken into consideration. Based on the above said parameters Inertsil C18 (250 mm x 4.6 mm x 5 µm) column was finalized for simultaneous analysis of emtricitabine, tenofovir and efavirenz. Different composition of mobile phases containing a mixture (v/v) of 0.1M CH₃COONH₄, methanol and 0.1% OPA were assessed in order to get apt composition of mobile phase. Finally the mixture of 0.1% OPA and methanol in the ratio of 55:45(v/v) was selected as optimal as it produced well defined and well resolved peaks of emtricitabine, tenofovir and efavirenz at a flow rate of 1.0 ml/min and with column temperature of 30°C. For the detection and quantification of emtricitabine, tenofovir and efavirenz, 260 nm was selected as the optimum wavelength. The retention time for emtricitabine, tenofovir and efavirenz was found to be 2.993, 7.129 min and 9.096 min, respectively. A typical chromatogram is given in Figure 4.

**Method validation**
The optimized RP-HPLC method for simultaneous assay of emtricitabine, tenofovir and efavirenz was validated according to ICH guidelines [16, 17] with respect to system suitability, linearity, sensitivity, accuracy, precision and robustness.

**System suitability**
Prior to analysis, the chromatographic system must satisfy system suitability test requirements. System suitability test was assessed from five replicate injections of the standard solution containing 200, 300 and 600 µg/ml emtricitabine, tenofovir and efavirenz, respectively. All the three peaks were well resolved and the precision of injections for all the peaks were acceptable. The percent relative standard deviation of the emtricitabine, tenofovir
and efavirenz peaks area responses were determined to be less than 1. The USP tailing factor and USP plate count were also calculated. The results of system suitability in comparison with the required limits are shown in Table 1 and are found to be within the accepted limits.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Results</th>
<th>Recommended limits</th>
</tr>
</thead>
<tbody>
<tr>
<td>Retention time</td>
<td>Emtricitabine</td>
<td>2.993</td>
</tr>
<tr>
<td></td>
<td>Tenofovir</td>
<td>7.129</td>
</tr>
<tr>
<td></td>
<td>Efavirenz</td>
<td>9.096</td>
</tr>
<tr>
<td>Peak area</td>
<td>Emtricitabine</td>
<td>6516518 (%RSD – 0.5)</td>
</tr>
<tr>
<td></td>
<td>Tenofovir</td>
<td>9209798 (%RSD – 0.6)</td>
</tr>
<tr>
<td></td>
<td>Efavirenz</td>
<td>10064097 (%RSD – 0.6)</td>
</tr>
<tr>
<td>USP resolution</td>
<td>-</td>
<td>13.67</td>
</tr>
<tr>
<td>USP plate count</td>
<td>4290</td>
<td>4894</td>
</tr>
<tr>
<td>USP tailing factor</td>
<td>1.37</td>
<td>1.35</td>
</tr>
</tbody>
</table>

**Linearity and range**

The linearity of the HPLC method was determined, for the simultaneous assay of emtricitabine, tenofovir and efavirenz, by analyzing five different concentrations of each drug. The calibration curve was plotted by area under the peak responses of the three drugs against their corresponding concentrations. Calibration curves were linear over the concentration range of 200 µg/ml emtricitabine, 300 µg/ml tenofovir and 600 µg/ml efavirenz. The linearity parameters such as regression equations and regression coefficients are given in Figures 5, 6 and 7. The results show a good correlation between the peak areas of the three drugs and their corresponding concentrations.

![Figure 5: Linearity curve of emtricitabine](image1)

![Figure 6: Linearity curve of tenofovir](image2)
Sensitivity
The sensitivity of the method was assessed by calculating limit of detection (LOD) and limit of quantification (LOQ). LOD was found to be 0.066 µg/ml, 0.1540 µg/ml and 0.353 µg/ml for emtricitabine, tenofovir and efavirenz, respectively (signal to noise ratio of 3:1). LOQ was found to be 0.220 µg/ml, 0.5133 µg/ml and 1.176 µg/ml for emtricitabine, tenofovir and efavirenz, respectively (signal to noise ratio of 10:1). The low values of LOD and LOQ demonstrate sufficient sensitivity of the HPLC method.

Precision
Precision was determined by injecting six standard solutions of emtricitabine, tenofovir and efavirenz with 200, 300 and 600 µg/ml concentrations, respectively. The peak areas were determined. Relative standard deviation of emtricitabine, tenofovir and efavirenz peaks was then calculated to represent precision. The results are summarized in Table 2. The low % RSD values indicated that the method is precise.

Accuracy
Accuracy of the method was evaluated by recovery studies at three concentration (50%, 100%, and 150%) levels by standard addition method. The mean percentage recoveries obtained were in the range of 99-100% for all the three drugs (Table 3). The good % recovery values showed the method to be highly accurate.
Robustness

In order to show the robustness of the method, system suitability parameters were evaluated by slightly varying flow rate and column temperature. The parameters used to define robustness were retention time, USP tailing factor and USP plate count. The results showed (Table 4) that slight variations in method parameters had a negligible effect on the analysis.

Table 4: Robustness of the method

<table>
<thead>
<tr>
<th>Drug</th>
<th>Parameter</th>
<th>Retention time</th>
<th>Peak area</th>
<th>USP Plate Count</th>
<th>USP Tailing</th>
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<tr>
<td></td>
<td>Flow 1</td>
<td>3.678</td>
<td>8154266</td>
<td>4585</td>
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<tr>
<td></td>
<td>Flow 2</td>
<td>2.537</td>
<td>5602490</td>
<td>3917</td>
<td>1.44</td>
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<tr>
<td>Emtricitabine</td>
<td>Temperature 1</td>
<td>3.671</td>
<td>8062743</td>
<td>4445</td>
<td>1.43</td>
</tr>
<tr>
<td></td>
<td>Temperature 2</td>
<td>2.530</td>
<td>5514877</td>
<td>3879</td>
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<tr>
<td></td>
<td>Flow 1</td>
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<td>11425345</td>
<td>5482</td>
<td>1.38</td>
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<tr>
<td>Tenofovir</td>
<td>Flow 2</td>
<td>6.190</td>
<td>709337</td>
<td>4584</td>
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<td>8.857</td>
<td>11350965</td>
<td>5586</td>
<td>1.35</td>
</tr>
<tr>
<td></td>
<td>Temperature 2</td>
<td>6.176</td>
<td>7795871</td>
<td>4521</td>
<td>1.37</td>
</tr>
<tr>
<td></td>
<td>Flow 1</td>
<td>11.250</td>
<td>12522932</td>
<td>5370</td>
<td>1.16</td>
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<tr>
<td>Efavirenz</td>
<td>Flow 2</td>
<td>7.909</td>
<td>8701290</td>
<td>4197</td>
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<tr>
<td></td>
<td>Temperature 1</td>
<td>11.256</td>
<td>12429136</td>
<td>5670</td>
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<tr>
<td></td>
<td>Temperature 2</td>
<td>7.896</td>
<td>8555025</td>
<td>4371</td>
<td>1.20</td>
</tr>
</tbody>
</table>

CONCLUSION

A RP-HPLC method has been reported for simultaneous estimation emtricitabine, tenofovir and efavirenz. The proposed method gives good resolution of the above said drugs. The validation of developed method was done as per ICH guidelines and proved that method to be simple, sensitive, precise, accurate and robust. The validated method was successfully applied to the determination of commercially available pharmaceutical dosage form. Hence, the method can be used for the routine quality control analysis of pharmaceutical dosage forms containing emtricitabine, tenofovir and efavirenz.
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REFERENCES