A NEW STABILITY INDICATING ANALYTICAL METHOD DEVELOPMENT AND VALIDATION FOR THE QUANTITATIVE DETERMINATION OF EMTRICITABINE AND TENOFOVIR DISPROXIMAL FUMARATE BY RP-HPLC

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ABSTRACT
A novel stability indicating, precise, accurate and ecofriendly reverse phase high performance liquid chromatographic method was developed and validated for the quantitative determination of Emtricitabine and Tenofovir disproximal fumarate in pure and pharmaceutical dosage forms. Estimation of drugs in this combination was done with a C18 column [Kromasil C18 column. 5µm, 4.6×250 mm]using mobile phase of composition Methanol and phosphate buffer (40:60 v/v, pH 4). The flow rate was 1.0 ml/min and the effluents were monitored at 261 nm. The retention time of Tenofovir disoproxil fumarate and Emtricitabine were 2.810 min and 4.727 min respectively. The linearity was found to be 40-80µg/ml for Tenofovir and 40-80µg/ml for Emtricitabine. The stability parameters were evaluated by injecting the stressed sample and it was proved that there was no degradants. The established method was validated according to ICH guidelines.

KEYWORDS: Emtricitabine, Tenofovir disoproxil fumarate, RP-HPLC, Stability and Method validation.

INTRODUCTION
Emtricitabine is chemically designated as 5-fluoro-1-[(2R,5S)-2-(hydroxymethyl)-1,3-oxathiolan-5-yl]cytosine. Emtricitabine is a nucleoside analogue and reverse transcriptase inhibitor used in combination with other agents for treatment and prevention of human immunodeficiency virus (HIV) infection and the acquired immunodeficiency syndrome.
(AIDS). That is soluble in water, methanol, acetone, formaldehyde, and ethyl acetate. It is official drug in Martindale, Merck index and Indian Pharmacopeia 2007 and 2014.

Tenofovir disoproxil fumarate is chemically designated as (9-[(R)-2-[[bis[(isopropoxycarbonyl) oxy]methoxy]phosphinyl]methoxy]propyl] adenine fumarate 1:1, tenofovir DF) is an orally bioavailable ester prodrug of tenofovir (also known as PMPA), an acyclic nucleotide analog with activity in vitro against retroviruses, including HIV-1, HIV-2, and hepatitis B virus (HBV). That is soluble in methanol, acetone, acetonitrile, formaldehyde, slightly soluble in water and ethyl acetate. It is official drug in Martindale, Merck index and Indian Pharmacopeia 2007 and 2014.

After literature survey it was proved that there were few HPLC methods reported for the estimation of selected drugs of interest.[4,25] So in the present investigation we tried to establish a novel, stable and sensitive chromatographic method for the estimation of selected drugs.

MATERIALS AND METHODS

Equipment used
The chromatographic separation was performed on Agilent 1120 compact liquid chromatographic system integrated with a variable wavelength programmable UV detector and a Rheodyne injector equipped with 20µl fixed loop. A reverse phase C18 [Kromasil ODS UG 5 column, 250mm × 4.5mm] was used. Elico SL-210 double beam UV visible spectrophotometer and Axis AGN204-PO electronic balances were used for spectrophotometric determinations and weighing purposes respectively.

Reagents and chemicals
Pharmaceutical grade pure Tenofovir disoproxil fumarate and Emtricitabine gift samples were procured from Mylan Laboratories, Hyderabad. Marketed formulation Tablets with dose of 300mg of Tenofovir disoproxil fumarate and 200mg of Emtricitabine were procured from local market. (Mfd.by Emcure® Pharmaceuticals ltd). HPLC grade Methanol and Water were procured from Merck specialties private limited, Mumbai.

Chromatographic conditions
Kromasil C18 (2) column 5µm [250mm x 4.6mm] was used for the chromatographic separation at a detection wave length of 261 nm. Mobile phase of composition Methanol and
Phosphate buffer pH 4 in a ratio of 40:60 v/v was selected for elution and same mixture was used in the preparation of standard and sample solutions. Flow rate was adjusted to 1.0 ml/min and the injection volume was 20 µl.

**Preparation of Mobile phase**
Phosphate buffer pH 4 was prepared by dissolve 0.504gm of disodium hydrogen phosphate and 0.301gm of Potassium dihydrogen phosphate of HPLC grade water and adjusts the pH to 4.0 with glacial acetic acid and sufficient water was added to produce 100 ml filtered through 0.45 µm membrane filter and sonicated for 10 minutes.

**Preparation of Standard solutions**
25mg each of Tenofovir disoproxil fumarate and Emtricitabine were accurately weighed and transferred into two 25ml volumetric flasks, dissolved using mobile phase and the volume was made up with the same solvent to obtain primary stock solutions A (Tenofovir disoproxil fumarate) and B (Emtricitabine) of concentration 1000 µg/ml of each drug. From the primary stock solutions, 0.4ml and 0.4ml were pipette out from A and B respectively, transferred to a 10ml volumetric flask and the volume was made up with the mobile phase to obtain final concentrations of 40 µg/ml and 40 µg/ml of Tenofovir disoproxil fumarate and Emtricitabine respectively and this solution is (working stock solution A).

**Preparation of Sample Solution**
Twenty tablets of Tenofovir disoproxil fumarate and Emtricitabine were weighed and crushed. Tablet powder equivalent to 300mg of Tenofovir disoproxil fumarate and 200mg of Emtricitabine was weighed accurately and transferred to a 25ml volumetric flask. The content was dissolved with 10ml of mobile phase and then sonicated for 15min. The volume was made up with the mobile phase and filtered with 0.45 µm membrane filter and sonicated for 20min. 0.8ml of this solution was pipette out and transferred to a 10ml volumetric flask and the volume was made up with the mobile phase to obtain a concentration of 80 µg/ml of Tenofovir disoproxil fumarate and 80 µg/ml of Emtricitabine (working stock solution B).

**Optimization of RP-HPLC method**
The HPLC method was optimized with an aim to develop a simultaneous estimation procedure for the assay of Tenofovir disoproxil fumarate and Emtricitabine. For the method optimization, different mobile phases were tried, but acceptable retention times, theoretical
plates and good resolution were observed with Methanol, Phosphate buffer pH 4 (40:60 v/v) using Kromasil C18 (2) column 5µm [250mm x 4.6mm].

Validation of the RP-HPLC method
Validation of the optimized method was performed according to the ICH Q2 (B) guidelines.

System suitability
System suitability was carried out with five injections of solution of 100% concentration having 80µg/ml of Tenofovir disoproxil fumarate and 80µg/ml of Emtricitabine in to the chromatographic system. Number of theoretical plates (N) obtained and calculated tailing factors (T) were reported in table 1.

Linearity
For the determination of linearity, appropriate aliquots were pipette out from working stock solution A to a series of 10ml volumetric flasks and volume was made up with the solvent to obtain concentration ranging from 40-80µg/ml of Tenofovir disoproxil fumarate and 40-80µg/ml of Emtricitabine. Each solution was injected in triplicate. Calibration curves were plotted with observed peak areas against concentration followed by the determination of regression equations and calculation of the correlation coefficients. The calibration curves for Tenofovir disoproxil fumarate and Emtricitabine were shown in figure 3 and figure 4 their corresponding linearity parameters were given in table 2.

Limit of Detection (LOD) and Limit of Quantitation (LOQ)
The LOD and LOQ were calculated from the slope(s) of the calibration plot and the standard deviation (SD) of the peak areas using the formulae LOD = 3.3 σ/s and LOQ = 10 σ/s. The results were given in table 2.

Precision
The repeatability of the method was verified by calculating the %RSD of six replicate injections of 100% concentration (80µg/ml of Tenofovir disoproxil fumarate and 80µg/ml of Emtricitabine) on the same day and for intermediate precision % RSD was calculated from repeated studies on different days. The results were given in table 3.
Accuracy
To ensure the reliability and accuracy of the method recovery studies were carried out by standard addition method. A known quantity of pure drug was added to pre-analyzed sample and contents were reanalyzed by the proposed method and the percent recovery was reported. The results were given in table 4.

Specificity
Specificity of a method was determined by testing standard substances against potential interferences. The method was found to be specific when the test solution was injected and no interferences were found because of the presence of excipients. The optimized chromatogram of Tenofovir disoproxil fumarate and Emtricitabine without any interference was shown in figure 2.

Robustness
Robustness of the method was verified by altering the chromatographic conditions like mobile phase composition, wavelength detection, flow rate, etc. and the % RSD should be reported. Small changes in the operational conditions were allowed and the extent to which the method was robust was determined. A deviation of ±2nm in the detection wave length and ±0.2ml/min in the flow rate, were tried individually. A solution of 100% test concentration with the specified changes in the operational conditions was injected to the instrument in triplicate. %RSD was reported in the table 5.

Assay of Marketed Formulations
20μl of sample solution of concentration 80μg/ml of Tenofovir disoproxil fumarate and 80μg/ml of Emtricitabine was injected into chromatographic system and the peak responses were measured. The solution was injected three times in to the column. The amount of drug present and percentage purity was calculated by comparing the peak areas of the standards with that of test samples. A typical chromatogram for assay of marketed formulation was shown in figure 5 and the obtained values were reported in the table 6.

Stability Studies
Acid degradation studies
Prepared each 1mg/ml stock solution of Tenofovir disoproxil fumarate and Emtricitabine by using mobile phase as solvent, and then filtered through 0.45μm membrane filter paper. Stock solutions of 0.8 ml and 0.8ml of Tenofovir disoproxil fumarate and Emtricitabine stock
solution was transferred into 10ml volumetric flask and added 1 ml of 0.1N HCL and diluted to volume with mobile phase. The resultant solution was injected into the system; there was no acid degradation products were found. The obtained chromatogram was shown in figure 6.

Alkaline degradation studies
Prepared each 1mg/ml of stock solution with Tenofovir disoproxil fumarate and Emtricitabine then filtered through 0.45μm membrane filter paper. Stock solutions of 0.8ml and 0.8ml of Tenofovir disoproxil fumarate and Emtricitabine stock solution was transferred into 10ml volumetric flask and added 1 ml of 0.1N NaOH and diluted to volume with mobile phase. The obtained non interfered chromatogram was represented in figure 7.

Oxide degradation studies
Prepared each 1mg/ml of stock solution of Tenofovir disoproxil fumarate and Emtricitabine then filtered through 0.45µm membrane filter paper. Stock solutions of 0.8ml and 0.8ml of Tenofovir disoproxil fumarate and Emtricitabine stock solution was transferred into 10ml volumetric flask and added 1 ml of H2O2 and diluted to volume with mobile phase. In this investigation no identifiable oxidative degradants were found and the chromatogram was shown in figure 8.

Thermal degradation studies
Prepared each 1mg/ml of stock solution with Tenofovir disoproxil fumarate and Emtricitabine and then filtered through 0.45μm membrane filter paper. Stock solutions of 0.8 ml and 0.8 ml of Tenofovir disoproxil fumarate and Emtricitabine 10ml volumetric flask and diluted to volume with mobile phase and kept for 60min at 60℃ in hot air oven. From the obtained chromatogram it was proved that the selected samples were stable against thermal conditions. The chromatogram was shown in figure 8.

RESULTS AND DISCUSSION
After a number of trials with mobile phases of different composition, Methanol, Phosphate buffer pH 4.0 in the ratio 40:60v/v was selected as mobile phase because of better resolution and symmetric peaks. Tenofovir disoproxil fumarate and Emtricitabine were found to show appreciable absorbance at 261nm when determined spectrophotometrically and hence it was selected as the detection wavelength. An optimized chromatogram showing the separation of Tenofovir disoproxil fumarate and Emtricitabine at different Rf's was shown in figure 2.
System suitability was carried out by injecting 5 replicate injections of 100% test concentration, number of theoretical plates, HETP and resolution were satisfactory. The chromatograms confirm the presence of Tenofovir disoproxil fumarate and Emtricitabine at 2.8min and 4.7min respectively without any interference. The parameters were given in table 1.

Concentration range of 40-80µg/ml for Tenofovir disoproxil fumarate and 40-80µg/ml of Emtricitabine were found to be linear with correlation coefficients 0.999 and 0.999 for Tenofovir disoproxil fumarate and Emtricitabine respectively. The results were given in table 2.

The limits of detection for Tenofovir disoproxil fumarate and Emtricitabine were found to be 1.1µg/ml and 3.63µg/ml respectively and the limit of Quantitation were 2.7µg/ml and 8.91µg/ml respectively. Values were represented in table 2.

The proposed method was found to be precise and reproducible with %RSD of 0.65 and 0.9 for Tenofovir disoproxil fumarate and Emtricitabine respectively. %RSD was reported in table 3.

Accuracy of the method was verified by performing recovery studies by standard addition method. The percent recovery of the standard added to the pre-analysed sample was calculated and it was found to be 98.2% for Tenofovir disoproxil fumarate and 98.3% for Emtricitabine. This indicates that the method was accurate. Values obtained were given in table 4.

The method was found to be robust after changing the conditions like detection wavelength (± 2nm) and flow rate (± 0.2 ml). %RSD was calculated for each variation and reported. Values obtained were given in table 5.

The method was found to be specific for the combination of interest after verifying the chromatograms showing no interference of the excipients present. Hence, the method was well suitable for the estimation of the commercial formulations of the selected combination with a percentage purity of 98.1% for Tenofovir disoproxil fumarate and 98.3% for Emtricitabine. The typical chromatogram for assay of marketed formulations was shown in figure 5 and Values obtained were given in table 6.
Forced Degradation Study
Degradation studies indicated the specificity of developed method in presence of degradation products. Degradation was carried out in combination of two drugs and purity of drug peaks was confirmed by purity angles. Their combination drug products were exposed to acid, base, oxidative and thermal stress conditions. Then found to be no degradable substances presence and proved that the proposed method was stable towards acid, alkali, peroxide and thermal conditions. The obtained values were reported in table 7.

Figures and Tables

![Chemical Structures](image)

a. Tenofovir disoproxil fumarate

b. Emtricitabine

Fig. 1: Chemical Structures of a) Tenofovir disoproxil fumarate and b) Emtricitabine.
Fig. 2: Optimized chromatogram of Tenofovir disoproxil fumarate and Emtricitabine.

Fig. 3: Calibration plot of Tenofovir disoproxil fumarate.

Fig. 4: Calibration plot of Emtricitabine.
Figure 5: Chromatogram for assay of marketed formulation.

Figure 6: Chromatogram of acid degradation.

Figure 7: Chromatogram of alkaline degradation.

Figure 8: Chromatogram of Hydrogen peroxide degradation.
Figure 9: Chromatogram of thermal degradation.

### Table 1: System Suitability Parameters.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Tenofovir disoproxil fumarate</th>
<th>Emtricitabine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Retention time (min)</td>
<td>2.8</td>
<td>4.7</td>
</tr>
<tr>
<td>Theoretical plates (N)</td>
<td>8596</td>
<td>9542</td>
</tr>
<tr>
<td>Tailing factor (T)</td>
<td>1.2</td>
<td>1.1</td>
</tr>
<tr>
<td>Resolution ($R_s$)</td>
<td>1.9</td>
<td></td>
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### Table 2: Results for Linearity.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Tenofovir disoproxil fumarate</th>
<th>Emtricitabine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Slope</td>
<td>28995</td>
<td>45641</td>
</tr>
<tr>
<td>y intercept</td>
<td>13581</td>
<td>15870</td>
</tr>
<tr>
<td>Correlation coefficient $r^2$</td>
<td>0.999</td>
<td>0.999</td>
</tr>
<tr>
<td>Regression Equation</td>
<td>$y = 28995x + 13581$</td>
<td>$y = 45641x + 15870$</td>
</tr>
<tr>
<td>Linearity range</td>
<td>40-80 μg/ml</td>
<td>40-80 μg/ml</td>
</tr>
<tr>
<td>LOD</td>
<td>1.1 μg/ml</td>
<td>2.7 μg/ml</td>
</tr>
<tr>
<td>LOQ</td>
<td>3.63 μg/ml</td>
<td>8.91 μg/ml</td>
</tr>
</tbody>
</table>

### Table 3: Results of Precision.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Intraday Precision (%RSD)</th>
<th>Interday Precision (%RSD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tenofovir disoproxil fumarate</td>
<td>1.12</td>
<td>0.65</td>
</tr>
<tr>
<td>Emtricitabine</td>
<td>0.97</td>
<td>0.9</td>
</tr>
</tbody>
</table>

### Table 4: Results for Accuracy.

<table>
<thead>
<tr>
<th>Recovery level</th>
<th>Tenofovir disoproxil fumarate</th>
<th>Emtricitabine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Amount Added (µg/ml)</td>
<td>Amount Found (µg/ml)</td>
</tr>
<tr>
<td></td>
<td>std</td>
<td>test</td>
</tr>
<tr>
<td>50%</td>
<td>10</td>
<td>20</td>
</tr>
<tr>
<td>100%</td>
<td>40</td>
<td>20</td>
</tr>
<tr>
<td>150%</td>
<td>70</td>
<td>20</td>
</tr>
<tr>
<td>Mean recovery</td>
<td>98.2%</td>
<td></td>
</tr>
</tbody>
</table>
Table 5: Results for Robustness.

<table>
<thead>
<tr>
<th>Parameters (n=3)</th>
<th>Tenofovir disoproxil fumarate</th>
<th>Emtricitabine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Detection wavelength at 263nm</td>
<td>0.31</td>
<td>0.39</td>
</tr>
<tr>
<td>Detection wavelength at 259nm</td>
<td>0.25</td>
<td>0.64</td>
</tr>
<tr>
<td>Flow rate 0.8ml/min</td>
<td>0.74</td>
<td>0.85</td>
</tr>
<tr>
<td>Flow rate 1.2ml/min</td>
<td>0.89</td>
<td>0.99</td>
</tr>
</tbody>
</table>

Table 6: Results for Assay of Marketed formulation.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Label claim (mg/tab)</th>
<th>Amount recovered</th>
<th>% Amount found in drug</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tenofovir disoproxil fumarate</td>
<td>300</td>
<td>294.5</td>
<td>98.1%</td>
</tr>
<tr>
<td>Emtricitabine</td>
<td>200</td>
<td>196.7</td>
<td>98.3%</td>
</tr>
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</table>

Table 7: Results for Stability studies.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>% of degradation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tenofovir disoproxil fumarate</td>
</tr>
<tr>
<td>Acid degradation</td>
<td>0.21</td>
</tr>
<tr>
<td>Alkali degradation</td>
<td>0.5</td>
</tr>
<tr>
<td>Peroxide degradation</td>
<td>0.1</td>
</tr>
<tr>
<td>Thermal degradation</td>
<td>0.32</td>
</tr>
</tbody>
</table>

CONCLUSION

The proposed liquid chromatographic method allows a specific and rapid quantitative estimation of Emtricitabine and Tenofovir disoproxil fumarate in bulk and marketed formulations. It was proved that all the validation parameters were fall in acceptance limits as per ICH guidelines. The established and validated method was proved to be sensitive and selective for the determination of drugs of present investigation. This was a most accurate, specific, precise and stable under different degradation conditions. Hence it can be utilized in the routine estimations of the dosage forms.

ACKNOWLEDGMENTS

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REFERENCES


