Simultaneous estimation of emtricitabine, tenofovir disoproxil fumarate, and rilpivirine in bulk form by RP-HPLC method

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ABSTRACT

A new simple, accurate, rapid and precise isocratic High performance liquid chromatographic (HPLC) method was developed and validated for the determination of Emtricitabine, Tenofovir disoproxil fumarate, Rilpivirine. The Method employs Thermo Hypersil ODS C-18 column (150×4.6mm, 5µ) and flow rate of 1 ml/min with a load of 20µl. Acetonitrile and Phosphate buffer pH 3 was used as mobile phase in the composition of 60:40. The Detection was carried out at 260 nm. Linearity ranges for Emtricitabine, Tenofovir disoproxil fumarate, Rilpivirine were 8-48µg/ml, 12-72µg/ml, 1-6µg/ml respectively. Retention Time of Emtricitabine, Tenofovir disoproxil fumarate, Rilpivirine were found to be 2.408 min, 3.664 min, 12.062 min respectively. Percent Recovery study values of Emtricitabine, Tenofovir disoproxil fumarate, Rilpivirine were found to be within 98-102%. This newly developed method was successfully utilized for the Quantitative estimation of Emtricitabine, Tenofovir disoproxil fumarate, Rilpivirine in bulk form. This method was validated for accuracy, precision, linearity and Robustness as per ICH guidelines.

Keywords: Emtricitabine, Tenofovir disoproxil fumarate, Rilpivirine, RP-HPLC, validation, simultaneous estimation.

EXPERIMENT

Materials and methods

Collection of drugs:
Emtricitabine of purity 99% w/w, Tenofovir of purity 99% w/w, Rilpivirine of purity 99% w/w procured from Hetero Labs, Hyderabad.
Chemicals and reagents:
Acetonitrile-HPLC grade (merck) water for HPLC –milli-Q grade (merck), Potassium dihydrogen phosphate (merck) O-phosphoric acid.

Apparatus:
HPLC system (Analytical Technologies Limited, Baroda, Gujarat, INDIA) used in this method was equipped with UV-Visible detector. Empower chromatography software was used for liquid chromatogram peak integration. Data Apex- CLARITY software was used for data acquisition and data processing. Chromatographic separations were performed on X tetra C-18 column (150x4.6mm, 5µ). Samples were injected by means of a rheodyne injector fitted with a 20µL loop. Ultrasonic bath (Analytical Technologies Limited, Gujarat, INDIA) was used for sonication for degassing of mobile phase. UV-1601, UV-Visible spectrophotometer (Shimadzu, Japan) with Ext Trans (RS-232C port) software was used to obtain the overlay spectra of the drugs to determine the analytical wavelength. Other instruments included Shimadzu electronic balance (type BL-220H), micropipettes and micro-pore filtration assembly etc.

Mobile phase: Acetonitrile and phosphate buffer pH 3 adjusted with phosphoric acid(40:60). Preparation of 0.05 M phosphate buffer solution: 2.95 gm of potassium dihydrogen phosphate and 0.545 mg of Dihydrogen phosphate were weighed and made up to 500ml with water (pH 3).

Preparation of standard solutions:
Accurately weighed quantity of 40 mg of Emtricitabine and 60 mg of Tenofovirdisoproxil fumarate, 5 mg of Rilpivirine was transferred to a 100 ml volumetric flask, dissolved it in 10ml of mobile phase, sonicated for 15 min and the volume was made up with mobile phase. From the standard stock preparation 10ml of solution was taken in 100ml flask further diluted with mobile phase.

Procedure:
Inject 20µl of the standard into the chromatographic system and measure the areas for the Emtricitabine, Tenofovir, Rilpivirine peaks and calculate the %Assay by using the formulae.

Optimized chromatographic conditions:
The following parameters were used for HPLC analysis for the estimation of bulk form.
Stationary phase : X tetra C-18 column (150x4.6mm, 5µ)
Mobile phase : Acetonitrile:phosphate buffer pH 3 40: 60
Software : EMPOWER 2 software
Mode of Operation : Isocratic
pH : 3
Flow rate : 1 ml/min
Column Temperature : Room temperature
Volume of Injection loop : 20µl
Detection of Wavelength : 260 nm
Run time : 22 min

Method validation
The proposed chromatographic method was validated with respect to following parameters, as per ICH guidelines (13). Calibration curves were prepared in the concentration range of 0-48µg/ml for Emtricitabine, 0-72µg/ml for Tenofovir and 0-6 µg/ml for Tenofovir disoproxil fumarate. Linearity was demonstrated by analysing six different concentrations of active compound. Peak areas were recorded for all the peaks and calibration plot was constructed by plotting peak area Vs concentrations of active compound. Accuracy was determined by recovery studies with three standard solutions containing known concentration of drugs and the percentage recoveries of the added drugs were determined. Precision was evaluated in terms of intra-day and inter-day precision. The intra-day precision was investigated using six replicates of same concentration of standard solutions. The intra-day and inter-day precision of the proposed method was determined by analysing the corresponding concentration six times on the same day and six times on the different days and the results were reported. LOD and LOQ values were calculated from the calibration curves. Robustness of the method was determined by deliberately varying certain parameters like flow-rate, analytical wavelength and composition of mobile phase etc.

RESULTS AND DISCUSSION
Working conditions for the HPLC method were determined with Emtricitabine, Tenofovir disoproxil fumarate and Rilpivirine bulk drug forms. Under the experimental conditions investigated, the retention times for Emtricitabine,Tenofovir disoproxil fumarate and Rilpivirine were 2.40min, 3.66min, and 12.06min respectively.
The proposed method was found to be selective for the estimation of three drugs.

**Linearity:**
For the construction of calibration curve six standard solutions were prepared over the concentration ranges of 0-48 µg/ml, 0-72 µg/ml and 0-6µg/ml for Emtricitabine, Tenofovir disoproxil fumarate and Rilpivirine respectively. The areas exhibited linear responses with r² values 1 for Emtricitabine, Tenofovir disoproxil fumarate and Rilpivirine respectively. All the validation parameters such as correlation coefficients, concentration ranges, detection limits are summarised in the table.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Emtricitabine</th>
<th>Tenofovir</th>
<th>Rilpivirine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linearity Dynamic Range</td>
<td>0-48µg/ml</td>
<td>0-72µg/ml</td>
<td>0-6µg/ml</td>
</tr>
<tr>
<td>Correlation Coefficient</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Slope (m)</td>
<td>11884.2</td>
<td>24146.6</td>
<td>200006.3</td>
</tr>
<tr>
<td>Intercept</td>
<td>9983.3</td>
<td>1494.5</td>
<td>17641.4</td>
</tr>
</tbody>
</table>

**Accuracy and precision**
Accuracy and precision was determined with three replicates of QC samples. QC standards were prepared in the mobile phase and are dilutions from weightings independent of those used for the preparation of calibration curves. The recoveries ranged from 100.3-100% for Emtricitabine, 100.2-100% for Tenofovir disoproxil fumarate and 99.6-99.58% for Rilpivirine was determined. The RSD values obtained were < 2. The results were mentioned in the table.

**Accuracy table**

<table>
<thead>
<tr>
<th>Accuracy level</th>
<th>Emtricitabine</th>
<th>Tenofovir</th>
<th>Rilpivirine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Accuracy 50 %</td>
<td>100.31</td>
<td>100.13</td>
<td>100.06</td>
</tr>
<tr>
<td>Accuracy 100 %</td>
<td>100.29</td>
<td>100.17</td>
<td>100.07</td>
</tr>
<tr>
<td>Accuracy 150 %</td>
<td>99.60</td>
<td>99.23</td>
<td>99.58</td>
</tr>
</tbody>
</table>

**Precision table**

<table>
<thead>
<tr>
<th>Sample no:</th>
<th>Area of Emtricitabine (mV)</th>
<th>Area of Tenofovir (mV)</th>
<th>Area of Rilpivirine (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3855762</td>
<td>1172873</td>
<td>7318793</td>
</tr>
<tr>
<td>2</td>
<td>3827278</td>
<td>1164008</td>
<td>7297357</td>
</tr>
<tr>
<td>3</td>
<td>3822781</td>
<td>1164008</td>
<td>7297357</td>
</tr>
<tr>
<td>4</td>
<td>3825781</td>
<td>1163008</td>
<td>7295357</td>
</tr>
<tr>
<td>5</td>
<td>3826781</td>
<td>1166008</td>
<td>7296357</td>
</tr>
<tr>
<td>MEAN</td>
<td>3831945</td>
<td>1165652</td>
<td>7300430</td>
</tr>
<tr>
<td>SD</td>
<td>11695.54</td>
<td>3670.654</td>
<td>9031.66</td>
</tr>
<tr>
<td>% RSD</td>
<td>0.31</td>
<td>0.31</td>
<td>0.12</td>
</tr>
</tbody>
</table>

**Robustness:**
The robustness of the developed method was investigated by evaluating the influence of small deliberate variations in procedure variables like flow rate (±5%) and change in buffer concentration (±5%). Minor changes did not significantly affect the recoveries, peak area and retention time of all the above drugs indicating that the proposed method is robust.

**Ruggedness:**
The method is rugged by different analyst, different time intervals and the method did not significantly affect the recoveries, peak area and retention time of all the above drugs indicating that the proposed method is rugged.

**LOD and LOQ**
LOD and LOQ values decide about the sensitivity of the method. LOD is the lowest detectable concentration of the analyte, while LOQ is the lowest quantifiable concentration. The results for LOD & LOQ values shows that the method is quite sensitive for Emtricitabine, Tenofovir disoproxil fumarate and Rilpivirine and the results are given in the table.

<table>
<thead>
<tr>
<th>Sample</th>
<th>LOD</th>
<th>LOQ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Emtricitabine</td>
<td>0.3247</td>
<td>0.99000</td>
</tr>
<tr>
<td>Tenofovir</td>
<td>0.50166</td>
<td>1.53530</td>
</tr>
<tr>
<td>Rilpivirine</td>
<td>0.14900</td>
<td>0.14900</td>
</tr>
</tbody>
</table>

**CONCLUSION**
The proposed method gives a good resolution between three drugs Efavirenz, Lamivudine and Tenofovir disoproxil fumarate. The developed RP-HPLC method met the criteria of ICH guidelines for the method validation. The method requires no extraction procedure or sample pre-treatment, less costly solvents are used with good resolution is obtained. Therefore, this method is recommended for routine quality control analysis of investigated drugs to provide simple, accurate, less economic and reproducible quantitative analysis for the determination of Efavirenz, Lamivudine and Tenofovir disoproxil fumarate in anti-retroviral fixed dose combination.

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