

ANALYTICAL METHOD DEVELOPMENT AND VALIDATION FOR THE SIMULTANEOUS ESTIMATION OF EMTRICITABINE AND TENOFOVIR BY REVERSED-PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY IN BULK AND TABLET DOSAGE FORMS

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ABSTRACT

Objective: A simple rapid, accurate, precise, and reproducible validated reversed-phase high performance liquid chromatography method was developed for the determination of emtricitabine (EMB) and tenofovir (TEN) in bulk and tablet dosage forms.

Methods: The quantification was carried out using symmetry Premsil C₁₈ (250 mm×4.6 mm, 5 μm) Younglin (S.K.) gradient way using mobile phase comprising of methanol:water (70:30 v/v) pH 3 and a detection wavelength of 273 nm, and injection volume of 20 μL, with a flow rate of 1 ml/minutes.

Results: In the developed method, the retention time of EMB and TEN were found to be 3.1667 minutes and 7.5000 minutes. The developed method was validated according to the International Conference on Harmonization (ICH) guidelines.

Conclusion: The linearity, precision, range, robustness was within the limits as specified by the ICH guidelines. Hence, the method was found to be simple, accurate, precise, economic, and reproducible. Hence, it is worthwhile that the proposed methods can be successfully utilized for the routine quality control analysis EMB and TEN in bulk drug as well as in formulations.

Keywords: Emtricitabine and tenofovir, Method development, Validation, Simultaneous estimation, High performance liquid chromatography.

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INTRODUCTION

Emtricitabine (EMB) and tenofovir (TEN) are antiretroviral drugs used for the treatment of Human Immune Syndrome [1]. Forstavir - EM is the combination of the two drugs containing 150 mg of EMB and 300 mg of TEN. EMB is chemically 4-Amino-5-fluoro-1-[2-(hydroxyl methyl)-1,3-oxathiolan-5-yl]-pyrimidin-2-one [2,3]. It is a nucleoside reverse transcriptase inhibitor (Fig. 1). Chemically TEN is 1-(6-aminopurin-9-yl)-propan-2-yl-oxymethylphosphonic acid [3,4]. It is a nucleotide analogue reverse transcriptase inhibitor (Fig. 2). Extensive literature survey revealed that only liquid chromatography mass spectroscopy (LC-MS/MS) and reversed-phase high performance liquid chromatography (RP-HPLC) [5,6] methods for the determination of EMB and TEN in human plasma, RP-HPLC [7] for determination of TEN in plasma, LC/MS/MS for determination of plasma TEN concentrations [8], LC-MS method for determination of plasma TEN concentrations [9], and HPLC with fluorimetric detection for determination of EMB in human plasma [10] have been reported so far. There is no evidence of determination of the drug combination by HPLC. Thus, this study is to develop simple, precise, and accurate HPLC methods for the quantification of EMB and TEN in combined dosage form.

METHODS

Reagents and materials

The analysis of the drug was performed on Youngline (S.K.) gradient system ultraviolet (UV) detector. Equipped with Reverse Phase (premsil) c18 column (4.6 mm×250 mm; 5 μm), a SP930D pump, a 20 μl injection loop and UV730D Absorbance detector and running autochro-3000 software.

EMB and TEN in the form of gift samples were kindly supplied by R. S. I. T. C, Jalgaon. AR grade methanol used for HPLC method and methanol: Water (0.1% orthophosphoric acid [OPA]), prepared in

solvent double distilled water was used as solvent throughout the study. A combination of EMB (20 mg) and TEN (30 mg) in tablet formulation was procured from local pharmacy (Travin-EM, Emcure Pvt., Ltd).

Chromatographic conditions

Column C18 (250 mm×4.6 mm); particle size packing 5 μm; detection wavelength 271 nm; flow rate 0.5 ml/minutes; temperature ambient; sample size 20 μl; mobile phase acetonitrile: Water (0.05% OPA with pH 3) (50+50% v/v); run time 10 minutes.

Preparation of standard stock solution

Preparation of standard EMB solution: (Stock I)

From the freshly prepared standard stock solution (1000 μg/ml), 0.1 ml stock solution was pipette out in 10 ml of volumetric flask and volume was made up to 10 ml with mobile phase to get final concentration of 10 μg/ml (Fig. 3).

Preparation of standard TEN solution: (Stock II)

From the freshly prepared standard stock solution (1000 μg/ml), 0.1 ml stock solution was pipette out in 10 ml of volumetric flask and volume was made up to 10 ml with mobile phase to get final concentration 10 μg/ml (Fig. 4).

Preparation of standard EMB and TEN solution: (Stock III)

From the freshly prepared standard stock solution (1000 μg/ml), 0.1 ml stock solution was pipette out in 10 ml of volumetric flask and volume was made up to 10 ml with mobile phase to get final concentration 10 μg/ml. In the standard mixture of EMB and TEN theoretical plates (TP) were found above 2000, i.e., for EMB 4085.3 and TEN 11229.0 at minimum retention time (RT) 3.1667 and 7.500, respectively (Fig. 5 and Table 1).

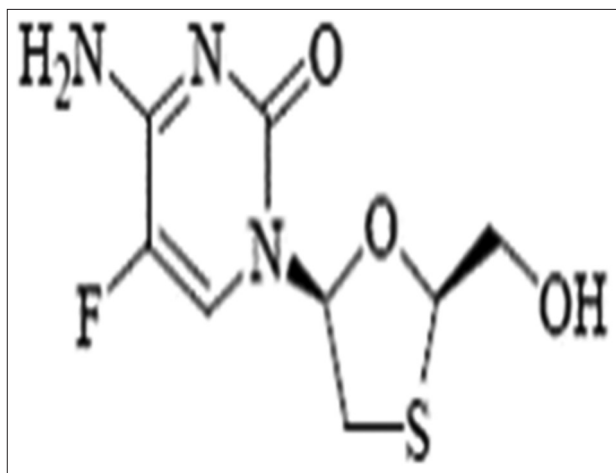


Fig. 1: Structure of emtricitabine

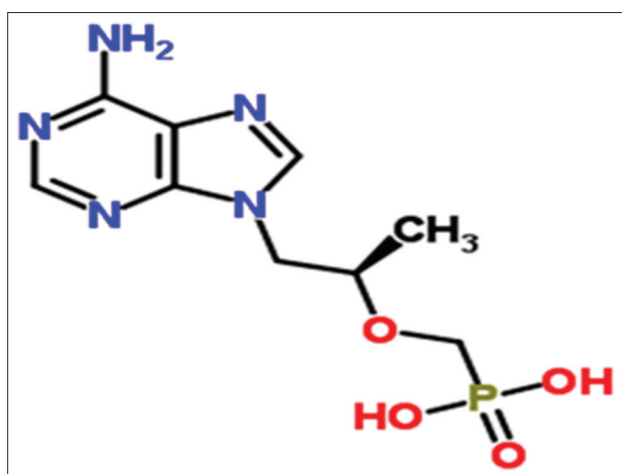


Fig. 2: Structure of tenofovir

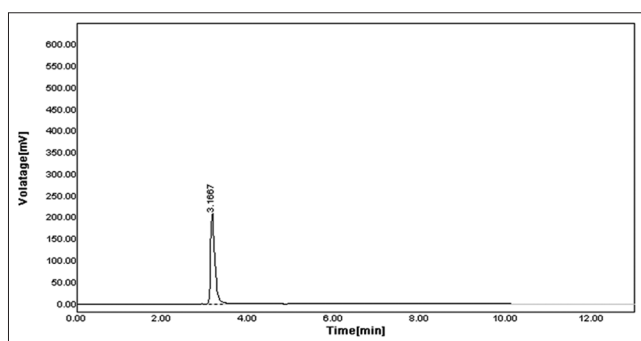


Fig. 3: Chromatogram of standard emtricitabine

Method development and validation

Working standard of concentrations was prepared by taking aliquots of standard solution and diluted to get required concentration for calibration plot and which was injected [11,12].

Assay preparation for commercial formulation

Weigh 20 EMB and TEN combination tablets weigh 14.98 g and calculated the average weight of powder 0.749 g accurately weigh and transfer the sample equivalent to 49.93 mg EMB and TEN into 10 ml volumetric flask. Add about 10 ml methanol of diluents and sonicate to dissolve it completely and make volume up to the mark with diluents. Mix well and filter through 0.45 μm filter. Further pipette 0.1 ml of the

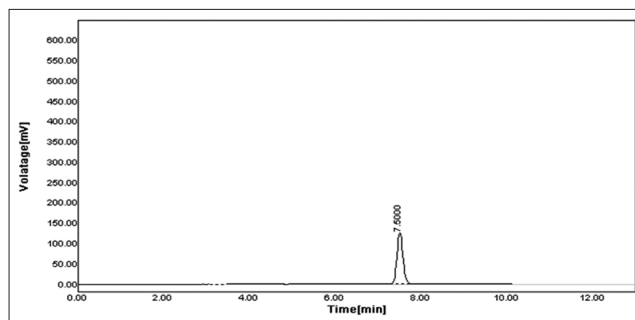


Fig. 4: Chromatogram of standard tenofovir

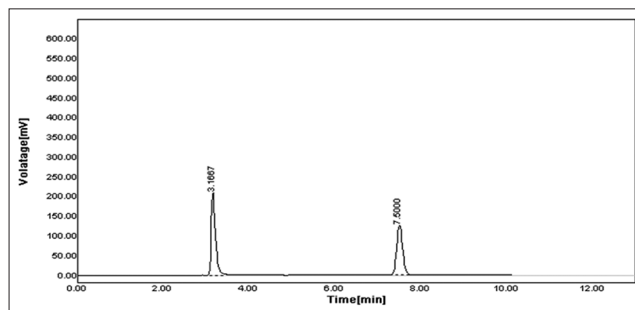


Fig. 5: Chromatogram of standard combination of emtricitabine and tenofovir

Table 1: Details of chromatogram of standard combination containing EMB and TEN

| RT (minutes) | Area (mV*s) | Area (%) | TP | TF | Resolution |
|--------------|-------------|----------|---------|--------|------------|
| 3.1667 | 1470.3370 | 54.64 | 4085.3 | 1.3333 | 0.0000 |
| 7.5000 | 1220.3994 | 45.36 | 11229.0 | 1.1667 | 15.2941 |
| | 2690.7363 | | | | |

EMB: Emtricitabine, TEN: Tenofovir, RT: Retention time, TP: Theoretical plates, TF: Tailing factor

above stock solution into a 10 ml volumetric flask and dilute up to the mark with diluents. (10 $\mu\text{g}/\text{ml}$). The simple chromatogram of test EMB and TEN shown in Fig. 6. The amounts of EMB and TEN per tablet were calculated by extrapolating the value of area from the calibration curve. Analysis procedure was repeated five times with tablet formulation. Analysis of marketed formulation was also % Label Claim was found to be 99-101% satisfactory are concluded (Table 2).

RESULTS

Linearity and range

The data obtained in the calibration experiments when subjected to linear regression analysis showed a linear relationship between peak areas and concentrations in the range 10-50 $\mu\text{g}/\text{ml}$ for EMB and 15-75 $\mu\text{g}/\text{ml}$ for TEN (Tables 3 and 4) depict the calibration data of EMB and TEN. The respective linear equation for EMB was $y=62.06x+0.546$ and TEN equation $y=47.73x+55.11$ where x is the concentration and y is area of peak. The correlation coefficient was 0.999. The calibration curve of EMB and TEN is depicted in Figs. 7 and 8.

Accuracy

It is defined as closeness of agreement between the actual (true) value and analytical value and obtained by applying test method for a number of times. The accuracy of the methods was determined at three different concentration levels, i.e. 80%, 100%, and 120% (Figs. 9-11) in triplicate for each drug as per International Conference on Harmonization guidelines. From the total amount of drug found, the percentage recovery was found in range of 99-101% (Tables 5 and 6).

Table 2: Analysis of marketed formulation

| Assay | Drug | Label claimed | Amount found | % Label claim | SD | % RSD |
|----------------|------|---------------|--------------|---------------|------|-------|
| RP-HPLC method | EMB | 10 | 9.90 | 99.0 | 0.15 | 0.37 |
| | TEN | 15 | 15.28 | 101.87 | 0.13 | 0.27 |
| | EMB | 10 | 10.11 | 101.10 | 0.27 | 0.27 |
| | TEN | 15 | 15.09 | 100.60 | 0.34 | 0.27 |

EMB: Emtricitabine, TEN: Tenofovir, SD: Standard deviation, RSD: Relative standard deviation, RP-HPLC: Reversed-phase high performance liquid chromatographic, RT: Retention time

Table 3: Linearity data for EMB

| Method | Concentration µg/ml | Peak area (µV.seconds) | | Average peak area (µV.seconds) | SD of peak area | % RSD of peak area |
|----------------|---------------------|------------------------|---------|--------------------------------|-----------------|--------------------|
| | | 1 | 2 | | | |
| RP-HPLC method | 10 | 623.5154 | 600.25 | 611.8876 | 16.44 | 2.69 |
| | 20 | 1220.399 | 1298.35 | 1259.379 | 55.13 | 4.38 |
| | 30 | 1819.49 | 1902.35 | 1860.924 | 58.60 | 3.15 |
| | 40 | 2441.527 | 2498.36 | 2469.947 | 40.19 | 1.63 |
| | 50 | 3050.95 | 3168.26 | 3109.607 | 82.95 | 2.67 |
| | Equation | y=62.06x+0.546 | | | | |
| | R ² | 0.999 | | | | |

RP-HPLC: Reversed-phase high performance liquid chromatographic, RSD: Relative standard deviation, SD: Standard deviation, EMB: Emtricitabine

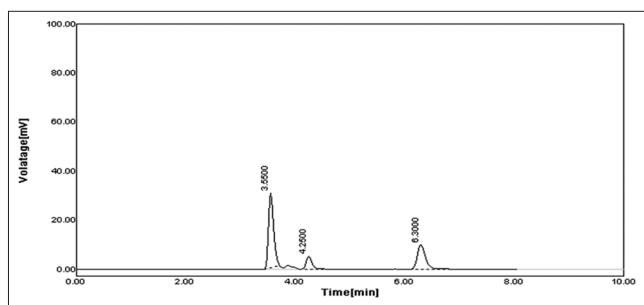


Fig. 6: Chromatogram for marketed formulation

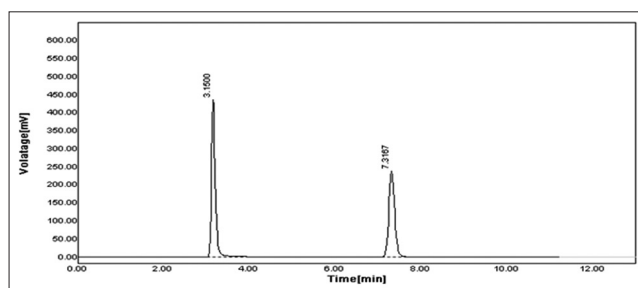


Fig. 9: Chromatogram of accuracy 80%

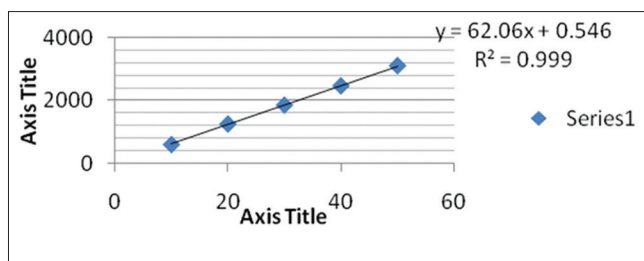


Fig. 7: Calibration curve of emtricitabine

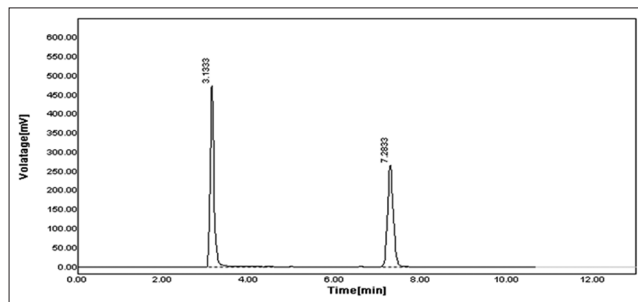


Fig. 10: Chromatogram of accuracy 100%

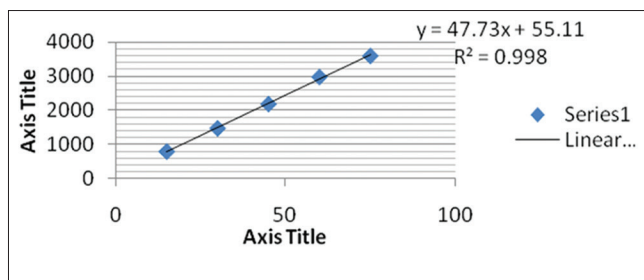


Fig. 8: Calibration curve of tenofovir

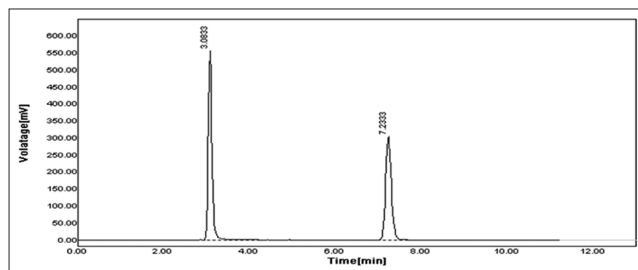


Fig. 11: Chromatogram of accuracy 120%

Precision

Precision was studied to find out intra- and interday variations in the test method of EMB and TEN. Intraday precision was determined by analyzing three concentration in three replicate measurements of

within linearity range of drugs on three different times in the same day. Interday precision was conducted during routine operation of the system over a period of 3 consecutive days. Intra- and Interday

Table 4: Linearity data for TEN

| Method | Concentration µg/ml | Peak area (µV.seconds) | | Average peak area (µV.seconds) | SD of peak area | % RSD of peak area |
|----------------|---------------------|------------------------|----------|--------------------------------|-----------------|--------------------|
| | | 1 | 2 | | | |
| RP-HPLC method | 15 | 775.2776 | 788.365 | 781.8213 | 9.25 | 1.18 |
| | 30 | 1470.337 | 1459.327 | 1464.832 | 7.79 | 0.53 |
| | 45 | 2202.554 | 2165.296 | 2183.925 | 26.34 | 1.21 |
| | 60 | 3008.865 | 2956.354 | 2982.609 | 37.13 | 1.24 |
| | 75 | 3638.126 | 3568.265 | 3603.195 | 49.40 | 1.37 |
| | Equation | y=47.73x+55.11 | | | | |
| | R ² | 0.998 | | | | |

RP-HPLC: Reversed-phase high performance liquid chromatographic, SD: Standard deviation, RSD: Relative standard deviation, TEN: Tenofovir

Table 5: Recovery data for EMB and TEN

| Method | Drug | Level (%) | Amount taken (µg/ml) | Amount added (µg/ml) | Mean*±SD | | |
|----------------|------|-----------|----------------------|----------------------|------------|------------------|-------------|
| | | | | | Absorbance | Amount recovered | % Recovery |
| RP-HPLC method | EMB | 80 | 20 | 10 | 35.99±0.27 | 15.99±0.27 | 99.96±1.70 |
| | | 100 | 10 | 20 | 39.94±0.30 | 20.58±0.30 | 99.72±1.48 |
| | | 120 | 20 | 24 | 43.85±0.27 | 23.85±0.27 | 101.58±1.15 |
| | TEN | 80 | 30 | 24 | 54.20±0.42 | 24.20±0.42 | 100.8±1.75 |
| | | 100 | 30 | 30 | 60.46±0.34 | 20.58±0.34 | 101.5±1.74 |
| | | 120 | 30 | 36 | 66.40±0.30 | 36.40±0.30 | 101.5±0.84 |

*Mean of each 3 reading for RP-HPLC method. RP-HPLC: Reversed-phase high performance liquid chromatographic, SD: Standard deviation, EMB: Emtricitabine, TEN: Tenofovir

Table 6: Statistical validation of recovery studies EMB and TEN

| Method | Level of recovery (%) | Drug | Mean % recovery | SD* | % RSD |
|----------------|-----------------------|-------|-----------------|------|-------|
| RP-HPLC method | 80 | EMTRI | 99.96 | 1.70 | 1.70 |
| | | TENO | 100.83 | 1.75 | 1.74 |
| | 100 | EMTRI | 99.72 | 1.48 | 1.49 |
| | | TENO | 101.53 | 1.74 | 1.12 |
| | 120 | EMTRI | 101.58 | 1.15 | 1.93 |
| | | TENO | 101.58 | 0.84 | 0.84 |

*Denotes average of three determinations for RP-HPLC. RP-HPLC: Reversed-phase high performance liquid chromatographic, SD: Standard deviation, RSD: Relative standard deviation, EMB: Emtricitabine, TEN: Tenofovir

precision studies on HPLC method for EMB and TEN which shows the high precision % amount in between 98% and 100% indicates to analytical method that concluded (Table 7 and Figs. 12-14).

Limit of detection (LOD) and limit of quantification (LOQ)

LOD is the lowest amount of analyte in a sample that can be detected but not necessarily quantify under the stated experimental conditions. LOQ is the lowest concentration of analyte in a sample that can be determined with the acceptable precision and accuracy under stated experimental conditions.

Robustness

The robustness is measure of its capacity to remain unaffected by small and deliberate variations in method parameters and provides an indication of its reliability during normal usage; hence, the following are performed by slight variations in parameters. The changes were did flow rate (±1 ml/minute⁻¹), pH of mobile phase composition (±1 ml/minute⁻¹), and Wavelength (±1 ml/minute⁻¹). % relative standard deviation (RSD) for peak area was calculated which should be <2%. The result is shown in Tables 8 and 9.

Repeatability

Repeatability studies on HPLC method for EMB and TEN were found to be, the % RSD was <2%, which shows high percentage amount found in

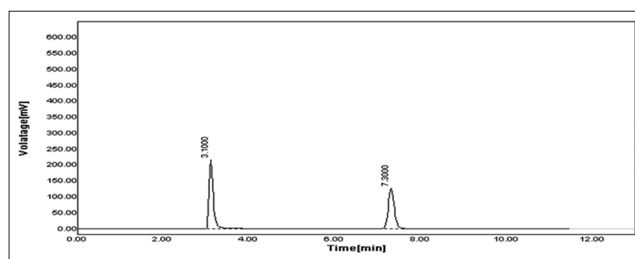


Fig. 12: Chromatogram of precision

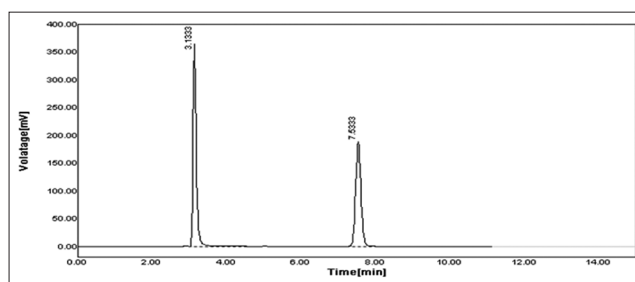


Fig. 13: Chromatogram intraday precision

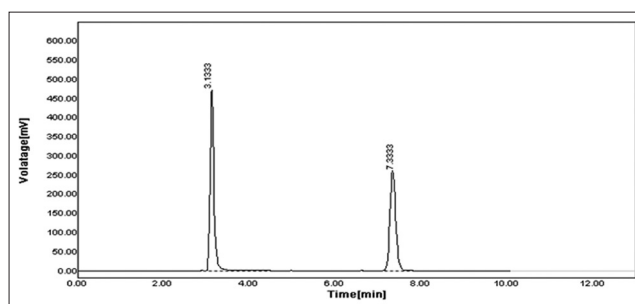


Fig. 14: Chromatogram interday precision

Table 7: Intra- and interday precision studies on RP-HPLC method for EMB and TEN

| Method | Drug | Concentration ($\mu\text{g/ml}$) | Intraday precision | | Inter-day precision | |
|----------------|------|------------------------------------|--------------------|----------------|---------------------|----------------|
| | | | Mean \pm SD | % Amount found | Mean \pm SD | % Amount found |
| RP-HPLC method | EMB | 20 | 19.74 \pm 21.74 | 97.77 | 1475.3 \pm 29.75 | 102.70 |
| | | 30 | 29.64 \pm 28.43 | 102.18 | 2245.1 \pm 45.88 | 98.20 |
| | | 40 | 39.26 \pm 26.54 | 102.8 | 39.30 \pm 38.77 | 98.25 |
| | TEN | 30 | 145.0 \pm 19.52 | 97.77 | 142.30 \pm 29.75 | 99.19 |
| | | 45 | 224.7 \pm 14.80 | 102.18 | 2245.1 \pm 45.88 | 101.96 |
| | | 60 | 298.6 \pm 44.18 | 102.38 | 259.57 \pm 61.58 | 102.63 |

Mean of each 3 reading for RP-HPLC method. RP-HPLC: Reversed-phase high performance liquid chromatographic, SD: Standard deviation, EMB: Emtricitabine, TEN: Tenofovir

Table 8: Robustness study of EMB

| Parameters | Concentration ($\mu\text{g/ml}$) | Amount of detected (mean \pm SD) | % RSD |
|---|------------------------------------|------------------------------------|-------|
| Chromatogram of flow change 0.9 ml | 50 | 2544.24 \pm 39.39 | 1.55 |
| Chromatogram of flow change 1.1 ml | 50 | 2331.6 \pm 21.55 | 0.92 |
| Chromatogram of composition change 69 ml MEOH+21 ml water | 50 | 1113.7 \pm 17.27 | 1.55 |
| Chromatogram of composition change 71 ml MEOH+37 ml water | 50 | 1182 \pm 2002 | 1.69 |
| Chromatogram of composition change wavelength change 272 nm | 50 | 3135.9 \pm 33.46 | 1.07 |
| Chromatogram of composition change wavelength change 274 nm | 50 | 3016.2 \pm 21.10 | 0.70 |

RSD: Relative standard deviation, SD: Standard deviation, EMB: Emtricitabine

Table 9: Robustness study of TEN

| Parameters | Concentration ($\mu\text{g/ml}$) | Amount of detected (mean \pm SD) | % RSD |
|---|------------------------------------|------------------------------------|-------|
| Chromatogram of flow change 0.9 ml | 75 | 3487.84 \pm 30.90 | 0.89 |
| Chromatogram of flow change 1.1 ml | 75 | 3547.77 \pm 30.24 | 0.85 |
| Chromatogram of composition change 69 ml MEOH+21 ml water | 75 | 1154.7 \pm 12.50 | 1.08 |
| Chromatogram of composition change 71 ml MEOH+37 ml water | 75 | 1282.80 \pm 20.02 | 1.56 |
| Chromatogram of composition change wavelength change 272 nm | 75 | 3628.8 \pm 23.31 | 0.64 |
| Chromatogram of composition change wavelength change 274 nm | 75 | 3562.41 \pm 51.13 | 1.44 |

RSD: Relative standard deviation, SD: Standard deviation, TEN: Tenofovir

Table 10: Repeatability studies on RP-HPLC for EMB and TEN

| Method | Concentration of EMB and TEN (mg/ml) | Peak area | Amount found (mg) | % Amount found |
|------------------------|--------------------------------------|---------------|-------------------|----------------|
| RP-HPLC method for EMB | 50 | 3054.69 | 49.21 | 98.42 |
| | 50 | 3055.69 | 49.22 | 99.00 |
| | 50 | 3059.70 | 40.23 | 96.00 |
| | 50 | 3058.72 | 50.10 | 101.00 |
| | 50 | 3059.73 | 50.20 | 98.00 |
| | | Mean \pm SD | 49.56 \pm 1.23 | |
| | % RSD | 0.23 | | |
| RP-HPLC method for TEN | 75 | 3661.05 | 75.54 | 100.72 |
| | 75 | 3662.05 | 76.55 | 100.00 |
| | 75 | 3660.04 | 75.50 | 101.02 |
| | 75 | 3666.01 | 76.49 | 102.96 |
| | 75 | 3659.06 | 75.48 | 101.40 |
| | | Mean \pm SD | 75.20 \pm 2.20 | |
| | % RSD | 0.30 | | |

RSD: Relative standard deviation, SD: Standard deviation, RP-HPLC: Reversed-phase high performance liquid chromatographic, EMB: Emtricitabine, TEN: Tenofovir

between 98% and 102% indicates the analytical method that concluded (Table 10).

DISCUSSION

The proposed method utilize two medium, i.e., methanol + water (acetic acid 0.1% [OPA]) (70: 30%) v/v) 273 nm, 1.0 ml, pH 3.0 gave adequate RT at 3.166 minutes and 7.500 minutes with good peak shape (TP of 4085.3 of EMB and 11229.0 of TEN. The values of % RSD are within the prescribed limit of 2%, showing high precision of methods and recovery was close to 100% for both the drugs. The comparison of method with already published two methods shows that the developed method is more accurate and economic as compared to other two methods further the method complies

with detection of drugs as per their label claim also no further derivatization or modification in spectra is required so the proposed method can be said as simple accurate and economic as compared to other published method.

CONCLUSION

The developed HPLC methods were found to be more accurate, precise, and reproducible. The analysis of tablets containing two drugs gave the satisfactory results. The statistical parameter of these methods showed good results. The recovery studies revealed excellent accuracy and high precision of the method. The methods were found to be simple and timesaving. All proposed methods could be applied for routine analysis in quality control laboratories.

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