CODEN [USA]: IAJPBB ISSN: 2349-7750

INDO AMERICAN JOURNAL OF
PHARMACEUTICAL SCIENCE

http://doi.org/10.5281/zenodo.821044

**Available online at:** http://www.iajps.com *Research Article*

# DEVELOPMENT AND VALIDATION OF A NEW RP-HPLC METHOD FOR SIMULTANEOUS ESTIMATION OF EMTRICITABINE, TENOFOVIR, COBICISTAT, ELVITEGRAVIR AND ITS COMPARISION WITH A REPORTED METHOD

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**Abstract:**

*A new stability indicating RP HPLC method has been developed and validated for simultaneous estimation of Emtricitabine, Tenofovir, Cobicistat and Elvitegravir in bulk and dosage forms. The method involves separation on Kromasil C18 column (250mm x 4.6mm x5µm particle size). The optimized mobile phase consists of 0.01N $KH_2PO_4$(pH 2.5) and Acetonitrile (43:57v/v) with a flow rate of 1ml/min and UV detection at 254nm. Retention time was 2.286 min for Emtricitabine,3.308 min for Tenofovir,5.316 min for Cobicistat,6.638 min for Elvitegravir. RP-HPLC method for the simultaneous estimation of Emtricitabine, Tenofovir, Cobicistat and Elvitegravir in their combine dosage form was developed and validated as per the ICH guidelines. Linearity was observed in the range of 20-120µg/ml for Emtricitabine, 30-180µg/ml for Tenofovir, 15-90µg/ml for Cobicistat and 15-90µg/ml for Elvitegravir with correlation coefficients ($r^2$=0.999).The percentage recoveries of Emtricitabine, Tenofovir, Cobicistat and Elvitegravir were in the range of 98.55-101.4% which was within the acceptance criteria. The percentage RSD was NMT 2% which proved the precision of the developed method. The developed method is simple, sensitive, rapid, linear, precise, rugged, accurate, specific, and robust. The developed method was found superior in certain respects such as RT and Accuracy. The method was more economical when compared to reported method.*

**Keywords:** *Emtricitabine, Tenofovir, Cobicistat, Elvitegravir, RP-HPLC Method, Simultaneous estimation, Validation as per ICH guidelines, Forced degradation studies.*

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*Please cite this article in press as K.Mangamma et al,* ***Development and Validation of A New RP-HPLC Method for Simultaneous Estimation of Emtricitabine, Tenofovir , Cobicistat , Elvitegravir and Its Comparison with A Reported Method****, Indo Am. J. P. Sci, 2017; 4(06).*

## INTRODUCTION:

Emtricitabine is an antiretroviral agent belonging to the class of nucleotide reverse transcriptase inhibitor for the treatment of HIV infection in adults. Chemically, it is 5-fluoro-1-(2R,5S)-[2-(hydroxymethyl)-1,3-oxathiolan-5-yl] cytosine. Tenofovir disoproxil fumarate (a prodrug of tenofovir) belongs to a class of antiretroviral drugs known as nucleotide analogue reverse transcriptase inhibitors. Chemically, it is 9-[(R)-2-[[bis[(isopropoxycarbonyl)oxy]methoxy]phosphinyl]methoxy]propyl]adenine fumarate. Cobicistat is chemically, 1,3-thiazol-5-ylmethyl N-[(2R,5R)-5-[[(2S)-2-[[methyl-[(2-propan-2-yl-1,3-thiazol-4-yl)methyl]carbomyl]amino]-4-morpholin-4-ylbutanoyl]amino]-1,6-diphenylhexan-2-yl]carbamate. Elvitegravir is used for the treatment of HIV infection. It acts as an integrase inhibitor. Chemically, it is 6-[(3-chloro-2-fluorophenyl) methyl]-1-(2S)-1-hydroxy-3-methyl butan-2-yl]-7-methoxy-4-oxoquinoline-3-carboxylic acid.

Though several methods are reported [1-6] in literature for the estimation of Emtricitabine, Tenofovir, Cobicistat and Elvitegravir individually, there are only a few HPLC methods are reported [7-8] for the simultaneous estimation of the four drugs in combination. The objective of the study is to develop and validate a new RP-HPLC method for simultaneous estimation of Emtricitabine, Tenofovir, Cobicistat, Elvitegravir and its comparison with the earlier reported methods.

## EXPERIMENTAL:

### Materials and Reagents

Acetonitrile (Rankem, avantor performance material India limited), HPLC water (Rankem, avantor performance material India limited), $KH_2PO_4$ (Molychem), were used in the study. The working standards of Emtricitabine, Tenofovir, Cobicistat and Elvitegravir were generous gift obtained from Hetero Pharma Ltd., Hyderabad, India. Stribild tablet containing Emtricitabine 200mg, Tenofovir 300mg, Cobicistat 150mg and Elvitegravir 150mg was kindly supplied by Janssen pharmaceuticals, Inc.

### Instrumentation

Chromatography was performed on a WATERS 2695 HPLC column (Alliance) with an auto sampler and equipped with a 2996 series of PDA detector with a spectral bandpass of 1.2nm. Components were detected using UV and that processing was achieved by Empower 2 software. A hot air oven was used for thermal degradation of the samples and a UV cross inker, with series of 23400 model UV chamber, equipped with a UV fluorescence lamp with the wavelength range between 200 & 300nm was selected for photolytic degradation. Ultrasonic bath (Labman), digital Ph meter (Metsar) were used in the study.

### Chromatography Conditions

The chromatographic condition was performed on Kromosil $C_{18}$ column (250 X 4.6mm,5µm particle size) at an ambient column temperature. The samples were eluted using 0.01N $KH_2PO_4$ (pH adjusted to 2.5): Acetonitrile(43:57v/v) as the mobile phase at a flow rate of 1 ml/min the mobile phase and samples were degassed by ultra-sonication for 20 min and filtered through 0.45µm Nylon(N66)47mm membrane filter. The measurements were carried out with an injection volume of 10µL, flow rate was set to 1.0 mL/min, and UV detection was carried out at 254 nm. All determinations were done at ambient column temperature (30°C). The chromatograms of the prepared standard stock solutions of Emtricitabine, Tenofovir, Cobicistat and Elvitegravir were recorded under optimized chromatographic conditions (**Fig. 1**).

Diluent: Water and Acetonitrile in 50:50 v/v ratio.

### Preparation of Standard Solution

#### Stock solution of Emtricitabine

Standard stock solution of Emtricitabine was prepared by dissolving 8 mg of Emtricitabine in 7ml of diluent (Water: Acetonitrile, 50:50v/v) in a 10ml clean dry volumetric flask and the solution was sonicated for 30 minutes and filter through 0.45µm nylon membrane filter and make up to the final volume with diluent to get the concentration of 80µg/ml of Emtricitabine. The above standard stock solution was suitably diluted with diluent to obtain various concentrations of Emtricitabine.

#### Stock solution of Tenofovir

Standard stock solution of Tenofovir was prepared by dissolving 12 mg of Tenofovir in 7ml of diluent (Water: Acetonitrile, 50:50v/v) in a 10ml clean dry volumetric flask and the solution was sonicated for 30 minutes and filter through 0.45µm nylon membrane filter and make up to the final volume with diluent to get the concentration of 120µg/ml of Tenofovir. The above standard stock solution was suitably diluted with diluent to obtain various concentrations of Tenofovir.

#### Stock solution of Cobicistat

Standard stock solution of Cobicistat was prepared by dissolving 6mg of Cobicistat in 7ml of diluent (Water: Acetonitrile, 50:50v/v) in a 10ml clean dry volumetric flask and the solution was sonicated for 30 minutes and filter through 0.45µm nylon membrane filter and make up to the final volume with diluent to get the concentration of 60µg/ml of Cobicistat. The above standard stock solution was suitably diluted with diluent to obtain various concentrations of Cobicistat.

#### Stock solution of Elvitegravir

Standard stock solution of Elvitegravir was prepared by dissolving 6mg of Elvitegravir in 7ml of diluent (Water: Acetonitrile, 50:50v/v) in a

10ml clean dry volumetric flask and the solution was sonicated for 30 minutes and filter through 0.45µm nylon membrane filter and make up to the final volume with diluent to get the concentration of 60µg/ml of Elvitegravir. The above standard stock solution was suitably diluted with diluent to obtain various concentrations of Elvitegravir**.**

**Working Standard Solution**

Working standard solutions of Emtricitabine, Tenofovir, Cobicistat and Elvitegravir was prepared by taking 1ml of stock solutions of Emtricitabine, Tenofovir, Cobicistat and Elvitegravir in to clean dry 10ml volumetric flask and make up volume with diluent to get a concentration of 80µg/ml of Emtricitabine, 120µg/ml of Tenofovir, 60µg/ml of Cobicistat and 60µg/ml of Elvitegravir.

**Preparation of Sample Solutions of Emtricitabine, Tenofovir, Cobicistat and Elvitegravir**

Twenty tablets were accurately weighed and calculate the average weight of each tablet then the weight equivalent to one tablet was transferred into 100ml volumetric flask, 70ml of diluent added and sonicated for 25 minutes, further the volume made up with diluent and filtered. From the filtered solution 0.4 ml was pippeted out into a 10ml volumetric flask and made up to 10ml diluent.

## RESULTS AND DISCUSSION:

**Optimization of chromatographic conditions**

Proper selection of the method depends upon the nature of the sample (ionic or ionizable or neutral molecule), its molecular weight and solubility. Emtricitabine, Tenofovir, Cobicistat and Elvitegravir were dissolved in polar solvents, so the developed method of estimation was carried out on reverse phase high performance liquid chromatography. To develop a rugged and suitable HPLC method for the quantitative determination of Emtricitabine, Tenofovir, Cobicistat and Elvitegravir, the analytical conditions were selected after the consideration of different parameters such as diluent, buffer, buffer concentration, organic solvent for mobile phase and mobile phase composition, and other chromatographic conditions. Preliminary trials were taken with different composition of buffer and organic phase of mobile phases with pH range of 2.5–5. The column selection has been done by backpressure, resolution, peak shape, theoretical plates and day-to-day reproducibility of the retention time and resolution between Emtricitabine, Tenofovir, Cobicistat and Elvitegravir peaks. After evaluating all these factors, a Kromasil $C_{18}$ column was found to be giving satisfactory results. The selection of acetonitrile and buffer were based on chemical structure of both the drugs. The acidic pH range was found suitable for solubility, resolution, stability, theoretical plates, and peak shape of both components. Best results were obtained with 0.01N $KH_2PO_4$ pH adjusted to 2.5 that improved the peak shapes of Emtricitabine, Tenofovir, Cobicistat and Elvitegravir. For the selection of organic constituent of mobile phase, acetonitrile was chosen to reduce the longer retention time and to attain good peak shape. Therefore, final mobile phase composition consisting of a mixture of buffer-pH 2.5 (0.01N $KH_2PO_4$): Acetonitrile (43:57v/v). Flow rates between 0.5 to 1.2ml/min were tried. Flow rate of 1ml/min was observed to be enough to get all the drugs eluted within less than 10min. The column temperature was set at $30^oC$. Optimized method was providing good resolution and peak shape for Emtricitabine, Tenofovir, Cobicistat and Elvitegravir. Under above described experimental conditions, all the peaks were well defined and free from tailing. The concern of small deliberate changes in the mobile phase composition, flow rates, and column temperature on results were evaluated as a part of testing for methods robustness.

**Validation of Method Developed**

The proposed method was validated according to the ICH guidelines for system suitability, specificity, recovery, precision, linearity, robustness, limit of detection (LOD) and limit of quantification (LOQ). Under the validation study, the following parameters were studied.

**System suitability**

The Retention time of Emtricitabine, Tenofovir, Cobicistat and Elvitegravir using optimum conditions was 2.28min, 3.30min, 5.31min and 6.63min respectively. For all of them, the peak symmetries were <1.5 and the theoretical plates numbers were >2000 and %RSD of areas of six standard injections of Emtricitabine, Tenofovir, Cobicistat and Elvitegravir were less than 2. These values are within the acceptable range of United States pharmacopoeia definition and the chromatographic conditions. The results obtained are shown in **Table 1.**

**Specificity**

The specificity of the method was evaluated by assessing interference from excipients in the pharmaceutical dosage form prepared as a placebo solution. Optimized Chromatogram of Emtricitabine, Tenofovir, Cobicistat and Elvitegravir is shown in **Fig. 1** clearly shows the ability of the method to assess the analyte in the presence of other excipients.

**Table 1: System suitability results of Emtricitabine, Tenofovir, Cobicistat and Elvitegravir**

| Parameter | Emtricitabine | Tenofovir | Cobicistat | Elvitegravir |
|---|---|---|---|---|
| Peak area | 1012865 | 1118501 | 50381 | 2620610 |
| Theoretical plates | 2862.66 | 6402.16 | 6433 | 22287.5 |
| Retention time | 2.286 | 3.308 | 5.316 | 6.638 |
| Tailing factor | 0.96 | 1.335 | 1.285 | 1.22 |

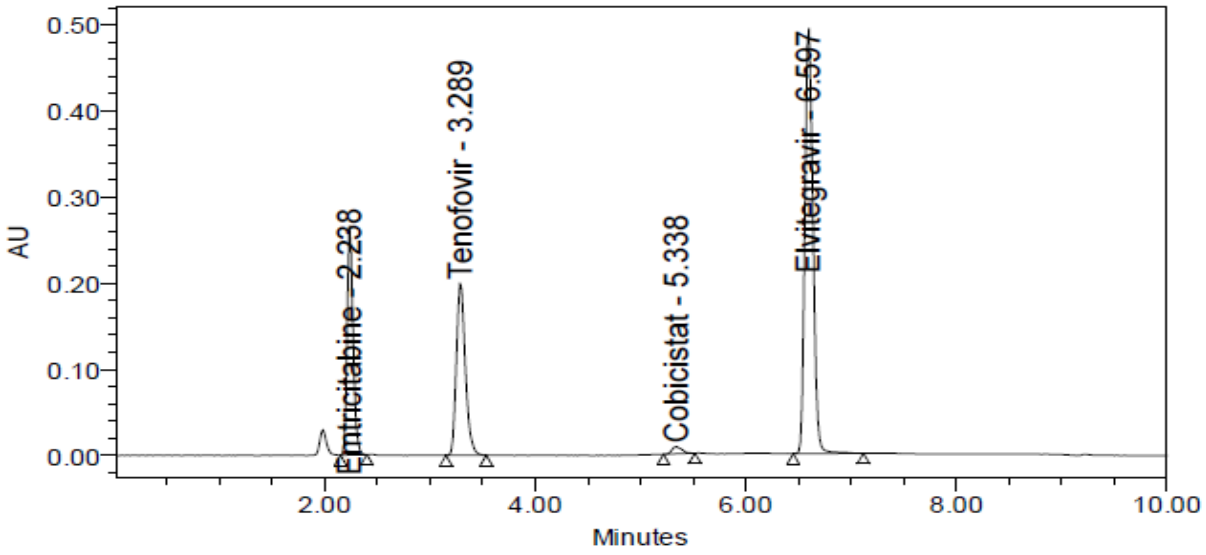


**Fig. 1: Optimized Chromatogram of Emtricitabine, Tenofovir, Cobicistat, Elvitegravir**

**Linearity and Range**

Linearity was assessed for all the four drugs at concentration ranges 20-120µg/ml for Emtricitabine, 30-180µg/ml for Tenofovir, 15-90µg/ml for Cobicistat and 15-90µg/ml for Elvitegravir. The Chromatograms of level 1 and level 6 are shown in **Fig.2 and Fig.3**. A linear relationship was established at these ranges between Area under the peak (AUP) and concentration. Good linearity was proved by high values of coefficient of determinations (**Fig.4, Fig.5, Fig.6 and Fig.7**). The results were tabulated in **Table 2.**

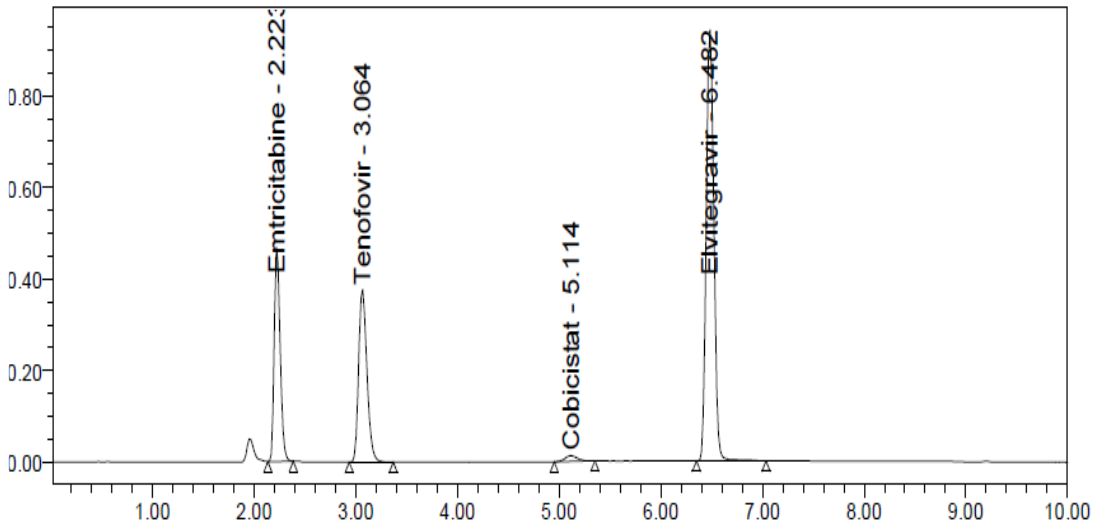


**Fig.2: Chromatogram of Level 1**

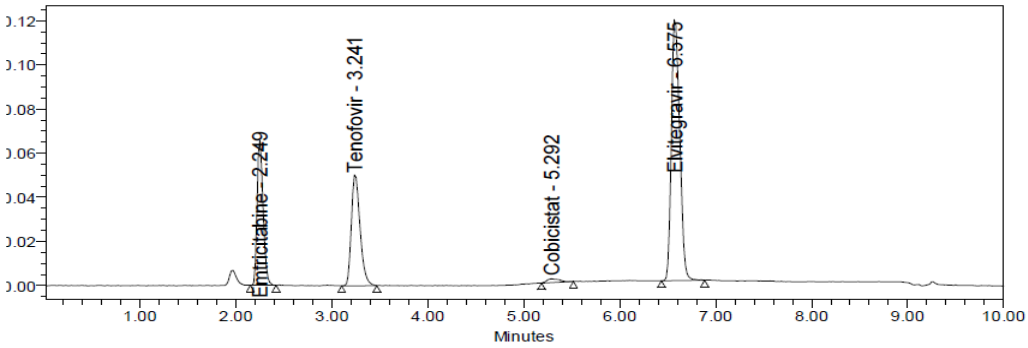


**Fig.3: Chromatogram of Level 2**

**Table 2: Linearity data of Emtricitabine, Tenofovir, Cobicistat and Elvitegravir**

| Level | Concentration of Emtricitabine(µg/ml) | Peak area | Concentration of Tenofovir (µg/ml) | Peak area |
|---|---|---|---|---|
| 1 | 20 | 283034 | 30 | 317539 |
| 2 | 40 | 534553 | 60 | 587945 |
| 3 | 60 | 822169 | 90 | 836659 |
| 4 | 80 | 1095483 | 120 | 1105179 |
| 5 | 100 | 1358475 | 150 | 1372882 |
| 6 | 120 | 1617736 | 180 | 1621268 |

| Level | Concentration of Cobicistat (µg/ml) | Peak area | Concentration of Elvitegravir (µg/ml) | Peak area |
|---|---|---|---|---|
| 1 | 15 | 14527 | 15 | 704888 |
| 2 | 30 | 28265 | 30 | 1385700 |
| 3 | 45 | 41812 | 45 | 2075179 |
| 4 | 6 | 55228 | 60 | 2662006 |
| 5 | 75 | 70894 | 75 | 3430322 |
| 6 | 90 | 84297 | 90 | 4056307 |

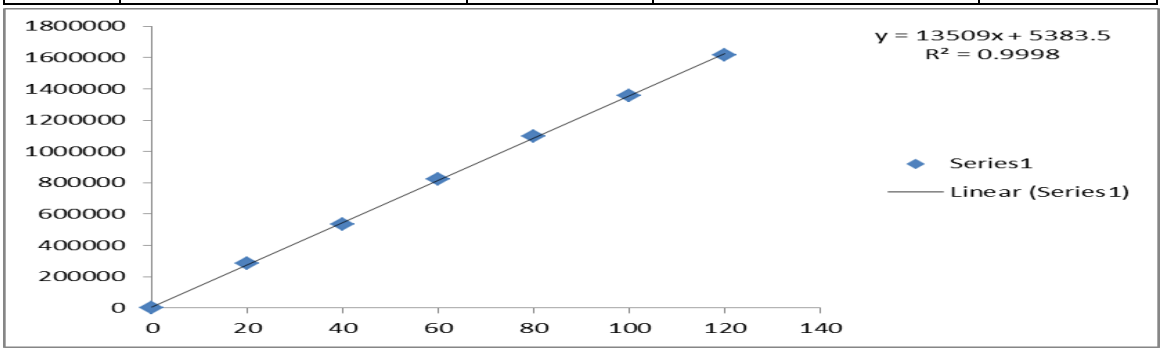


**Fig.4: Linearity graph of Emtricitabine**

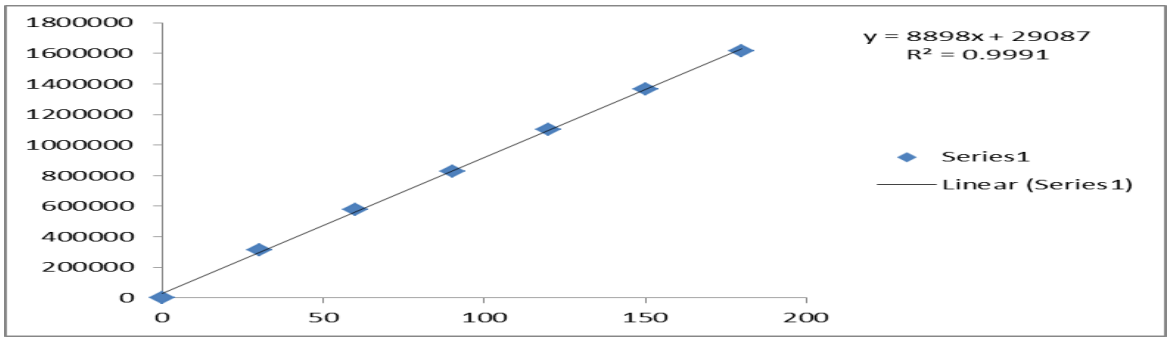


**Fig.5: Linearity graph of Tenofovir**

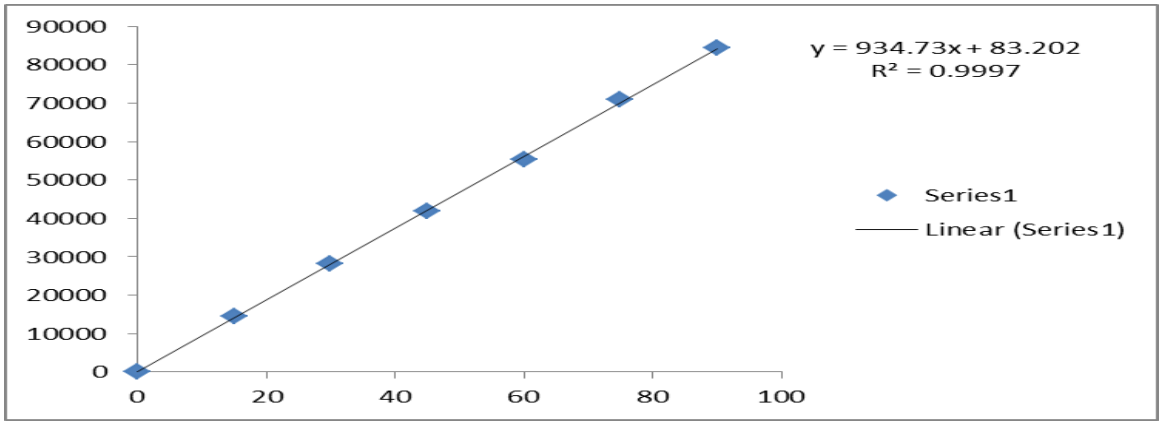


**Fig.6: Linearity graph of Cobicistat**

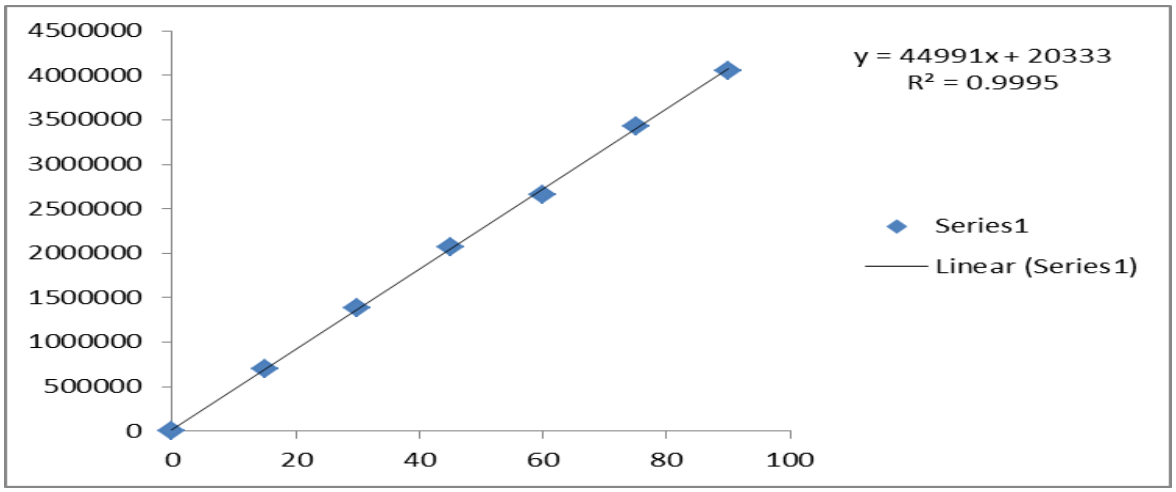


**Fig.7: Linearity graph of Elvitegravir**

**Limit of Detection (LOD)/Limit of Quantitation (LOQ)**

The LOD was determined on the basis of signal to noise ratios and was determined using analytical response of three times the background noise. LOQ was determined as the lowest amount of analyte that was reproducibly quantified above the baseline noise following triplicate injections. Both LOQ and LOD were calculated on the peak area using the following equations:

LOQ= 10 x N/ B LOD= 3 x N/ B

The limit of detection and limit of quantification were evaluated by serial dilutions of Emtricitabine, Tenofovir, Cobicistat and Elvitegravir stock solution in order to obtain signal to noise ratio of 3:1 for LOD and 10:1 for LOQ. The LOD value for Emtricitabine, Tenofovir, Cobicistat and Elvitegravir were found to be 0.92µg/mL, 1.04µg/mL,0.66µg/mL and 0.37µg/mL, respectively, and the LOQ value were found to be 2.78µg/mL, 3.14µg/mL, 2.01µg/mL and 1.12µg/mL, respectively.

**Precision:**

**System Precision**

System Precision was carried to ensure analytical system is working properly. One dilution of all the drugs in six replicates was injected into HPLC system & was analyzed and the results were found within the acceptance limits (RSD<2) as shown in the **Table 3**.

**Table 3: System Precision data for Emtricitabine, Tenofovir, Cobicistat and Elvitegravir**

| Emtricitabine | | | | Tenofovir | | |
|---|---|---|---|---|---|---|
| **S. No** | **Concentration (µg/ml)** | **Retention time (min)** | **Peak Area** | **Concentration (µg/ml)** | **Retention time (min)** | **Peak Area** |
| 1 | 80 | 2.261 | 1014502 | 120 | 3.238 | 1106208 |
| 2 | 80 | 2.273 | 1002497 | 120 | 3.250 | 1125384 |
| 3 | 80 | 2.289 | 1015381 | 120 | 3.282 | 1118334 |
| 4 | 80 | 2.292 | 1029147 | 120 | 3.338 | 1134718 |
| 5 | 80 | 2.298 | 1011444 | 120 | 3.366 | 1109557 |
| 6 | 80 | 2.305 | 1004221 | 120 | 3.376 | 1116805 |
| Average | | | 1012865 | Average | | 1118501 |
| SD | | | 9574.4 | SD | | 10434.4 |
| %RSD | | | 0.9 | %RSD | | 0.9 |

| Cobicistat | | | | Elvitegravir | | |
|---|---|---|---|---|---|---|
| **S. No** | **Concentration (µg/ml)** | **Retention time (min)** | **Peak Area** | **Concentration (µg/ml)** | **Retention time (min)** | **Peak Area** |
| 1 | 60 | 5.242 | 50564 | 60 | 6.558 | 2624766 |
| 2 | 60 | 5.261 | 50522 | 60 | 6.571 | 2618119 |
| 3 | 60 | 5.274 | 50234 | 60 | 6.577 | 2603467 |
| 4 | 60 | 5.345 | 50413 | 60 | 6.672 | 2621553 |
| 5 | 60 | 5.378 | 50060 | 60 | 6.715 | 2617026 |
| 6 | 60 | 5.401 | 50490 | 60 | 6.739 | 2638726 |
| Average | | | 50381 | Average | | 2620610 |
| SD | | | 195.4 | SD | | 11482.9 |
| %RSD | | | 0.4 | %RSD | | 0.4 |

**Method Precision (Repeatability)**

Precision is expressed as the closeness of agreement between a series of measurements obtaining from multiple sampling of the same homogeneous sample. Six replicate injections of a known concentration of sample preparation of Emtricitabine, Tenofovir, Cobicistat and Elvitegravir have been analyzed by injecting them into a HPLC column on the same day and on consecutive days. From the results obtained, %RSD was calculated and was found to be within the limits (<2). The results of precision are given in **Table 4**.

**Ruggedness**

Intermediate precision was accessed injecting sample preparation of Emtricitabine, Tenofovir,Cobicistat and Elvitegravir in six replicates in to HPLC column on the same day and on consecutive days and in different laboratories by different analysts. Results were found within the acceptance limits (RSD<2) as shown in the **Tables 5,6,7,8** below.

**Table 4: Method Precision data for Emtricitabine and Tenofovir**

| Emtricitabine | | | | Tenofovir | | |
|---|---|---|---|---|---|---|
| **S. No** | **Concentration (µg/ml)** | **Peak Area** | **% Assay** | **Concentration (µg/ml)** | **Peak Area** | **% Assay** |
| 1 | 80 | 1016702 | 100.03 | 120 | 1107208 | 98.31 |
| 2 | 80 | 1001497 | 98.54 | 120 | 1135384 | 100.81 |
| 3 | 80 | 1015281 | 99.89 | 120 | 1119443 | 99.40 |
| 4 | 80 | 1019147 | 100.28 | 120 | 1134818 | 100.76 |
| 5 | 80 | 1021444 | 100.50 | 120 | 1109997 | 98.56 |
| 6 | 80 | 1004321 | 98.82 | 120 | 1117697 | 99.24 |
| Average | | 1013065 | 99.67 | Average | 1120758 | 99.51 |
| SD | | 8192.83 | 0.80 | SD | 12017.52 | 1.06 |
| %RSD | | 0.80 | 0.80 | %RSD | 1.07 | 1.06 |

| Elvitegravir | | | | Cobicistat | | |
|---|---|---|---|---|---|---|
| **S. No** | **Concentration (µg/ml)** | **Peak Area** | **% Assay** | **Concentration (µg/ml)** | **Peak Area** | **% Assay** |
| 1 | 60 | 2625866 | 99.62 | 60 | 51264 | 101.29 |
| 2 | 60 | 2617113 | 99.29 | 60 | 50544 | 99.87 |
| 3 | 60 | 2642408 | 100.24 | 60 | 50352 | 99.49 |
| 4 | 60 | 2630554 | 99.79 | 60 | 50653 | 100.08 |
| 5 | 60 | 2623886 | 99.54 | 60 | 50080 | 98.95 |
| 6 | 60 | 2636743 | 100.03 | 60 | 50490 | 99.76 |
| Average | | 2629428 | 99.75 | Average | 50563.83 | 99.90 |
| SD | | 9146.78 | 0.34 | SD | 395.72 | 0.78 |
| %RSD | | 0.34 | 0.34 | %RSD | 0.78 | 0.78 |

**Table 5: Ruggedness Data for Emtricitabine**

| Laboratory-1 (% Assay)-HPLC-1 | | | | | Laboratory-2 (% Assay)-HPLC-2 | | | |
|---|---|---|---|---|---|---|---|---|
| | **Analyst-1** | | **Analyst-2** | | **Analyst-1** | | **Analyst-2** | |
| **Concentration (µg/ml)** | **Day-1** | **Day-2** | **Day-1** | **Day-2** | **Day-1** | **Day-2** | **Day-1** | **Day-2** |
| 80 | 79.28 | 79.68 | 79.67 | 79.45 | 79.43 | 79.64 | 80.02 | 79.86 |
| 80 | 79.46 | 80.21 | 79.41 | 79.42 | 79.54 | 79.43 | 79.42 | 80.02 |
| 80 | 79.54 | 79.62 | 79.46 | 79.34 | 79.28 | 79.01 | 79.27 | 79.52 |
| 80 | 79.89 | 79.46 | 80.14 | 79.86 | 79.45 | 79.45 | 79.48 | 79.84 |
| 80 | 80.29 | 79.84 | 80.07 | 80.23 | 80.23 | 80.08 | 79.59 | 80.05 |
| 80 | 80.03 | 80.14 | 79.21 | 80.14 | 80.03 | 80.12 | 79.76 | 79.82 |
| **Average** | 79.74 | 79.82 | 79.66 | 79.74 | 79.66 | 79.62 | 79.59 | 79.85 |
| **SD** | 0.38 | 0.29 | 0.37 | 0.39 | 0.13 | 0.42 | 0.26 | 0.18 |
| **%RSD** | 0.47 | 0.36 | 0.46 | 0.48 | 0.16 | 0.52 | 0.32 | 0.22 |

**Table 6: Ruggedness Data for Tenofovir**

| Laboratory-1 (% Assay)-HPLC-1 | | | | | Laboratory-2 (% Assay)-HPLC-2 | | | |
|---|---|---|---|---|---|---|---|---|
| | Analyst-1 | | Analyst-2 | | Analyst-1 | | Analyst-2 | |
| Concentration (µg/ml) | Day-1 | Day-2 | Day-1 | Day-2 | Day-1 | Day-2 | Day-1 | Day-2 |
| 120 | 119.42 | 119.24 | 119.42 | 119.43 | 119.84 | 119.29 | 119.41 | 119.21 |
| 120 | 120.01 | 119.46 | 119.84 | 119.86 | 119.49 | 119.45 | 119.64 | 119.48 |
| 120 | 119.78 | 120.01 | 119.56 | 119.45 | 119.34 | 119.81 | 119.21 | 119.42 |
| 120 | 119.83 | 119.89 | 120.04 | 119.62 | 119.89 | 119.42 | 119.89 | 119.61 |
| 120 | 119.65 | 120.23 | 119.48 | 120.23 | 119.94 | 119.19 | 120.21 | 119.49 |
| 120 | 120.14 | 120.43 | 119.46 | 120.42 | 119.44 | 119.12 | 119.37 | 120.21 |
| Average | 119.80 | 119.87 | 119.63 | 119.83 | 119.65 | 119.38 | 119.62 | 119.57 |
| SD | 0.25 | 0.45 | 0.25 | 0.40 | 0.26 | 0.24 | 0.37 | 0.33 |
| %RSD | 0.20 | 0.37 | 0.20 | 0.33 | 0.21 | 0.20 | 0.30 | 0.27 |

**Accuracy**

The percentage recovery was calculated by preparing standard drug concentrations of Emtricitabine, Tenofovir, Cobicistat and Elvitegravir with concentration levels of 50%, 100% and 150%. A known amount of the standard drug was added to the blank sample at each level.

Good recovery of the spiked drugs was obtained at each added concentration, and the mean percentage recovery of Emtricitabine, Tenofovir, Cobicistat and Elvitegravir was achieved between 99.42–100.81 ± 0.753% and 99.53-100.16±0.327.The results are given in **Tables 9, 10, 11, 12** below.

**Table 7: Ruggedness data for Elvitegravir**

| Laboratory-1 (% Assay)-HPLC-1 | | | | | Laboratory-2 (% Assay)-HPLC-2 | | | |
|---|---|---|---|---|---|---|---|---|
| | Analyst-1 | | Analyst-2 | | Analyst-1 | | Analyst-2 | |
| Concentration (µg/ml) | Day-1 | Day-2 | Day-1 | Day-2 | Day-1 | Day-2 | Day-1 | Day-2 |
| 60 | 59.52 | 59.34 | 59.52 | 59.53 | 59.94 | 59.39 | 59.51 | 59.31 |
| 60 | 60.11 | 59.56 | 59.94 | 59.96 | 59.59 | 59.55 | 59.74 | 59.58 |
| 60 | 59.88 | 60.11 | 59.66 | 59.55 | 59.44 | 59.91 | 59.31 | 59.52 |
| 60 | 59.93 | 59.99 | 60.14 | 59.72 | 59.99 | 59.52 | 59.99 | 59.62 |
| 60 | 59.75 | 60.33 | 59.58 | 59.33 | 60.04 | 59.29 | 60.31 | 59.59 |
| 60 | 60.24 | 60.53 | 59.56 | 59.52 | 59.54 | 59.22 | 59.47 | 60.31 |
| **Average** | 59.90 | 59.97 | 59.73 | 59.60 | 59.73 | 59.48 | 59.72 | 59.65 |
| **SD** | 0.25 | 0.45 | 0.25 | 0.21 | 0.26 | 0.24 | 0.37 | 0.33 |
| **%RSD** | 0.41 | 0.75 | 0.41 | 0.35 | 0.43 | 0.40 | 0.61 | 0.55 |

**Table 8: Ruggedness Data for Cobicistat**

| Laboratory-1 (% Assay)-HPLC-1 | | | | | Laboratory-2 (% Assay)-HPLC-2 | | | |
|---|---|---|---|---|---|---|---|---|
| | Analyst-1 | | Analyst-2 | | Analyst-1 | | Analyst-2 | |
| Concentration (µg/ml) | Day-1 | Day-2 | Day-1 | Day-2 | Day-1 | Day-2 | Day-1 | Day-2 |
| 60 | 59.38 | 59.78 | 59.77 | 59.55 | 59.53 | 59.74 | 60.12 | 59.96 |
| 60 | 59.56 | 60.31 | 59.41 | 59.52 | 59.64 | 59.53 | 59.52 | 60.12 |
| 60 | 59.64 | 59.72 | 59.56 | 59.44 | 59.38 | 59.11 | 59.37 | 59.62 |
| 60 | 59.99 | 59.56 | 60.24 | 59.96 | 59.55 | 59.55 | 59.58 | 59.64 |
| 60 | 60.39 | 59.94 | 60.17 | 60.33 | 60.33 | 60.18 | 59.69 | 60.15 |
| 60 | 60.13 | 60.24 | 59.31 | 60.24 | 60.13 | 60.22 | 59.86 | 60.02 |
| Average | 59.84 | 59.92 | 59.86 | 59.84 | 59.76 | 59.72 | 59.69 | 59.91 |
| SD | 0.38 | 0.29 | 0.61 | 0.39 | 0.37 | 0.42 | 0.26 | 0.26 |
| %RSD | 0.63 | 0.48 | 1.01 | 0.65 | 0.61 | 0.70 | 0.43 | 0.43 |

**Table 9: Recovery data of Emtricitabine**

| Sample name | Amount added (µg/ml) | Amount found (µg/ml) | % Recovery | Statistical Analysis |
|---|---|---|---|---|
| **S1:50%** | 40 | 39.58 | 98.97 | Mean=99.28 |
| **S2:50%** | 40 | 39.27 | 98.19 | S.D=1.273 |
| **S3:50%** | 40 | 40.27 | 100.68 | %RSD=1.28 |
| **S4:100%** | 80 | 80.66 | 80.66 | Mean=79.92 |
| **S5:100%** | 80 | 78.91 | 78.91 | S. D=0.905 |
| **S6:100%** | 80 | 80.19 | 80.19 | %RSD=1.13 |
| **S7:150%** | 120 | 119.57 | 99.65 | Mean=100.13 |
| **S8:150%** | 120 | 120.72 | 100.61 | S. D=0.480 |
| **S9 :150%** | 120 | 120.18 | 100.15 | %RSD=0.47 |

**Table 10: Recovery data of Tenofovir**

| Sample Name | Amount added (µg/ml) | Amount found (µg/ml) | %Recovery | Statistical Analysis |
|---|---|---|---|---|
| **S1:50%** | 60 | 60.36 | 100.66 | Mean=100.44 |
| **S2:50%** | 60 | 59.92 | 99.92 | S.D=0.455 |
| **S3:50%** | 60 | 60.42 | 100.75 | %RSD=0.45 |
| **S4:100%** | 120 | 121.10 | 100.92 | Mean=100.73 |
| **S5:100%** | 120 | 119.83 | 99.86 | S.D=0,792 |
| **S6:100%** | 120 | 121.69 | 101.41 | %RSD=0.78 |
| **S7:150%** | 180 | 181.99 | 101.11 | Mean=100.33 |
| **S8:150%** | 180 | 181.07 | 100.60 | S.D=0.933 |
| **S9 :150%** | 180 | 178.73 | 99.30 | %RSD=0.92 |

**Table 11: Recovery data of Cobicistat**

| Sample name | Amount added (µg/ml) | Amount found (µg/ml) | % Recovery | Statistical Analysis |
|---|---|---|---|---|
| **S1:50%** | 30 | 30.22 | 100.74 | Mean=100.55% (n=3) |
| **S2:50%** | 30 | 30.13 | 100.43 | S.D=0.132 |
| **S3:50%** | 30 | 30.15 | 100.50 | %RSD=0.162 |
| **S4:100%** | 60 | 59.74 | 99.57 | Mean=100.44 |
| **S5:100%** | 60 | 60.58 | 100.97 | S.D=0.761 |
| **S6:100%** | 60 | 60.00 | 100.79 | %RSD=0.75 |
| **S7:150%** | 90 | 90.28 | 100.31 | Mean=100.00 |
| **S8:150%** | 90 | 89.85 | 99.83 | S.D=0.266 |
| **S9 :150%** | 90 | 89.89 | 99.87 | %RSD=0.26 |

**Table 12: Recovery data of Elvitegravir**

| Sample name | Amount added (µg/ml) | Amount found (µg/ml) | % Recovery | Statistical Analysis |
|---|---|---|---|---|
| **S1:50%** | 30 | 30.06 | 100.20 | Mean=99.92% (n=3) |
| **S2:50%** | 30 | 29.62 | 98.72 | S.D=0.891 |
| **S3:50%** | 30 | 30.26 | 100.85 | %RSD=1.091 |
| **S4:100%** | 60 | 59.81 | 99.69 | Mean=100.36% |
| **S5:100%** | 60 | 60.86 | 101.44 | S.D=0.769 |
| **S6:100%** | 60 | 59.98 | 99.96 | %RSD=0.942 |
| **S7:150%** | 90 | 89.20 | 99.11 | Mean=100.35% |
| **S8:150%** | 90 | 90.80 | 100.89 | S.D=0.881 |
| **S9 :150%** | 90 | 90.95 | 101.-06 | %RSD=1.080 |

**Robustness**

Robustness of the proposed analytical method is a measure of its capacity to remain unaffected, and it reflects the reliability of the analysis with respect to deliberate changes in the parameters such as flow rate (1.0 ± 0.2 mL), column temperature (30 ± 5°C), mobile phase ratio of the mobile phase. The result of robustness study of the developed assay method was established in **Tables 13, 14, 15**. The result shown that during all variance conditions, assay value of the test preparation solution was not affected and it was in accordance with that of actual. System suitability parameters were also found satisfactory; hence the analytical method would be concluded as robust.

**Table: 13: Robustness (change in flow rate) for Emtricitabine, Tenofovir, Elvitegravir and Cobicistat**

| Drug | Change in Flow rate (ml/min) | Change in flow Rate (0.8ml/min to 1.2 ml/min) | | |
|---|---|---|---|---|
| | | %Assay | SD | % RSD |
| Emtricitabine | 0.9 | 80.99 | | |
| | 100000 | 81.99 | 0.60 | 0.74 |
| | 1.1 | 80.89 | | |
| Tenofovir | 0.9 | 90.06 | | |
| | 10 | 91.06 | 1.05 | 1.16 |
| | 1.1 | 88.96 | | |
| Elvitegravir | 0.9 | 85.07 | | |
| | 1 | 86.07 | 1.05 | 1.23 |
| | 1.1 | 83.97 | | |
| Cobicistat | 0.9 | 143.19 | | |
| | 1 | 144.19 | 1.05 | 0.73 |
| | 1.1 | 142.09 | | |

**Table 14: Robustness (change in Mobile phase composition) for Emtricitabine , Tenofovir, Elvitegravir and Cobicistat**

| Drug | Change in mobile phase | Change in Mobile phase (0.8ml/min to 1.2 ml/min) | | |
|---|---|---|---|---|
| | | %Assay | SD | % RSD |
| Emtricitabine | 5% less organic phase | 114.92 | | |
| | Actual | 113.6 | 0.68 | 0.59 |
| | 5% more organic phase | 114.60 | | |
| Tenofovir | 5% less organic phase | 112.93 | | |
| | Actual | 111.81 | 0.87 | 0.77 |
| | 5%more organic phase | 113.54 | | |
| Elvitegravir | 5% less organic phase | 109.64 | | |
| | Actual | 108.52 | 0.69 | 0.63 |
| | 5% more organic phase | 109.79 | | |
| Cobicistat | 5% less organic phase | 137.37 | | |
| | Actual | 136.25 | 2.30 | 1.66 |
| | 5%more organic phase | 140.68 | | |

**Table 15: Robustness (change in column Temparature) for Emtricitabine, Tenofovir, Elvitegravir and Cobicistat**

| Drug | Change in column temperature | Change in column temperature | | |
|---|---|---|---|---|
| | | %Assay | SD | % RSD |
| Emtricitabine | 25°C | 107.07 | | |
| | 30°C | 107.10 | 0.03 | 0.02 |
| | 35°C | 107.13 | | |
| Tenofovir | 25°C | 105.40 | | |
| | 30°C | 105.65 | 0.21 | 1.98 |
| | 35°C | 105.83 | | |
| Elvitegravir | 25°C | 103.78 | | |
| | 30°C | 103.90 | 0.14 | 1.34 |
| | 35°C | 104.06 | | |
| Cobicistat | 25°C | 135.23 | | |
| | 30°C | 138.45 | 2.92 | 0.02 |
| | 35°C | 141.08 | | |

**Forced degradation studies**

The assay method was used to test the drug stability by conducting forced degradation studies for the drug substances under various stress conditions. Stress degradation studies were carried out for acid hydrolysis (1M HCl heated for 30 min at 60°C), alkali hydrolysis (2 N NaOH heated for 30 min at 60°C), oxidative degradation (20%H2O2 heated at 60°C for 30 min) and thermal degradation (samples placed in an oven at 105°C for 6 h). For photolytic stress studies, samples were exposed to UV light by keeping them in a UV chamber for 7 days.

The retention time of Emtricitabine, Tenofovir, Cobicistat and Elvitegravir were found to be 2.28min, 3.30min, 5.31min and 6.63 min respectively. Linearity was established for Emtricitabine, Tenofovir, Cobicistat and Elvitegravir in the range of 20-120µg/ml for Emtricitabine, 30-180µg/ml for Tenofovir, 15-90µg/ml for Cobicistat and 15-90µg/ml for Elvitegravir with correlation coefficients ($r^2$=0.999) and the percentage recoveries were between 79.92 – 100.13%, 100.33 – 100.73%, 100.00 – 100.55% and 99.92 – 100.36% for Emtricitabine, Tenofovir, Cobicistat, Elvitegravir respectively, which indicate accuracy of the proposed method. The % RSD values of accuracy for Emtricitabine, Tenofovir, Cobicistat and Elvitegravir were found to be < 2%. The % RSD values of method precision are 0.80%, 1.07%, 0.78%, 0.34% for Emtricitabine, Tenofovir, Cobicistat and Elvitegravir respectively and % RSD values of system precision are 0.9% for Emtricitabine, Tenofovir and 0.4% for Cobicistat, Elvitegravir. The % RSD values of reproducibility are 0.80, 1.07, 0.78 and 0.34 for Emtricitabine, Tenofovir, Cobicistat and Elvitegravir respectively, reveal that the proposed method is precise. LOD values for Emtricitabine, Tenofovir, Cobicistat and Elvitegravir were found to be 0.92µg/ml, 1.04µg/ml, 0.66µg/ml and 0.37µg/ml respectively and LOQ values for Emtricitabine, Tenofovir, Cobicistat and Elvitegravir were found to be 2.78µg/ml, 3.14µg/ml, 2.01µg/ml and 1.12µg/ml respectively was shown. The % RSD values of robustness studies were found to be < 2% reveal that the method is robust enough was shown in (Table 13,14,15). These data show that the proposed method is specific and sensitive for the determination of Emtricitabine, Tenofovir, Cobicistat and Elvitegravir.

**Comparison of developed method with the earlier reported methods**

The validation parameters of the new method developed are compared to those of earlier reported methods in Table 16.

When the validation parameters of the method developed are compared with those of the earlier reported method [8] the developed method was found superior in certain respects such as RT and accuracy. The method was more economical when compared to others. Other parameters were similar to earlier reported methods.

**Table 16: Comparison of developed method with the earlier reported methods [8]**

| **Validation parameters** | **Method developed** | | | | **Reported method[8]** | | | |
|---|---|---|---|---|---|---|---|---|
| Specificity: i.Retention time (min) | **EMT** 2.28 | **TEN** 3.30 | **COB** 5.31 | **ELVI** 6.63 | **EMT** 0.13 | **TEN** 0.03 | **COB** 0.05 | **ELVI** 0.07 |
| **Linearity (µg/ml)** | 20-120 | 30-180 | 15-90 | 15-90 | 10-60 | 15-90 | 7.5-45 | 7.5-45 |
| **Regression equation** | Y=13509X +5383.5 | Y=8898X +29087 | Y=934.73X +83.202 | Y=44991x +20333 | Y=31567 +361947 | Y=7077.8 +209511 | Y=4807.9 +113788 | Y=39259 +548607 |
| **$r^2$** | 0.999 | 0.999 | 0.999 | 0.999 | 0.999 | 0.998 | 0.999 | 0.999 |
| **LOD (µg/ml)** | 0.92 | 1.04 | 0.66 | 0.37 | | | | |
| **LOQ(µg/ml)** | 2.78 | 3.14 | 2.01 | 1.12 | | | | |
| **Accuracy** | 99.28-100.13 | 100.44-100.33 | 100.55-100.00 | 99.92-100.35 | 98.95-99.96 | 98.38-99.71 | 98.89-100.14 | 99.32-100.91 |
| **Precision (%RSD)** Method Precision | 0.80 | 1.07 | 0.78 | 0.34 | 0.11 | 0.19 | 0.55 | 0.09 |
| **Ruggedness (%RSD)** | 0.16-0.52 | 0.20-0.37 | 0.43-0.70 | 0.40-0.75 | | | | |
| **Robustness (%RSD)** | 0.74-0.59 | 1.16-0.77 | 0.73-1.66 | 1.23-0.63 | | | | |

## CONCLUSIONS:

1.A new RP-HPLC method for the simultaneous estimation of Emtricitabine, Tenofovir, Cobicistat and Elvitegravir in their combine dosage form was developed and validated as per the ICH guidelines.

2. Linearity was observed in the range of 20-120µg/ml for Emtricitabine, 30-180µg/ml for Tenofovir, 15-90µg/ml for Cobicistat and 15-90µg/ml for Elvitegravir with correlation coefficients ($r^2$=0.999).

3. The percentage recoveries of Emtricitabine, Tenofovir, Cobicistat and Elvitegravir were in the range of 98.55-101.4% which was within the acceptance criteria.

4. The percentage RSD was NMT 2% which proved the precision of the developed method.

5. The developed method is simple, sensitive, rapid, linear, precise, rugged, accurate, specific, and robust.

6. The developed method was found superior in certain respects such as RT and Accuracy. The method was more economical when compared to reported method.

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