Original Article

# RP-HPLC method for simultaneous estimation of tenofovir disoproxil fumarate, lamivudine, and efavirenz in combined tablet dosage form

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*Abstract*

**Background:** A simple, precise, accurate, and rapid reverse phase-high performance liquid chromatography (RP-HPLC) method with UV-Visible detector has been developed and subsequently validated for the simultaneous determination of tenofovir disoproxil fumarate (TDF), lamivudine (LAMI), and efavirenz (EFV) in their combined tablet dosage form. **Materials and Methods:** The separation was based on the use of a Kromasil $C_{18}$ analytical column (150 × 4.6 mm, i.d., 5 µm). The mobile phase consisted of a mixture of 70 volumes of methanol and 30 volumes of 10 mM phosphate buffer (pH 5.0). The separation was carried out at 40°C temperature with a flow rate of 1 ml/min. **Results:** Quantitation was achieved with UV detection at 254 nm, with linear calibration curves at concentration ranges of 1–6 µg/ml for TDF and LAMI and 2–12 µg/ml for EFV. The recoveries obtained were 99.46–101.36% for LAMI, 99.57–101.42% for TDF, and 99.96–100.87 for EFV. **Conclusion:** The method was validated according to International conference of harmonisation guidelines in terms of accuracy, precision, specificity, robustness, limits of detection and quantitation, and other aspects of analytical validation.

**Key words:** Efavirenz, lamivudine, RP-HPLC, tenofovir disoproxil fumarate, validation

## INTRODUCTION

Tenofovir disoproxil fumarate (TDF) {9-[(*R*)-2-[[bis [[isopropoxycarbonyl] oxy] methoxy] phosphonyl] methoxy] popyl] adenine fumarate} [Figure 1] is a nucleotide analog reverse transcriptase inhibitor (NRTI) and is used for treating HIV infection in adults, in combination with other antiretroviral agents. Lamivudine (LAMI) {4-amino-1-[(2*R*,5*S*)-2-(hydroxyl methyl)-1,3-oxathiolan-5-yl]-1,2-dihydro pyrimidin-2-one} [Figure 2] is an NRTI used in the treatment of HIV infection and chronic hepatitis B virus (HBV). Efavirenz (EFV) [(4*S*)-6-chloro-4-(cyclopropylethynyl)-1,4-dihydro-4-(trifluoromethyl)-2H-3,1-benzoxazin-2-one] [Figure 3] is a non-nucleotide reverse transcriptase inhibitor (NNRTI) used in the combination treatment of HIV infection (AIDS).[1-6] The combination of TDF, LAMI, and EFV (300 mg TDF, 300 mg LAMI, and 600 mg EFV) was approved in July 2008 by Central Drug Standard Control Organization (CDSCO) and tentatively approved by US Food and Drug Administration (USFDA) on 9 March 2009 for the treatment of HIV infection in adults.[7,8] Literature survey reveals that TDF is estimated individually by titrimetric, UV, reverse phase-high performance liquid chromatography (RP-HPLC) in tablet formulation and by RP-HPLC methods in human plasma.[9-14] Few UV, RP-HPLC, high performance thin layer chromatography (HPTLC), and liquid chromatography with tandem mass spectrometry (LC/MS/MS) methods have been reported for simultaneous estimation of emtricitabine and TDF in pharmaceutical formulation.[15-18] Similarly, estimation of LAMI by titrimetric, UV, HPTLC, RP-HPLC methods in tablet formulation and by RP-HPLC method in human plasma has been reported.[19-23] Few UV, RP-HPLC, HPTLC, and LC/MS/MS methods have been reported for the simultaneous estimation of LAMI with nevirapine, stavudine,

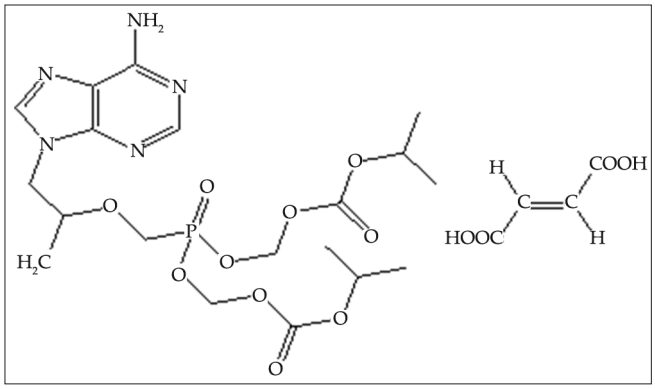

**Figure 1:** Chemical structure of tenofovir disoproxil fumarate

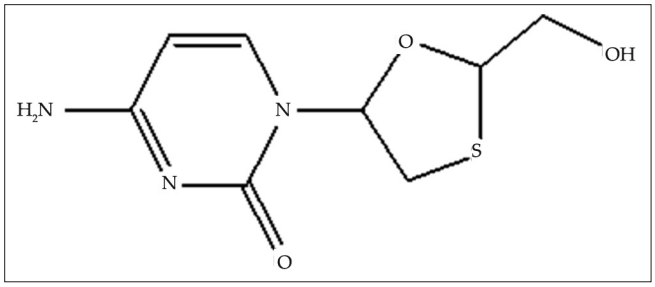

**Figure 2:** Chemical structure of lamivudine

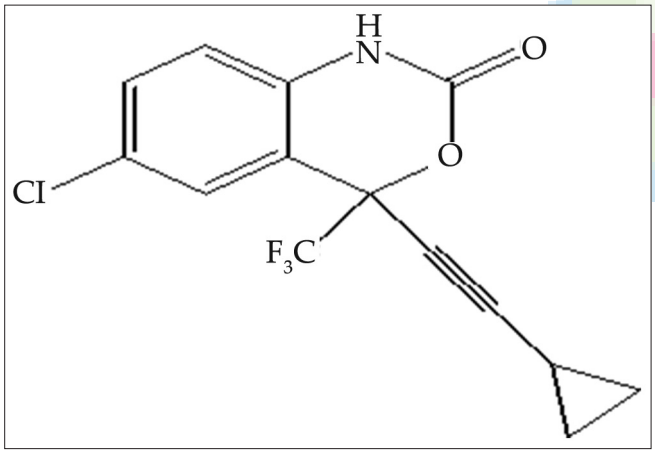

**Figure 3:** Chemical structure of efavirenz

and zidovudine.[24-28] Also, for the estimation of EFV, UV, HPTLC, and isocratic HPLC methods in tablet formulation and HPLC method in human plasma have been reported.[25-33] Also, RP-HPLC method is reported for the simultaneous estimation of EFV with TDF and emtricitabine.[34] Only one method, that is UV spectrophotometric method, is used for the simultaneous estimation of this combination of TDF, LAMI, and EFV in their combined tablet dosage form.[35] The purpose of this study was to develop a simple, rapid, precise, and accurate RP-HPLC method for the simultaneous estimation of these drugs in combined tablet dosage form.

## MATERIALS AND METHODS

### Instrumentation

Liquid chromatographic Shimadzu (LC-2010C$_{HT}$) system was manufactured by Shimadzu, Kyoto, Japan, and is equipped with auto-sampler, UV and Photodiode Array (PDA) detector, and Rheodyne injector with 20 µl loop volume. Weighing was done on a Digital Micro Balance an Acculab ALC 210.4 analytical balance, and pH of buffer was maintained by pH analyzer, Chemiline CL 180 µc based pH meter.

### Chemicals and reagents

Reference standards of TDF, LAMI, and EFV were obtained from Cipla Pharmaceuticals (Mumbai, India) and as a gift sample, whereas their combined tablet was obtained from local market. HPLC grade methanol, water (Finar Chemicals Pvt. Ltd., Ahmadabad, India), and HPLC grade orthophosphoric acid (80%) (Finar Chemicals Pvt. Ltd.) were also procured.

### Chromatographic conditions

The mobile phase consisted of methanol: Phosphate buffer (sodium dihydrogen orthophosphate, 10 mMol, pH 5.0) in the ratio of 70:30 (v/v) at a flow rate of 1.0 ml/min. Kromasil $C_{18}$ column (150 mm × 4.6 mm i.d., 5 µm) was used as the stationary phase. By considering the chromatographic parameter, sensitivity, and selectivity of the method for each of three drugs, 254 nm was selected as the detection wavelength for UV-PDA detector. The HPLC system was operated at a room temperature of 40°C.

### Preparation of standard solution

#### *Standard stock solution*

Standard stock solutions were prepared by dissolving separately 10 mg of LAMI and TDF and 20 mg of EFV in 100 ml volumetric flask. Dissolve and dilute with methanol up to the mark to get concentrations of 100 µg/ml of each of LAMI and TDF and 200 µg/ml of EFV.

#### *Working standard solution*

Working standard solutions were prepared by taking 0.1, 0.2, 0.3, 0.4, 0.5, and 0.6 ml into 10 ml volumetric flask and diluted up to the mark with the mobile phase to get 1–6 µg/ml each for LAMI and TDF and 2–12 µg/ml for EFV.

#### *Preparation of sample solution*

Twenty tablets of combined dosage form of LAMI, TDF, and EFV were weighed and ground to a fine

powder. Take powder equivalent to10 mg of LAMI, TDF, and 20 mg of EFV, mixed, and transferred to a 100-ml volumetric flask. The solution was sonicated to dissolve the powder in 60 ml methanol and diluted up to the mark with the same. The solution was filtered through a Whatman filter paper no. 41. Suitable dilutions with the diluent were made to prepare tablet solutions containing 3 μg/ml of each of LAMI and TDF and 6 μg/ml of EFV and then analyzed.

## RESULTS AND DISCUSSION

### Optimization of chromatographic condition

It was observed from the UV spectra that all the three drugs have considerable absorbances at 254 nm wavelength. So, 254 nm was selected as the detection wavelength. Various combinations of methanol, acetonitrile, and buffers of different pH were tried initially to separate LAMI, TDF, and EFV on $C_{18}$ column. Preliminary experiments indicated that use of different combinations of acetonitrile or methanol with water was not able to separate the peaks of LAMI, TDF, and EFV and to obtain suitable retention times and peak symmetry. In order to achieve acceptable peak symmetry and separation with good resolution, various buffer systems were tried systematically. Finally, a mobile phase consisting of methanol and phosphate buffer of pH 5.0 (adjusted with 10% solution of orthophosphoric acid) in a ratio of 70:30 v/v and a Kromasil $C_{18}$ column (150 mm × 4.6 mm i.d., 5 μm particle size) were selected to achieve good resolution and acceptable peak symmetry. Flow rates between 0.5-and 1.2 ml/min were tried. Flow rate of 1.0 ml/min was observed to be enough to get both the drugs eluted within less than 10 min. The column temperature was set at 40°C.

### System suitability

The retention times for LAMI, TDF, and EFV using optimum conditions were 2.76, 3.96, and 10.5 min, respectively. For three of them, the peak symmetries were <1.5 and the theoretical plates' numbers were >2000. These values are within the acceptable range of United state pharmacopoeia definition and the chromatograms obtained under optimized chromatographic conditions. Figure 4 clearly shows the ability of the method to assess the analyte in the presence of other excipients. The results obtained are shown in Table 1.

### Method validation[36]

The developed method for simultaneous estimation of LAMI, TDF, and EFV has been validated in accordance with the ICH guidelines.[11]

#### *Linearity*

Linearity was checked by preparing standard solutions at six different concentration levels of each of LAMI, TDF, and EFV, ranging from 1 to 6 μg/ml for each of LAMI and TDF and from 2 to 12 μg/ml for EFV. Triplicates of 20-μl injections were made for each concentration and were chromatographed under the chromatographic conditions mentioned above. Peak areas were plotted against the corresponding concentrations to obtain the

**Table 1: System suitability parameters**

| Parameter | LAMI | TDF | EFV |
|---|---|---|---|
| Retention time (min)* | 2.76±0.002 | 3.96±0.004 | 10.5±0.005 |
| Number of theoretical plates* | 2005±25.0 | 2056±38.0 | 5352±45.0 |
| Tailing factor* | 1.2±0.032 | 1.0±0.021 | 1.2±0.014 |
| HETP* | 116.23±0.34 | 77.40±0.42 | 28.02±0.25 |
| Resolution* | 3.37±0.05 | 3.51±0.01 | 3.21±0.01 |

*Each value is the mean±SD of six determinations

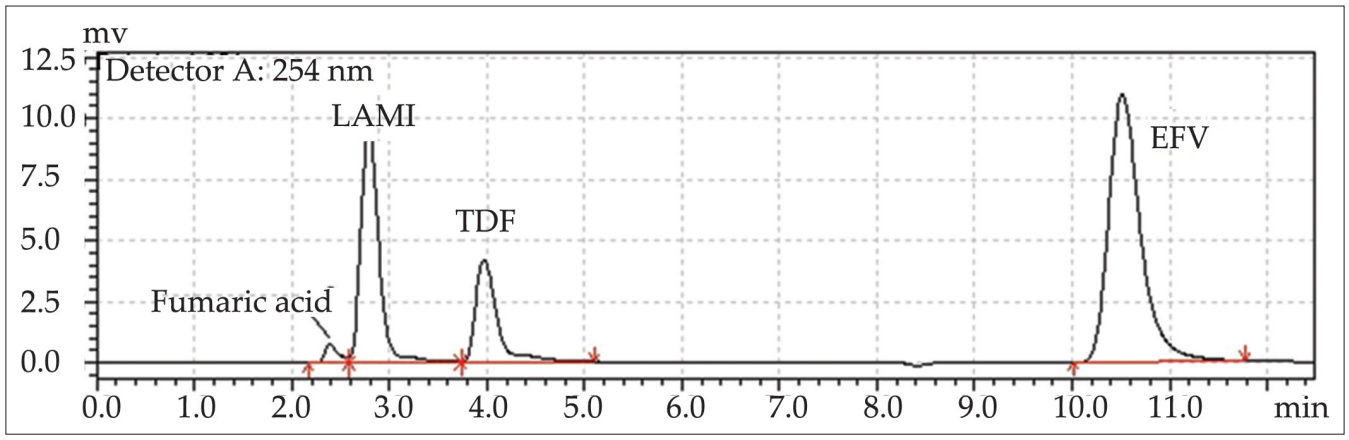


**Figure 4:** Chromatograms of LAMI (3 μg/ml), TDF (3 μg/ml), and EFV (6 μg/ml) reference substances

calibration graph for each compound. The regression analysis data are given in Table 2.

### *Sensitivity*

The sensitivity of the analytical method was evaluated by determining the limits of detection (LOD) and quantitation (LOQ). The values of LOD and LOQ for LAMI, TDF, and EFV are given in Table 2.

### *Accuracy*

The accuracy of the method for assay determination was checked at three concentration levels of 80%, 100%, and 120% for LAMI, TDF, and EFV. The percentage recoveries are tabulated in Table 3. The recovery was calculated from the slope and intercept of the calibration curve of each drug. As per the ICH guideline, the % recovery must be between 98% and 102%.

### *Precision*

*Repeatability:* System repeatability was determined by replicate applications and measurements of peak area for LAMI, TDF, and EFV. One dilution in six replicates was analyzed on the same day for repeatability and results were found within acceptable limits (RSD <2) as shown in Table 4.

*Intermediate precision:* Intermediate precision was assessed by the assay of sample sets on three different days (inter-day precision). Three dilutions in three replicates were analyzed and results were found within acceptable limits (RSD <2) as shown in Table 4.

### *Robustness*

As per the ICH norms, small, but deliberate variations are obtained by altering the temperature at 38°C and 42°C and changing the flow rate to 0.8 and 1.2 ml/min. The change in the flow rate of mobile phase does not affect the peak area; it may only change the retention time of peak of each drug. Results were found within acceptable limits (RSD <2), which are summarized in Table 5.

### *Stability of sample solution*

The sample solution stability was analyzed by injecting the same solution at 0, 6, 12, and 24 h. Identical change was not observed in the developed method. Also, results were found within acceptable limits (RSD <2), which are summarized in Table 6.

### *Specificity and selectivity*

The specificity test of the proposed method demonstrated that the excipients from sample do not interfere with the drug peak. Figure 5 shows the complete separation of LAMI, TDF, and EFV in the presence of tablet excipients.

**Table 2: Linearity data**

| Parameter | LAMI | TDF | EFV |
|---|---|---|---|
| Linearity range (μg/ml) | 1–6 | 1–6 | 2–12 |
| Regression equation | y = 36827x +20194 | y = 23574x – 3072 | y =45385x – 4786.2 |
| Correlation coefficient ($r^2$) | 0.9991 | 0.9992 | 0.9995 |
| LOD (μg/ml) | 0.018 | 0.01 | 0.02 |
| LOQ (μg/ml) | 0.05 | 0.1 | 0.07 |

**Table 3: Repeatability precision**

| Type of precision LAMI | % RSD | | |
|---|---|---|---|
| | TDF | EFV | |
| Repeatability | 0.03 | 0.013 | 0.005263 |
| Intermediate precision | | | |
| Intra-day | 0.64 | 0.92 | 1.0 |
| Inter-day | 0.61–1.04 | 1.24–1.47 | 0.90–1.64 |

**Table 4: Result of recovery studies with static evaluation**

| Drug | Amount taken (μg/ml) | Amount added (μg/ml) | Amount recovered (μg/ml) | %Recovery ± SD | %RSD |
|---|---|---|---|---|---|
| LAMI | 2.0 | 0 | -- | --- | --- |
| | 2.0 | 1.0 | 0.995 | 99.46±0.09 | 0.09 |
| | 2.0 | 2.0 | 1.999 | 99.97±0.05 | 0.15 |
| | 2.0 | 3.0 | 3.041 | 101.36±0.05 | 0.18 |
| TDF | 2.0 | 0 | -- | --- | --- |
| | 2.0 | 1.0 | 1.014 | 101.42±0.08 | 0.17 |
| | 2.0 | 2.0 | 2.018 | 100.89±0.07 | 0.25 |
| | 2.0 | 3.0 | 2.987 | 99.57±0.09 | 0.31 |
| EFV | 4.0 | 0 | -- | --- | --- |
| | 4.0 | 2.0 | 1.999 | 99.96±0.03 | 0.11 |
| | 4.0 | 4.0 | 4.035 | 100.87±0.06 | 0.14 |
| | 4.0 | 6.0 | 5.987 | 99.78±0.06 | 0.28 |

**Table 5: Robustness (column temperature related changes and mobile phase flow rate changes, concentration: 3 μg/ml for LAMI and TDF each and 6 μg/ml for EFV)**

| Type of precision | % RSD | | |
|---|---|---|---|
| | LAMI | TDF | EFV |
| Column temperature (±2°C) | | | |
| 38.0 | 0.30 | 0.40 | 0.11 |
| 40.0 | | | |
| 42.0 | | | |
| Flow rate of mobile phase (±0.2 ml/min) | | | |
| 0.8 | 0.20 | 0.18 | 0.17 |
| 1.0 | | | |
| 1.2 | | | |

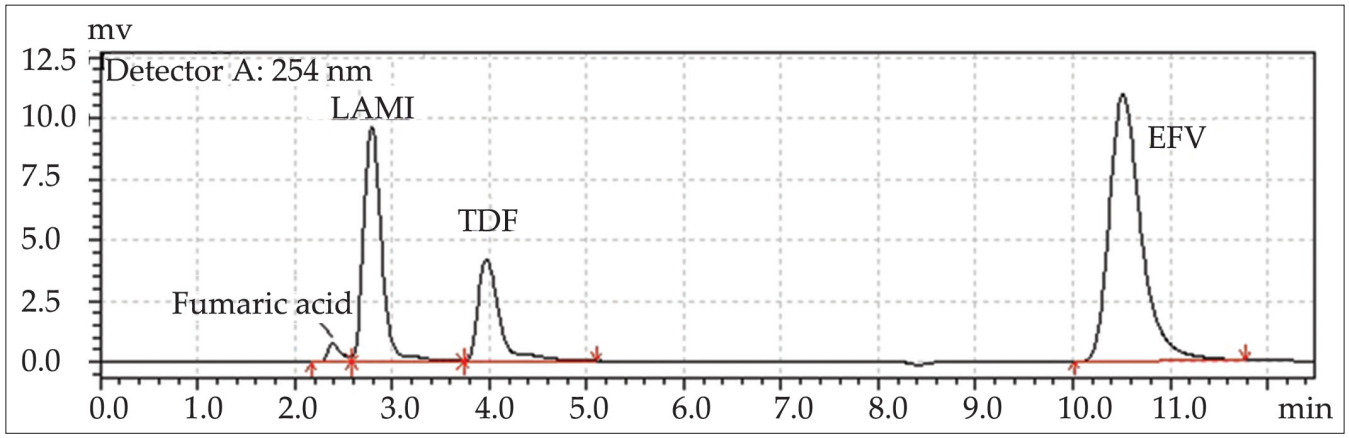


**Figure 5:** Chromatograms of LAMI (3 μg/ml), TDF (3 μg/ml), and EFV (6 μg/ml) in combined dosage formulation

**Table 6: Stability data of LAMI, TDF, and EFV (standard solutions)**

| Time | Assay (%) | | | %Difference | | |
|---|---|---|---|---|---|---|
| | LAMI (3 μg/ml) | TDF (3 μg/ml) | EFV (6 μg/ml) | LAMI (3 μg/ml) | TDF (3 μg/ml) | EFV (6 μg/ml) |
| Initial | 100.12 | 99.98 | 100.60 | --- | --- | --- |
| After 6 h | 100.05 | 99.86 | 100.54 | 0.08 | 0.12 | 0.12 |
| After 12 h | 99.96 | 99.66 | 100.17 | 0.16 | 0.32 | 0.49 |
| After 18 h | 99.92 | 99.30 | 100.11 | 0.21 | 0.68 | 0.55 |
| After 24 h | 99.73 | 99.14 | 100.02 | 0.39 | 0.84 | 0.64 |

### Assay

The % assay was found to be 100.13% for LAMI, 99.95% for TDF, and 101.14% for EFV.

## CONCLUSION

A simple, precise, reliable, sensitive, and accurate RP-HPLC method has been developed for the simultaneous determination of TDF, LAMI, and EFV. The developed method is suitable for the quantification of TDF, LAMI, and EFV in combined tablet dosage form.

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