Method Development for Quantification of (S) - Isomer in Tenofovir Disoproxil Fumarate Bulk Drug and Formulations using Meta Substituted Phenyl Carbamate of Amylose as Chiral Selector

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INTRODUCTION

The TDF is a fumarate of bis-iso-propoxy-carbonyloxy methyl ester derivative with IUPAC name 9-[(R)-2-[[bis [[isopropoxy-carbonyl] oxy] methyl] phosphinyl] propyl] adenine fumarate (Fig. 1). TDF is an orally available prodrug of tenofovir that is readily converted intracellularly to the diphosphate with greater plasma stability. The diphosphate halts the DNA synthesis of HIV via aggressive inhibition of reverse transcriptase and integration into viral DNA. It also retards the hepatitis B virus polymerase, thus inhibiting viral replication. Tenofovir has in vitro activity against HIV-1, HIV-2, and human hepatitis B virus.

Literature survey revealed that several techniques for determination of achiral impurity in formulations of TDF, HPLC coupled with fluorescence and UV detection, LCMS for plasma and pharmacokinetic...
syndromes,[14-16] and high-performance thin-layer liquid chromatography (HPTLCs).[17]

TDF has one asymmetric carbon giving two enantiomers, one being pharmacologically active R (+) isomer, while the other S (-) isomers being inactive, which might exert unwanted side effects. Hence, it is vital to develop separation methods to check the chiral purity of S (-) isomer in the drug and the final product of the drug. In order to quantify the enantiomer composition, few analytical methods were reported.[18-20] These methods were time-consuming with complex sample preparation techniques, including liquid chromatographic technique with chiral derivatizing reagents or traditional 5-micron column with extended run time.

Hence, there is a need to develop a quick and simple separation method for chiral purity control of S (-) isomer in bulk and formulations of TDF. The current research aimed to develop a chiral method for enantiomeric parting and estimating S (-) isomer in TDF using ultra-performance liquid chromatography (UPLC). This technique has minimal drawbacks from high column back-pressure compared to the same separations obtained with traditional HPLC instruments. With minimal void volumes and maximal sensitivity, faster separations can be achieved with higher efficiencies. It provides apt resolution with sharp peak shapes using low particle size columns and detects the low level of chiral impurities providing ready access for validation using compliance software.[21,22] The developed method was novel and unreported in the literature.

**Experimental**

**Chemicals Used**

TDF and S (-) isomer were procured from OTC. HPLC-grade Hexane, Diethyl amine, and ethyl alcohol were procured from Merck, India. All other chemicals were of analytical grade.

**Instrumentation and Chromatographic Conditions**

Waters Acquity UPLC instrument equipped with a binary solvent manager pump where Pump-A is used for Hexane and Pump-B is used as an organic solvent manager with four-line options, an auto sampler, a column manager with 4 column switch-over valve, and PDA detector operated with Empower-3 software. The enantiomeric separation was performed using ChiralpakIG-3 column (100 x 2.1 x 3.0). 0.5% Diethyl amine in Hexane and Ethanol (75:25 v/v) is used as a mobile phase. The column was maintained at 30˚C with a flow rate of 0.5 mL.min⁻¹, injection volume of 2.0 µL, and wave length of 259 nm. Ethanol was employed as diluent. The system allowed equilibrating using the mobile phase for 30 minutes before first injection. The method development, validation, and stress degradation samples were analyzed and reported using empower-3 software.

**Stock and Working Standard Solution Preparation Methods**

Racemic mixture of TDFR (+) and S (-) isomers (0.5 mg mL⁻¹ each) was prepared using diluent. The stock solutions of TDF R (+) isomer (10 mg mL⁻¹) and S (-) isomer (0.5 mg mL⁻¹) were prepared by diluting known quantities of isomer with diluents.

The concentration of analyte comprising R (+) isomer was maintained at 1.0 mg mL⁻¹. TDF and R (+) isomer solutions spiked with lower levels of S (-) isomer were prepared by mixing equivalent amount of S (-) isomer stock solution with known amounts of TDF R (+) stock solution, followed by dilution with diluent. Working standard solutions for carrying out validation were prepared by weighing accurately 50 mg of TDF (R isomer) into 50 mL standard flask and added 0.5 mL of S(-) isomer stock solution (1.0 mg mL⁻¹ of TDF-R isomer + 0.005 mg mL⁻¹ of TDF-S isomer) to give the final solution containing 0.5% of S Isomer in test concentration of R’ isomer.

**Specificity and Forced Degradation Studies (FDS)**

Specificity of the test method was evaluated by analyzing standard substances against potential interferences. The isomer samples and racemic mixture were injected individually. The specificity of the method was evaluated in the presence of enantiomer and degradants.

The stress conditions studied include acidic hydrolysis (0.5 N HCl), basic hydrolysis (0.5 N NaOH), peroxide oxidation (3% H2O2), thermal (60˚C), and photo light (254 nm). The study period for heat and light studies was 3 days, whereas, for acid, base, and peroxide, the test period was 4h. Peak purity of degradation samples of TDF was inspected using a photo diode array detector. TDF assays were performed by comparison with standard, and mass balance (%assay + %impurities + %DPs) were calculated for stress samples. The degradation samples were injected into a UPLC instrument equipped with a PDA detector to check the peak purity and homogeneity across the peak.

**Method Validation**

The developed method was validated by determining and quantifying the TDF (R) isomer in the presence of (S) isomer according ICH guidelines.

**System Suitability**

The system suitability test was performed throughout the validation studies by injecting 1.0 mg mL⁻¹ of TDF-R-isomer solution spiked with 0.005 mg mL⁻¹ (0.5%) of TDF-S-isomer. Resolution between the two isomers was checked on each parameter and each day of the validation.

**Precision**

The repeatability of the method analyzed by injecting six individual test preparations of TDF-R (+) isomer
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(1.0 mg.mL\(^{-1}\)) spiked with 0.5% of TDF S (-) isomer (0.005 mg.mL\(^{-1}\)). The intermediate precision was evaluated with same concentration solutions as used for method precision, but prepared separately on a different day by different analysts. This was checked for two days with two different analysts. Precision at LOQ levels was determined by injecting six preparations of R & S-Isomers at a concentration of LOQ level. The %RSD of areas of each isomer and resolution between two isomers were calculated for the precision studies.

**Limit of Quantification and Detection (LOQ & LOD)**

The signal defines the LOQ to noise ratio (S/N) that should be ≥ 10 for S (-) isomer. The LOD was identified by evaluating S/N ratio for diluted solutions with the criteria of S/N ≥ 3.0 for S (-) isomer. These values were evaluated by analyzing a series of diluted samples with known concentrations.

**Linearity**

Linearity of the proposed chiral method was established by analyzing series of 6 calibration samples of TDF S (-) enantiomer ranging from LOQ - 150% at specification level(0.5%) at different concentration ranges 0.25 µg.mL\(^{-1}\) (LOQ) - 7.5 µg. mL\(^{-1}\) (0.25, 2.5, 3.75, 5.0, 6.25, and 7.5 µg. mL\(^{-1}\)) and TDF R (+) isomer were prepared from 100 - 1500 µg.mL\(^{-1}\). The regression curve was plotted with peak area vs concentration, using the least square technique. The correlation coefficient (r\(^2\)), slope and Y-intercept of S (-) isomer was analyzed from each calibration plot.

**Accuracy**

This was determined by spiking known concentration of TDF S (-) isomer to TDF sample. The accuracy was evaluated as % recovery. The study was conducted in triplicates at 50, 100, and 150% (2.5, 5.0 and 7.5 µg.mL\(^{-1}\)) and % recovery of (S)-isomer evaluated.

**Robustness**

The robustness study was carried out to check the influence of small variations on the system suitability criteria in the optimized chromatographic conditions. The factors chosen for this study are flow rate (± 0.1 mL/min), Mobile phase composition of organic phase concentration (± 10.0%), and temperature variation (± 5°C). In all the above-mentioned conditions, the other parameters were kept constant. Standard solution of TDF R-Isomer (1.0 mg.mL\(^{-1}\)) spiked with 0.5% of TDF S-isomer (0.005 mg.mL\(^{-1}\)) was injected for six times in all the above-modified conditions and checked for resolution, retention, and %RSD for both the isomers.

**Solution and Mobile Phase Stability**

Solution stability of TDF was established by storing the solution(in capped container at room temperature and a standard solution of TDF R (+) isomer(1.0 mg.mL\(^{-1}\)) spiked with 0.5% S (-) isomer(0.005mg.mL\(^{-1}\)) for 72 hours. The impurity content and system suitability were checked. The mobile phase stability was studied by evaluation of the content of spiked S (-) isomer in TDF R (+) isomer solutions prepared freshly and analyzed at different time intervals (0, 24 and 48 hours), keeping mobile phase as constant.

**RESULTS**

**Method Development and Optimization**

In order to separate two enantiomers of TDF, a significant number of methods screenings were performed based on compound structure. During initial developments, different percentages of alcohol modifiers as mobile phase, various chiral stationary phases (CSPs) containing amylose and cellulose derivatives, and different flow rates were examined. Initial screening with mentioned parameters was carried out to determine the best suitable column and a mobile phase that provides an accurate method for routine analysis in the laboratory.

The separation of enantiomers was screened using six different columns, including Chiralpak IC-3, IG-3, IA-3) and Chiralcel (OD-3, OJ-3, OX-3). These columns were operated with hexane and ethanol with various ratios of solvents and additives.

Initial screening concluded that Chiralcel(OD-3, OJ-3, OX-3) and Chiralpak IC-3 could not generate desirable peak separation. Separation was observed on Chiralpak IA-3, but with resolution< 2.5. Hence Chiralpak IG-3 that provided good resolution with better peak shape, was chosen as a column. The parameters like the retention factor (k), the separation factor (βt), and resolution (Rs) were considered to analyze the separation of enantiomers. The Chiralpak IG-3 column demonstrated excellent resolution (>10) between the two enantiomers with the mobile phase consisting of hexane & ethanol with 0.5% Diethanolamine in 75:25 (%/v/v) ratio. The detection wavelength was 259 nm, and flow rate was maintained at 0.5 mL.min\(^{-1}\) and at column temperature of 30°C.

Under these conditions, the retention time of TDF and S (-) isomer were 1.35 and 2.65 minutes, respectively. Baseline separation of TDF and S (-) isomer was generated at a run time of 6 min. The final optimized method was applied to separate S (-) isomer from TDF and was proven reproducible and accurate. The separation of the racemic mixture, TDF drug, and TDF API spiked with 0.5% of S isomer was shown in Fig. 2.

**Method Validation**

**Specificity and FDS**

TDF- R (+) and S (-) isomers were injected separately to confirm the retention times. Racemic mixture was then injected. The specificity results are captured in Table 1.
During forced degradation studies TDF sample was exposed to 0.5N HCl, 3% H$_2$O$_2$, 0.5N NaOH at 60˚C with continuous constant stirring for 4 hours. The TDF R-isomer showed no degradation under thermal and UV conditions, while significant degradation was observed with 0.5 N HCl, 3% H$_2$O$_2$ and 0.5 N NaOH. Representative chromatograms for the degradation samples were shown in Fig. 3. The major degradation product was 55% at 1.54 minutes. Three degradants were observed at 1.0, 1.53, and 1.65 minutes, and the % assay of TDF peak in solution was 34%.

In acidic conditions, around 7% of degradants were observed. The primary impurity was about 5.0% at 0.95 minutes, and the wt% of TDF was 91.5%. Under peroxide stress conditions, 14.6% degradation was observed. All these degradation impurities were well separated from TDF peak, and the peak purity has been evaluated to check for any interference. TDF-S isomer was spiked in stressed samples, analyzed for peak purity, and confirmed that R and S isomer peaks were homogeneous and spectrally pure. It also showed that no degradants was co-eluted with the R & S enantiomer peaks of TDF, and also S (-) isomer was not observed in any stressed samples. The developed chiral technique was specific in the presence of degradants with S (-) isomer. The mass balance of all stress samples was close to 100.0%, indicating no other by-products formed during degradation apart from DP impurities (Table 2).

**System Suitability**

System suitability parameters were evaluated for both TDF (R) isomer, and the spiked (S) isomer indicated that the system was suitable for use. The tailing factor for the two isomers was less than 1.5, and the resolution between the isomers was 10.8. The USP plate count for two peaks was greater than 4000. These values were set as acceptance criteria for all the remaining studies.

**Precision**

The % RSD in repeatability of TDF (R) and spiked (S) isomer was 0.34 and 1.95, respectively. The % RSD for the method precision for TDF- R and S isomers were 0.27 and 2.71, respectively. The % RSD of two isomers obtained in the intermediate precision for two analysts on days 1 and 2 were between 0.32 and 3.70, respectively.

![Chiral SFC Chromatograms of TDF](image1)

**Fig. 2:** Chiral SFC Chromatograms of TDF (a) Racemic mixture of R&S isomers (b) TDF R isomer (API as such) (c) Spiking of 0.5% of TDF ‘S’ enantiomer in to the TDF ‘R’ enantiomer.

![Chromatograms of TDF degradation samples](image2)

**Fig. 3:** Chromatograms of TDF degradation samples: (a) As such (b) Acid stress sample (c) Base stress sample (d) Peroxide stress sample.

**Table 1:** Specificity Results

<table>
<thead>
<tr>
<th>Compound</th>
<th>RT</th>
<th>RRT</th>
<th>Purity angle</th>
<th>Purity threshold</th>
<th>Peak purity</th>
</tr>
</thead>
<tbody>
<tr>
<td>TDF R (+) enantiomer</td>
<td>1.4</td>
<td>1.00</td>
<td>0.62</td>
<td>3.29</td>
<td>Pass</td>
</tr>
<tr>
<td>TDF S (-) enantiomer</td>
<td>2.6</td>
<td>1.94</td>
<td>0.79</td>
<td>8.11</td>
<td>Pass</td>
</tr>
</tbody>
</table>
The repeatability at LOQ level was estimated by analyzing six samples of TDF 'R'&'S' isomers separately, and the %RSD was found to be 3.72 and 3.27, respectively. All the values were tabulated and presented in Table 3. The % RSD values for the repeatability and intermediate variations were ≤3.0, confirming that method was precise.

The LOD and LOQ
The detection and quantitation limits of S (-) enantiomer was 0.08 µg. mL⁻¹ and 0.25 µg. mL⁻¹ respectively as tabulated in Table 4. The LOD and LOQ for TDF R (+) isomer were not reported as it exists as a major component in real samples. This method was adequately sensitive towards detection and estimation of S (-) isomer in TDF.

**Linearity**
Linearity was evaluated by preparing six calibration sample solutions ranging between 0.25 µg. mL⁻¹ to 7.5 µg. mL⁻¹ for S (-) enantiomer and from 100 µg.mL⁻¹ to 1500 µg. mL⁻¹ for TDF R (+) isomer. The R² value was > 0.999 for both the enantiomers. These results indicate an excellent correlation between peak area and concentration, and described method is linear. The linearity is shown in Table 4.

**Accuracy**
The evaluation was carried out for S (-) isomer in bulk samples in triplicate between 2.5 µg.mL⁻¹ to 7.5 µg.mL⁻¹ (50%, 100%, and 150% of S (-) enantiomer specification level concerning test concentration). The %recovery was analyzed at every level in each sample. The recovery was >98.1% and not more than 100.1% (Table 4).

**Solution and Mobile Phase Stabilities**
No significant variations were observed for (S)-isomer in a spiked sample of TDF during stability study, %RSD was < 2.0 for (S)-enantiomer. No unknown peaks were observed in storage conditions hence found stable upto 72 h (Table-4).

**Robustness**
The robustness of this procedure was assessed by evaluating its ability to remain unaffected by deliberate variations such as organic modifier, flow rate and temperature. In all the modified conditions, the system suitability results (%RSD, Resolution and Retention) were in the acceptable range, indicating the proposed method's reliability (Table 5).

**Discussion**
The present work established the chiral UPLC method for the first time to determine the enantiomer separation.

### Table 2: Forced degradation results.

| Parameter & condition | Purity (%Area) | % of degradation | Purity angle | Purity threshold | Assay (Weight %) | Mass balance
<table>
<thead>
<tr>
<th></th>
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<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Acid (0.5N HCl) 60°C; 4 Hrs.</td>
<td>92.87</td>
<td>7.13</td>
<td>0.13</td>
<td>0.33</td>
<td>91.47</td>
<td>98.6</td>
</tr>
<tr>
<td>Base (0.5N NaOH) 60°C; 4 Hrs.</td>
<td>33.66</td>
<td>66.34</td>
<td>0.25</td>
<td>0.75</td>
<td>34.49</td>
<td>100.83</td>
</tr>
<tr>
<td>Peroxide (3%H2O2) 60°C; 4 Hrs.</td>
<td>85.38</td>
<td>14.62</td>
<td>0.13</td>
<td>0.32</td>
<td>83.15</td>
<td>97.77</td>
</tr>
<tr>
<td>Thermal 60°C; 4 days</td>
<td>100</td>
<td>ND</td>
<td>0.73</td>
<td>1.56</td>
<td>99.74</td>
<td>99.74</td>
</tr>
<tr>
<td>Photolytic (UV) 254nm; 4 days</td>
<td>100</td>
<td>ND</td>
<td>1.14</td>
<td>1.98</td>
<td>99.06</td>
<td>99.06</td>
</tr>
</tbody>
</table>

Table 3: Precision results

<table>
<thead>
<tr>
<th>Compound</th>
<th>System Precision</th>
<th>Method precision</th>
<th>LOQ precision</th>
<th>Intermediate precision</th>
</tr>
</thead>
<tbody>
<tr>
<td>TDF R (+) enantiomer</td>
<td>0.34</td>
<td>0.27</td>
<td>2.72</td>
<td>0.31</td>
</tr>
<tr>
<td>TDF S (-) enantiomer</td>
<td>1.95</td>
<td>2.71</td>
<td>2.27</td>
<td>2.90</td>
</tr>
</tbody>
</table>

Table 4: Method validation results

<table>
<thead>
<tr>
<th>Parameter</th>
<th>TDF (R isomer)</th>
<th>TDF (S isomer)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LOD(µg mL⁻¹)</td>
<td>0.08</td>
<td>0.08</td>
</tr>
<tr>
<td>LOQ(µg mL⁻¹)</td>
<td>0.25</td>
<td>0.25</td>
</tr>
<tr>
<td>Slope</td>
<td>3204.65</td>
<td>7943.93</td>
</tr>
<tr>
<td>Intercept</td>
<td>411369</td>
<td>3686.11</td>
</tr>
<tr>
<td>Correlation coefficient</td>
<td>0.999</td>
<td>0.998</td>
</tr>
<tr>
<td>Linearity range(µg mL⁻¹)</td>
<td>0.25–1500ᵃ</td>
<td>0.25–7.5ᵇ</td>
</tr>
<tr>
<td>% Recovery Amount spiked(%)</td>
<td>98.1</td>
<td>98.7</td>
</tr>
<tr>
<td>Solution stability %RSD</td>
<td>0.21</td>
<td>2.50</td>
</tr>
<tr>
<td>Resolution</td>
<td>10.42</td>
<td>10.65</td>
</tr>
<tr>
<td>Mobile phase stability %RSD</td>
<td>0.50</td>
<td>2.63</td>
</tr>
<tr>
<td>Resolution</td>
<td>10.65</td>
<td>10.52</td>
</tr>
</tbody>
</table>

ᵃValues obtained concerning target concentration of 1000 µg mL⁻¹ of TDF-R isomer
ᵇValues obtained concerning impurity concentration of 5.0 µg mL⁻¹ of TDF-S isomer
ᶜAmount of S-isomer spiked concerning 0.5% specification level individually to 1000 µg mL⁻¹ of TDF-R isomer.
and quantification of (R) TDF in the presence of other isomer (S) TDF. The developed chiral method showed the separation of unwanted enantiomer and degradation impurities from the peaks of interest (TDF-R isomer), ensuring the selectivity and accuracy of the detection. This method was able to detect all the degradation impurities. The proposed method was found chiral with good resolution and short run time (6 minutes). This method can be applied and used in process development, stability analysis, and quality control analysis of TDF bulk drugs and formulations for detecting stereoisomers and other possible degradation products. This enantiomer separation by UPLC is time and cost-effective, and environmentally eco-friendly than normal phase liquid chromatographic methods with longer run time.

REFERENCES


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