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A novel validated RP-UPLC-DAD method for the simultaneous estimation of Emtricitabine, Tenofovir Disoproxil Fumarate, Cobicistat and Elvitegravir in bulk and tablet dosage form with forced degradation studies

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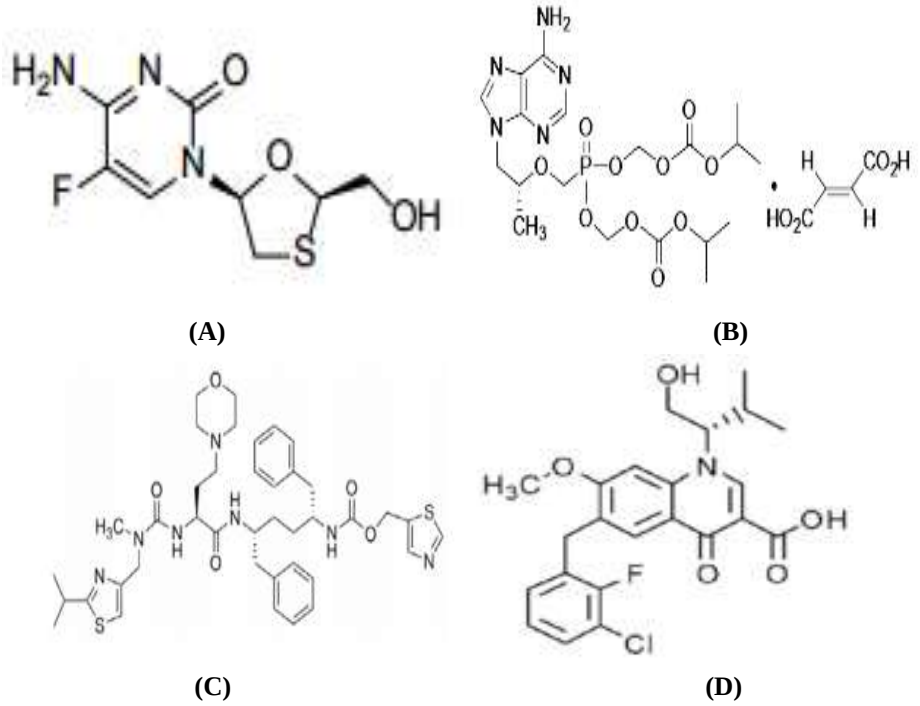
Abstract: *The aim of the present work was to develop and validate a rapid Reverse Phase Ultra Performance Liquid Chromatographic method for the simultaneous estimation of Emtricitabine, Tenofovir Disoproxil Fumarate, Cobicistat and Elvitegravir in its bulk and tablet dosage form with forced degradation studies. The separation was performed by ACQUITY UPLC BEH C_{18} (100 mm×2.1 mm, 1.7 µm particle size) column, Waters ACQUITY UPLC system with PDA detector and mobile phase contained a mixture of 0.01M Ammonium acetate (pH adjusted to 7.5 with ammonium hydroxide) and Acetonitrile (45:55, v/v). The flow rate was set to 0.25 mL/min with responses measured at 268 nm. The retention time of Emtricitabine, Tenofovir Disoproxil Fumarate, Cobicistat and Elvitegravir was 0.904 min, 1.240 min, 2.615 min and 3.801 min with resolution of 4.05, 13.02 and 8.27 respectively. Linearity was established in the range of 20-100 µg/mL for Emtricitabine, 30-150 µg/mL for Tenofovir Disoproxil Fumarate, 15-75 µg/mL for Cobicistat and 15-75 µg/mL for Elvitegravir with correlation coefficients (r^2=0.999). The percentage recoveries were between 99.55-99.96%, 100.04-100.07%, 99.86-100.09% and 99.95-100.19% for Emtricitabine, Tenofovir Disoproxil Fumarate, Cobicistat and Elvitegravir respectively. Validation parameters were evaluated according to the International Conference on Harmonization (ICH) Q2 R1 guidelines. The forced degradation studies were performed by using HCl, NaOH, H_2O_2, thermal and UV radiation. Emtricitabine are more sensitive towards alkaline hydrolysis degradation condition, Tenofovir Disoproxil Fumarate is more sensitive towards oxidative degradation condition, Cobicistat are more sensitive towards alkaline hydrolysis degradation condition and Elvitegravir are more sensitive towards acidic hydrolysis degradation condition. The developed method was successfully applied for the quantification and hyphenated instrumental analysis.*

Keywords: *Emtricitabine, Tenofovir Disoproxil Fumarate, Cobicistat, Elvitegravir, UPLC, PDA detector, Hyphenated and ICH.*

I. Introduction

Emtricitabine, Tenofovir Disoproxil Fumarate, Cobicistat and Elvitegravir combined dosage form is used for the treatment of HIV-1infection in adult patients[1]. Emtricitabine, a synthetic nucleoside analog of cytidine, is phosphorylated by cellular enzymes to form emtricitabine 5'-triphosphate. Emtricitabine is 5-fluoro-1-[(2R, 5S)-2- (hydroxyl methyl)-1, 3-oxathiolan-5-yl] cytosine were shown in figure 1A. Emtricitabine 5'-triphosphate inhibits the activity of the HIV-1 reverse transcriptase by competing with the natural substrate deoxycytidine 5'-triphosphate and by being incorporated into nascent viral DNA which results in chain termination. Emtricitabine 5′-triphosphate is a weak inhibitor of mammalian DNA polymerases α, β, ε, and mitochondrial DNA polymerase γ. Tenofovir Disoproxil Fumarate is a fumaric acid salt of the bis iso propoxy carbonyl oxy methyl ester derivative of tenofovir. Tenofovir Disoproxil Fumarate is 9-[(R)-2-[[bis [[(iso propoxy carbonyl) oxy] - methoxy] phosphinyl] methoxy] propyl] adenine fumarate were shown in figure 1B. Tenofovir Disoproxil Fumarate is an acyclic nucleoside phosphonate diester analog of adenosine monophosphate. Tenofovir Disoproxil Fumarate requires initial diester hydrolysis for conversion to tenofovir and subsequent phosphorylations by cellular enzymes to form tenofovir diphosphate. Tenofovir diphosphate inhibits the activity of HIV-1 reverse transcriptase by competing with the natural substrate deoxyadenosine 5′-triphosphate and after incorporation into DNA, by DNA chain termination. Tenofovir diphosphate is a weak inhibitor of mammalian DNA polymerases α, β, and mitochondrial DNA polymerase γ. Cobicistat is 1,3-thiazol-5-ylmethyl [(2R,5R)-5-{[(2S)-2-[(methyl{[2-(propan-2-yl)-1,3-thiazol-4-yl] methyl} carbamoyl) amino]-4-(morpholin-4yl) butanoyl] amino}-1,6-diphenylhexan-2-yl] carbamate were shown in figure 1C. Cobicistat is a

selective, mechanism-based inhibitor of cytochromes P450 of the CYP3A subfamily. Inhibition of CYP3A-mediated metabolism by cobicistat enhances the systemic exposure of CYP3A substrates, such as elvitegravir, where bioavailability is limited and half-life is shortened by CYP3A-dependent metabolism. Elvitegravir is 6-(3-Chloro-2-fluorobenzyl)-1-[(2S)-1-hydroxy-3-methylbutan-2-yl]-7-methoxy-4-oxo-1,4dihydro quinoline-3-carboxylic acid were shown in figure 1D. Elvitegravir inhibits the strand transfer activity of HIV-1 integrase (integrase strand transfer inhibitor; INSTI), an HIV-1 encoded enzyme that is required for viral replication. Inhibition of integrase prevents the integration of HIV-1 DNA into host genomic DNA, blocking the formation of the HIV-1 provirus and propagation of the viral infection. Elvitegravir does not inhibit human topoisomerases I or II [2].



(A) **(B)**

(C) **(D)**

Figure 1: Chemical structure of (A) Emtricitabine (B) Tenofovir Disoproxil Fumarate (C) Cobicistat (D) Elvitegravir

Literature survey reveals that many analytical methods are reported for determination of Emtricitabine, Tenofovir Disoproxil Fumarate, Cobicistat and Elvitegravir individually and with other combinations which includes high performance liquid chromatography (HPLC)[3-18], liquid chromatography-mass spectrophotometry (LC-MS)[19,20], UV-Spectrophotometry[21] and high performance thin layer chromatography (HPTLC)[22] methods. However, no method is reported for simultaneous estimation of Emtricitabine, Tenofovir Disoproxil Fumarate, Cobicistat and Elvitegravir in combined dosage form by Reversed Phase Ultra Performance Liquid Chromatography (UPLC) with forced degradation studies. The present study was aimed to develop a novel and validated Reversed Phase Ultra Performance Liquid Chromatography (UPLC) method for the simultaneous estimation of Emtricitabine, Tenofovir Disoproxil Fumarate, Cobicistat and Elvitegravir in bulk and pharmaceutical dosage form with forced degradation studies according to ICH guidelines [23].

1. Experimental

2.1 Chemicals and reagents

Emtricitabine (API) and Tenofovir Disoproxil Fumarate (API) were obtained from Hetero Drugs Limited, Hyderabad, India. Cobicistat (API) and Elvitegravir (API) were obtained from Shilpa Medicare Limited, India. HPLC grade of Ammonium Acetate was obtained from Rankem Ltd., India and HPLC grade of Acetonitrile was obtained from Merck Specialities Private Limited, India. HPLC grade of Water and Ammonium hydroxide was obtained from Rankem Ltd., India. Stribild (Emtricitabine, Tenofovir Disoproxil Fumarate, Cobicistat and Elvitegravir) contains 200 mg of Emtricitabine, 300 mg of Tenofovir Disoproxil Fumarate, 150 mg of Cobicistat and 150 mg of Elvitegravir were kindly supplied by Gilead Sciences, Inc.

2.2 Instrumentation

The analysis was performed by using a chromatographic system from Waters Acquity UPLC system with PDA detector. The UPLC system was equipped with Empower 2 software. Semi-micro analytical balance (India), Ultrasonic bath sonicator (Frontline FS 4, Mumbai, India), Digital pH meter (Systronics model 802) and Whatmann filter paper No. 41 (Whatmann International Ltd., England) were used in the study.

2.3 Selection of wavelength

In simultaneous estimation of Emtricitabine, Tenofovir Disoproxil Fumarate, Cobicistat and Elvitegravir isosbestic wavelength is used. Standard stock solutions of Emtricitabine, Tenofovir Disoproxil Fumarate, Cobicistat and Elvitegravir were prepared by dissolving 200 mg of Emtricitabine, 300 mg of Tenofovir Disoproxil Fumarate, 150 mg of Cobicistat and 150 mg of Elvitegravir in 100 ml of diluent into a 100 ml clean dry volumetric flask and the standard solutions was filtered through 0.45 µm nylon membrane filter and degassed by sonicator to get the concentration of 2000 µg/mL of Emtricitabine, 3000 µg/mL of Tenofovir Disoproxil Fumarate, 1500 µg/mL of Cobicistat and 1500 µg/mL of Elvitegravir. From the above standard stock solution of 2000 µg/mL of Emtricitabine, 3000 µg/mL of Tenofovir Disoproxil Fumarate, 1500 µg/mL of Cobicistat and 1500 µg/mL of Elvitegravir further pipette 1 mL and transferred into a 100 mL volumetric flask and dilute up to the mark with diluent to get the concentration of 20 µg/mL of Emtricitabine, 30 µg/mL of Tenofovir Disoproxil Fumarate, 15 µg/mL of Cobicistat and 15 µg/mL of Elvitegravir. The wavelength of maximum absorption (λmax) of 20 µg/mL of Emtricitabine, 30 µg/mL of Tenofovir Disoproxil Fumarate, 15 µg/mL of Cobicistat and 15 µg/mL of Elvitegravir were scanned using UV-Visible spectrophotometer within the wavelength region of 200–400 nm against mobile phase as blank. The isosbestic wavelength (λmax) was found to be 268 nm for the combination shown in figure 2.

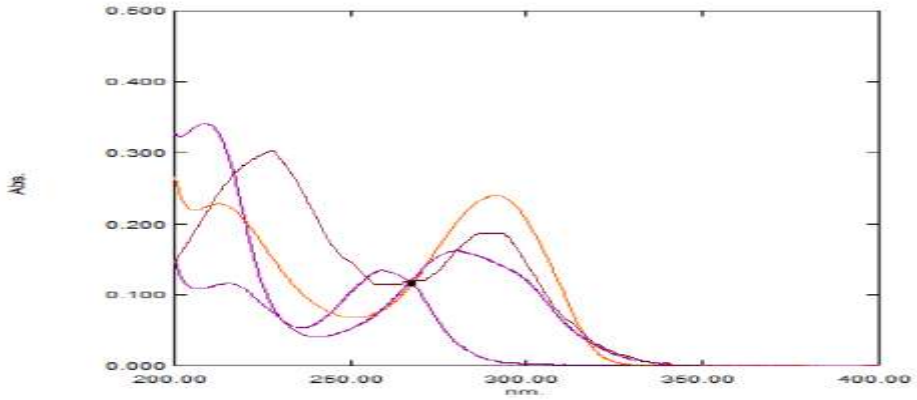


Figure 2: Isosbestic point of Emtricitabine, Tenofovir Disoproxil Fumarate, Cobicistat and Elvitegravir at 268 nm.

2.4 Chromatographic conditions

Emtricitabine, Tenofovir Disoproxil Fumarate, Cobicistat and Elvitegravir were analyzed in ACQUITY UPLC BEH C_{18} (100 mm×2.1 mm, 1.7 µm particle size) column for the chromatographic separation. The mobile phase was composed of 0.01M Ammonium acetate (pH adjusted to 7.5 with ammonium hydroxide) and Acetonitrile (45:55, v/v). Filtered through 0.45µm nylon membrane filter under vacuum filtration and pumped at ambient temperature, at a flow rate of 0.25 mL/min with UV detection wavelength at 268 nm. Injection volume was 20µL. The run time was 8 min and the retention time of Emtricitabine, Tenofovir Disoproxil Fumarate, Cobicistat and Elvitegravir was 0.904 min, 1.240 min, 2.615 min and 3.801 min with resolution of 4.05, 13.02 and 8.27 respectively.

Chromatographic Parameters:

Equipment : Waters Acquity UPLC system with PDA detector
Column : Acquity UPLC BEH C_{18} (100 mm×2.1 mm, 1.7 µm particle size)
Flow rate : 0.25 mL/min
Wavelength : 268 nm
Injection volume : 20 µL
Column oven : Ambient
Run time : 8 Minutes

2.5 Solutions and sample preparation

2.5.1 Preparation of Ammonium acetate buffer

A 0.01 M Ammonium acetate buffer was prepared by dissolving 0.77 gram of Ammonium acetate in 1000 mL of HPLC grade water and pH was adjusted to 7.5 with ammonium hydroxide. The buffer was filtered through 0.45 µm nylon membrane filter to remove all fine particles and gases.

2.5.2 Preparation of mobile phase

The above prepared 0.01 M Ammonium acetate buffer and Acetonitrile HPLC grade were mixed in the proportion of 45:55, v/v and was filtered through 0.45 µm nylon membrane filter and degassed by sonication.

2.5.3 Preparation of diluent

Mobile phase was used as diluent.

2.5.4 Preparation of standard stock solutions of Emtricitabine, Tenofovir Disoproxil Fumarate, Cobicistat and Elvitegravir

Standard stock solutions of Emtricitabine, Tenofovir Disoproxil Fumarate, Cobicistat and Elvitegravir were prepared by dissolving 200 mg of Emtricitabine, 300 mg of Tenofovir Disoproxil Fumarate, 150 mg of Cobicistat and 150 mg of Elvitegravir in 100 mL of diluent into a 100 mL clean dry volumetric flask and the standard solutions was filtered through 0.45 µm nylon membrane filter and degassed by sonicator to get the concentration of 2000 µg/mL of Emtricitabine, 3000 µg/mL of Tenofovir Disoproxil Fumarate, 1500 µg/mL of Cobicistat and 1500 µg/mL of Elvitegravir.

2.5.5 Preparation of standard solutions of Emtricitabine, Tenofovir Disoproxil Fumarate, Cobicistat and Elvitegravir for assay

From the above standard stock solution of 2000 µg/mL of Emtricitabine, 3000 µg/mL of Tenofovir Disoproxil Fumarate, 1500 µg/mL of Cobicistat and 1500 µg/mL of Elvitegravir further pipette 3 mL and transferred into a 100 mL volumetric flask and dilute up to the mark with diluent to get the concentration of 60 µg/mL of Emtricitabine, 90 µg/mL of Tenofovir Disoproxil Fumarate, 45 µg/mL of Cobicistat and 45 µg/mL of Elvitegravir.

2.5.6 Preparation of sample solutions of Emtricitabine, Tenofovir Disoproxil Fumarate, Cobicistat and Elvitegravir

Stribild (Emtricitabine, Tenofovir Disoproxil Fumarate, Cobicistat and Elvitegravir) contains equivalent amount of 200 mg of Emtricitabine, 300 mg of Tenofovir Disoproxil Fumarate, 150 mg of Cobicistat and 150 mg of Elvitegravir were taken into 100 mL clean dry volumetric flask, diluent was added and sonicated to dissolve it completely and was filtered through 0.45 µm nylon membrane filter and volume was made up to the mark with the same diluent. Further pipette out 3 mL from the above Emtricitabine, Tenofovir Disoproxil Fumarate, Cobicistat and Elvitegravir sample stock solution into a 100 mL volumetric flask and diluted up to the mark with diluent to get the concentration of 60 µg/mL of Emtricitabine, 90 µg/mL of Tenofovir Disoproxil Fumarate, 45 µg/mL of Cobicistat and 45 µg/mL of Elvitegravir. 20 µL from standard and sample solution were injected into the chromatographic system and the peak areas were measured for Emtricitabine, Tenofovir Disoproxil Fumarate, Cobicistat and Elvitegravir which was shown in figure 3 and 4 and the assay % was calculated by comparing the peak area of standard and sample chromatogram by using the formula given below and the assay results was shown in Table 1.

$$\text{Assay \%} = \frac{AT}{AS} \times \frac{WS}{DS} \times \frac{DT}{WT} \times \frac{P}{100} \times \frac{\text{Avg. Wt}}{\text{Label Claim}} \times 100$$

Where:

AT = Average peak area of sample preparation

AS= Average peak area of standard preparation

WS = Weight of standard taken in mg

WT=Weight of sample taken in mg

P = Percentage purity of working standard

DS= Dilution factor for standard preparation

DT=Dilution factor for sample preparation

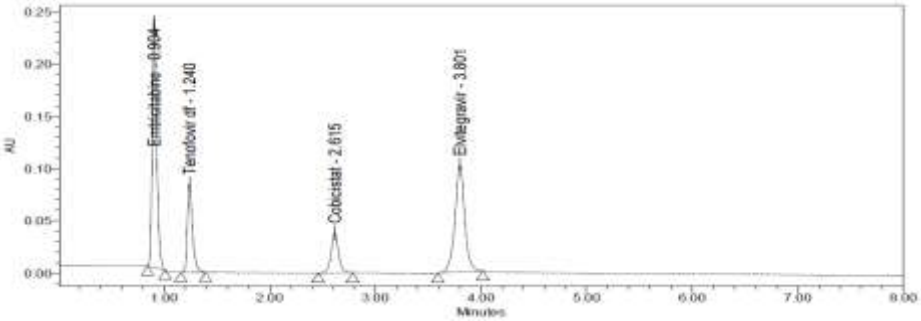


Figure 3: Standard chromatogram of Emtricitabine, Tenofovir Disoproxil Fumarate, Cobicistat and Elvitegravir.

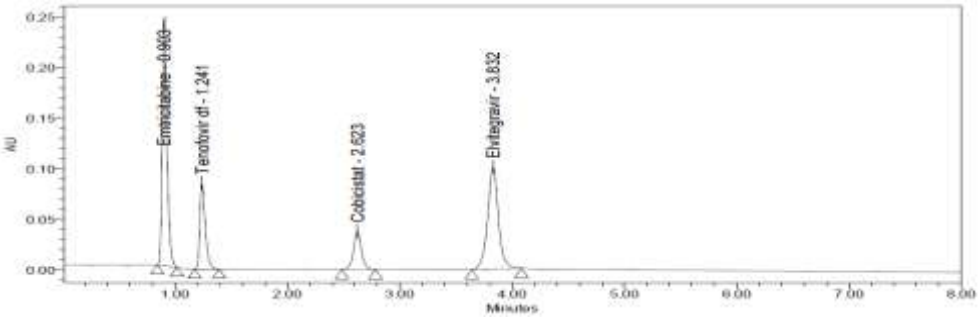


Figure 4: Sample chromatogram of Emtricitabine, Tenofovir Disoproxil Fumarate, Cobicistat and Elvitegravir.

Table 1. Assay of marketed formulation of Emtricitabine, Tenofovir Disoproxil Fumarate, Cobicistat and Elvitegravir.

Drug	**Stribild Label Claim (mg)**	**Amount Found (mg) (n=6)**	**Label Claim % ± RSD % (n=6)**
Emtricitabine	200	200.51	100.26± 0.4
Tenofovir Disoproxil Fumarate	300	298.66	99.55± 0.23
Cobicistat	150	149.95	99.96±0.43
Elvitegravir	150	150.10	100.07±1.00

2.6 Method validation

The developed method for the simultaneous estimation of Emtricitabine, Tenofovir Disoproxil Fumarate, Cobicistat and Elvitegravir was validated as per the ICH guidelines for the parameters like system suitability, specificity, linearity, accuracy, precision, ruggedness, robustness, limit of detection (LOD) and limit of quantitation (LOQ) [23].

II. Results

3.1 UPLC method development

To optimize the UPLC parameters, a number of commercially available UPLC columns and various mobile phases were evaluated for its chromatographic behavior of Emtricitabine, Tenofovir Disoproxil Fumarate, Cobicistat and Elvitegravir. A satisfactory separation and good peak symmetry for Emtricitabine, Tenofovir Disoproxil Fumarate, Cobicistat and Elvitegravir were obtained with ACQUITY UPLC BEH C_{18} (100 mm×2.1 mm, 1.7 µm particle size) column, Waters ACQUITY UPLC system with PDA detector and mobile phase contained a mixture of 0.01 M Ammonium acetate buffer (pH adjusted to 7.5 with ammonium hydroxide) and Acetonitrile (45:55, v/v) was delivered at a flow rate of 0.25 mL/min to get better reproducibility and repeatability. Quantification was achieved with PDA detection at 268 nm based on peak area.

3.2 UPLC method validation

3.2.1 System suitability

At first the UPLC system was optimized as per the chromatographic conditions. One blank followed by six replicates of a single calibration standard solution of 60 µg/mL of Emtricitabine, 90 µg/mL of Tenofovir Disoproxil Fumarate, 45 µg/mL of Cobicistat and 45 µg/mL of Elvitegravir was injected to check the system suitability. To ascertain the system suitability for the proposed method, the parameters such as retention time, theoretical plates, peak asymmetry and resolution were taken and results were presented in Table 2.

Table 2. System suitability parameters for Emtricitabine, Tenofovir Disoproxil Fumarate, Cobicistat and Elvitegravir.

Parameter (n=6)	Emtricitabine	Tenofovir Disoproxil Fumarate	Cobicistat	Elvitegravir
Retention Time (Minutes)	0.904	1.240	2.615	3.801
Theoretical plates	2117	3261	7064	8757
Tailing factor	1.41	1.46	1.02	0.98
Resolution		4.05	13.02	8.27

3.2.2 Specificity

The effect of excipients and other additives usually present in the combined dosage form of Emtricitabine, Tenofovir Disoproxil Fumarate, Cobicistat and Elvitegravir in the determination under optimum conditions was investigated. The specificity of the UPLC method was established by injecting the blank and placebo solution into the UPLC system. The representative chromatogram of blank and placebo was shown in figure 5 and 6.

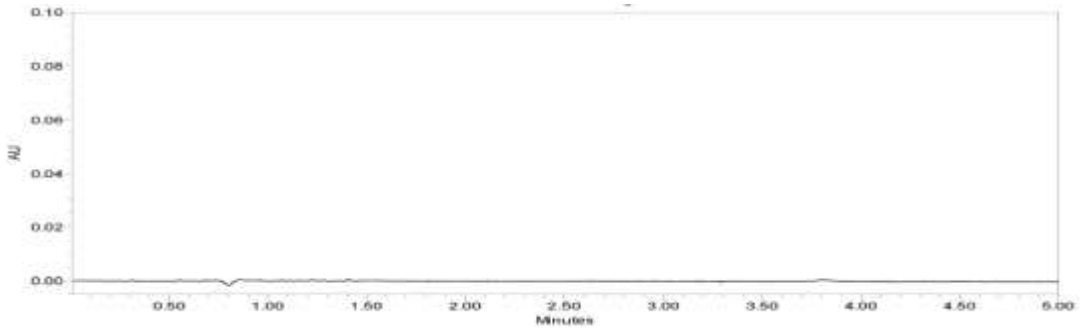


Figure 5: Chromatogram of blank.

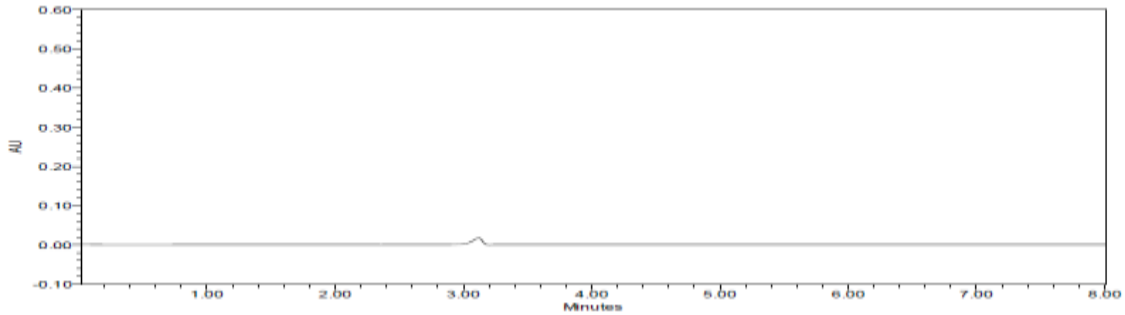


Figure 6: Chromatogram of placebo.

3.2.3 Linearity and range for Emtricitabine, Tenofovir Disoproxil Fumarate, Cobicistat and Elvitegravir

Aliquots of 0.1, 0.2, 0.3, 0.4 and 0.5 mL of mixed standard working solutions of Emtricitabine, Tenofovir Disoproxil Fumarate, Cobicistat and Elvitegravir was pipette out from the standard stock solution of 2000 µg/mL of Emtricitabine, 3000 µg/mL of Tenofovir Disoproxil Fumarate, 1500 µg/mL of Cobicistat and 1500 µg/mL of Elvitegravir and transferred into a series of 10ml clean dry volumetric flask and make volume up to the mark with the same diluent to get the concentration of 20, 40, 60, 80 and 100 µg/mL of Emtricitabine, 30, 60, 90, 120 and 150 µg/mL of Tenofovir Disoproxil Fumarate, 15, 30, 45, 60 and 75 µg/mL of Cobicistat and 15, 30, 45, 60 and 75 µg/mL of Elvitegravir. The calibration standard solutions of Emtricitabine, Tenofovir Disoproxil Fumarate, Cobicistat and Elvitegravir were injected using a 20 µL Hamilton Rheodyne injector and the chromatograms were recorded at 268 nm and a calibration graph was obtained by plotting peak area versus concentration of Emtricitabine, Tenofovir Disoproxil Fumarate, Cobicistat and Elvitegravir respectively. The linearity data is presented in figure 7 and Table 3. Acceptance Criteria: Correlation coefficient should be not less than 0.999.

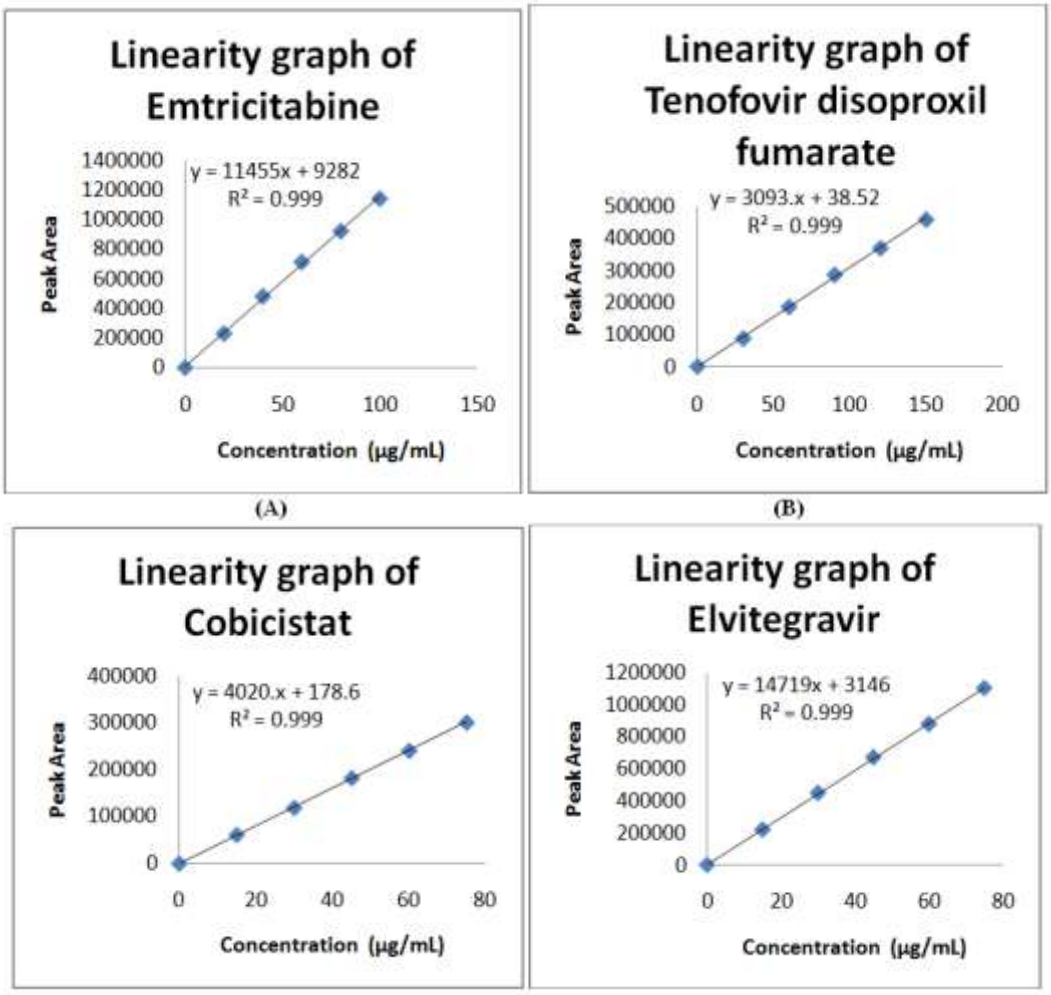


Figure 7: Linearity graph of (A) Emtricitabine (B) Tenofovir Disoproxil Fumarate (C) Cobicistat (D) Elvitegravir

Table 3. Linearity data for Emtricitabine, Tenofovir Disoproxil Fumarate, Cobicistat and Elvitegravir.

Linearity of Emtricitabine		**Linearity of Tenofovir Disoproxil Fumarate**	
Concentration (µg/mL)	Peak Area	Concentration (µg/mL)	Peak Area
20	230537	30	87796
40	482835	60	186776
60	713994	90	287190
80	922636	120	370559
100	1142222	150	459815
Linearity of Cobicistat		**Linearity of Elvitegravir**	
Concentration (µg/mL)	Peak Area	Concentration (µg/mL)	Peak Area
15	61237	15	221116
30	118996	30	450161
45	182791	45	673388
60	241282	60	881031
75	301356	75	1104853

3.2.4 Accuracy studies for Emtricitabine, Tenofovir Disoproxil Fumarate, Cobicistat and Elvitegravir

The accuracy of the method was determined by calculating recovery of Emtricitabine, Tenofovir Disoproxil Fumarate, Cobicistat and Elvitegravir by the method of standard addition. Known amount of standard solution of Emtricitabine, Tenofovir Disoproxil Fumarate, Cobicistat and Elvitegravir at 50%, 100% and 150% was added to a pre quantified sample solution and injected into the UPLC system. The mean percentage recovery of Emtricitabine, Tenofovir Disoproxil Fumarate, Cobicistat and Elvitegravir at each level was calculated and the results were presented in Table 4.

3.2.4.1 Preparation of pre quantified sample solution for accuracy studies

Stribild (Emtricitabine, Tenofovir Disoproxil Fumarate, Cobicistat and Elvitegravir) contains equivalent amount of 200 mg of Emtricitabine, 300 mg of Tenofovir Disoproxil Fumarate, 150 mg of Cobicistat and 150 mg of Elvitegravir were taken into 100 mL clean dry volumetric flask, diluent was added and sonicated to dissolve it completely and was filtered through 0.45 µm nylon membrane filter and volume was made up to the mark with the same diluent. Further pipette out 0.2 mL from the above Emtricitabine, Tenofovir Disoproxil Fumarate, Cobicistat and Elvitegravir sample stock solution into a 10 mL volumetric flask and diluted up to the mark with diluent to get the concentration of 40 µg/mL of Emtricitabine, 60 µg/mL of Tenofovir Disoproxil Fumarate, 30 µg/mL of Cobicistat and 30 µg/mL of Elvitegravir.

3.2.4.2 Preparation of standard solution of Emtricitabine, Tenofovir Disoproxil Fumarate, Cobicistat and Elvitegravir for accuracy studies

Standard stock solutions of Emtricitabine, Tenofovir Disoproxil Fumarate, Cobicistat and Elvitegravir were prepared by dissolving 200 mg of Emtricitabine, 300 mg of Tenofovir Disoproxil Fumarate, 150 mg of Cobicistat and 150 mg of Elvitegravir in 100 mL of diluent into a 100 mL clean dry volumetric flask and the standard solutions was filtered through 0.45 µm nylon membrane filter and degassed by sonicator to get the concentration of 2000 µg/mL of Emtricitabine, 3000 µg/mL of Tenofovir Disoproxil Fumarate, 1500 µg/mL of Cobicistat and 1500 µg/mL of Elvitegravir.

a.) Preparation of 50% standard solution

From the standard stock solution of 2000 µg/mL of Emtricitabine, 3000 µg/mL of Tenofovir Disoproxil Fumarate, 1500 µg/mL of Cobicistat and 1500 µg/mL of Elvitegravir further pipette 0.1 mL and transferred into a 10 mL volumetric flask and dilute up to the mark with diluent to get the concentration of 20 µg/mL of Emtricitabine, 30 µg/mL of Tenofovir Disoproxil Fumarate, 15 µg/mL of Cobicistat and 15 µg/mL of Elvitegravir.

b.) Preparation of 100% standard solution

From the standard stock solution of 2000 µg/mL of Emtricitabine, 3000 µg/mL of Tenofovir Disoproxil Fumarate, 1500 µg/mL of Cobicistat and 1500 µg/mL of Elvitegravir further pipette 0.2 mL and transferred into a 10 mL volumetric flask and dilute up to the mark with diluent to get the concentration of 40 µg/mL of Emtricitabine, 60 µg/mL of Tenofovir Disoproxil Fumarate, 30 µg/mL of Cobicistat and 30 µg/mL of Elvitegravir.

c.) Preparation of 150% standard solution

From the standard stock solution of 2000 µg/mL of Emtricitabine, 3000 µg/mL of Tenofovir Disoproxil Fumarate, 1500 µg/mL of Cobicistat and 1500 µg/mL of Elvitegravir further pipette 0.3 mL and transferred into a 10 mL volumetric flask and dilute up to the mark with diluent to get the concentration of 60 µg/mL of Emtricitabine, 90 µg/mL of Tenofovir Disoproxil Fumarate, 45 µg/mL of Cobicistat and 45 µg/mL of Elvitegravir. Acceptance Criteria**:** The Recovery % for each level should be between 98.0 to 102.0%.

Table 4. Recovery studies of Emtricitabine, Tenofovir DF, Cobicistat and Elvitegravir.

Recovery study data of Emtricitabine				
Sample name	Amount added (µg/mL)	Amount found (µg/mL)	Recovery %	Statistical Analysis
S_1:50%	20	19.97	99.86	Mean=99.83%(n=3)
S_2:50%	20	19.92	99.60	S.D=0.22
S_3:50%	20	20.01	100.03	RSD %=0.22
S_4:100%	40	40.02	100.04	Mean=99.96%(n=3)
S_5:100%	40	39.93	99.82	S.D=0.12
S_6:100%	40	40.01	100.02	RSD %=0.12
S_7:150%	60	59.77	99.61	Mean=99.55%(n=3)
S_8:150%	60	59.63	99.38	S.D=0.15
S_9 :150%	60	59.79	99.65	RSD %=0.15
Recovery study data of Tenofovir Disoproxil Fumarate				
Sample name	Amount added (µg/mL)	Amount found (µg/mL)	Recovery %	Statistical Analysis
S_1:50%	30	30.05	100.18	Mean=100.04%(n=3)
S_2:50%	30	29.93	99.78	S.D=0.22
S_3:50%	30	30.05	100.15	RSD %=0.22
S_4:100%	60	60.13	100.21	Mean=100.07%(n=3)
S_5:100%	60	59.96	99.93	S.D=0.14
S_6:100%	60	60.03	100.05	RSD %=0.14
S_7:150%	90	90.04	100.04	Mean=100.04%(n=3)
S_8:150%	90	90.06	100.07	S.D=0.03
S_9 :150%	90	90.01	100.02	RSD %=0.03

Recovery study data of Cobicistat				
Sample name	Amount added (µg/mL)	Amount found (µg/mL)	Recovery %	Statistical Analysis
S_1:50%	15	15.05	100.34	Mean=100.09%(n=3)
S_2:50%	15	14.98	99.85	S.D=0.24
S_3:50%	15	15.01	100.08	RSD %=0.24
S_4:100%	30	30.07	100.22	Mean=99.86%(n=3)
S_5:100%	30	29.87	99.57	S.D=0.33
S_6:100%	30	29.94	99.79	RSD %=0.33
S_7:150%	45	45.06	100.13	Mean=99.93%(n=3)
S_8:150%	45	45.02	100.03	S.D=0.28
S_9 :150%	45	44.82	99.61	RSD %=0.28
Recovery study data of Elvitegravir				
Sample name	Amount added (µg/mL)	Amount found (µg/mL)	Recovery %	Statistical Analysis
S_1:50%	15	14.98	99.89	Mean=100.06%(n=3)
S_2:50%	15	15.03	100.19	S.D=0.15
S_3:50%	15	15.01	100.09	RSD %=0.15
S_4:100%	30	30.01	100.05	Mean=99.95%(n=3)
S_5:100%	30	30.04	100.14	S.D=0.25
S_6:100%	30	29.90	99.67	RSD %=0.25
S_7:150%	45	44.98	99.96	Mean=100.19%(n=3)
S_8:150%	45	45.20	100.44	S.D=0.24
S_9 :150%	45	45.07	100.16	RSD %=0.24

3.2.5 Precision studies for Emtricitabine, Tenofovir Disoproxil Fumarate, Cobicistat and Elvitegravir

3.2.5.1 Method precision (Repeatability)

Stribild (Emtricitabine, Tenofovir Disoproxil Fumarate, Cobicistat and Elvitegravir) contains equivalent amount of 200 mg of Emtricitabine, 300 mg of Tenofovir Disoproxil Fumarate, 150 mg of Cobicistat and 150 mg of Elvitegravir were taken into 100 mL clean dry volumetric flask, diluent was added and sonicated to dissolve it completely and was filtered through 0.45 µm nylon membrane filter and volume was made up to the mark with the same diluent. Further pipette out 3 mL from the above Emtricitabine, Tenofovir Disoproxil Fumarate, Cobicistat and Elvitegravir sample stock solution into a 100 mL volumetric flask and diluted up to the mark with diluent to get the concentration of 60 µg/mL of Emtricitabine, 90 µg/mL of Tenofovir Disoproxil Fumarate, 45 µg/mL of Cobicistat and 45 µg/mL of Elvitegravir. A homogenous sample of a single batch is analyzed six times and was checked whether the method is giving consistent results. The RSD % for the assay of six replicate injections was calculated as mentioned in Table 5. Acceptance Criteria: The RSD % for the assay of six sample injections should not be more than 2%.

Table 5. Method precision data for Emtricitabine, Tenofovir Disoproxil Fumarate, Cobicistat and Elvitegravir.

Emtricitabine					**Tenofovir Disoproxil Fumarate**			
S.No.	Concentration (µg/mL)	Retention time (min)	Peak Area	Assay %	Concentration (µg/mL)	Retention time (min)	Peak Area	Assay %
1	60	0.926	726133	100.21	90	1.285	289566	99.77
2	60	0.924	723732	99.88	90	1.283	292125	100.65
3	60	0.921	722779	99.75	90	1.281	288450	99.39
4	60	0.923	723780	99.89	90	1.283	288621	99.44
5	60	0.919	723430	99.84	90	1.278	292676	100.84
6	60	0.923	722593	99.72	90	1.279	288870	99.53
Average		0.923	723741	99.88	**Average**	1.282	290051	99.94
SD		0.002422	1269.91	0.18	**SD**	0.002665	1867.09	0.64
RSD %		0.26	0.18	0.18	**RSD %**	0.21	0.64	0.64
Cobicistat					**Elvitegravir**			
S.No.	Concentration (µg/mL)	Retention time (min)	Peak Area	Assay %	Concentration (µg/mL)	Retention time (min)	Peak Area	Assay %
1	45	2.816	187393	99.20	45	4.146	692879	100.62
2	45	2.808	188609	99.84	45	4.142	687276	99.80
3	45	2.807	188603	99.84	45	4.139	696404	101.13
4	45	2.809	187952	99.49	45	4.145	684272	99.37
5	45	2.802	189421	100.27	45	4.139	686231	99.65
6	45	2.794	189247	100.18	45	4.135	682101	99.05
Average		2.806	188538	99.80	**Average**	4.141	688194	99.94
SD		0.007403	767.60	0.41	**SD**	0.004147	5413.98	0.79
RSD %		0.26	0.41	0.41	**RSD %**	0.1	0.79	0.79

3.2.5.2 System precision

The system precision was carried out to ensure that the analytical system is working properly. The standard preparation concentration of 60 µg/mL of Emtricitabine, 90 µg/mL of Tenofovir Disoproxil Fumarate,

45 µg/mL of Cobicistat and 45 µg/mL of Elvitegravir was injected six times into the UPLC system and the RSD % for the area of six replicate injections was calculated as mentioned in Table 6. Acceptance Criteria: The RSD % for the peak area of six standard injections should not be more than 2%.

Table 6. System precision data for Emtricitabine, Tenofovir Disoproxil Fumarate, Cobicistat and Elvitegravir.

Emtricitabine				**Tenofovir Disoproxil Fumarate**		
S.No.	Conc. (µg/mL)	Retention Time (min)	Peak Area	Conc. (µg/mL)	Retention time (min)	Peak Area
1	60	0.926	714733	90	1.285	288383
2	60	0.913	705793	90	1.268	282989
3	60	0.919	710858	90	1.278	288710
4	60	0.919	711211	90	1.278	288776
5	60	0.926	710008	90	1.286	288289
6	60	0.921	704761	90	1.281	284155
Average		0.921	709561	**Average**	1.279	286884
SD		0.004926	3703.8338	**SD**	0.006501	2598.208
RSD %		0.54	0.52	**RSD %**	0.51	0.91
Cobicistat				**Elvitegravir**		
S.No.	Conc. (µg/mL)	Retention Time (min)	Peak Area	Conc. (µg/mL)	Retention time (min)	Peak Area
1	45	2.816	188313	45	3.922	679833
2	45	2.751	186597	45	4.046	676216
3	45	2.802	186341	45	3.930	669763
4	45	2.802	186517	45	3.801	674183
5	45	2.828	187017	45	4.152	660703
6	45	2.807	183515	45	3.792	672501
Average		2.801	186383	**Average**	4.127	672200
SD		0.026427	1576	**SD**	0.039947	6581
RSD %		0.94	0.85	**RSD %**	1.0	0.98

3.2.5.3 Intermediate precision/ruggedness

The intermediate precision (also known as Ruggedness) of the method was evaluated by performing precision on different laboratories by different analysts and different days. The sample preparation concentration of 60 µg/mL of Emtricitabine, 90 µg/mL of Tenofovir Disoproxil Fumarate, 45 µg/mL of Cobicistat and 45 µg/mL of Elvitegravir was injected six times into the UPLC system and the RSD % for the assay of six replicate injections was calculated as mentioned in Table 7 and 8. Acceptance Criteria: The RSD % for the assay of six sample injections should not be more than 2%.

Table 7. Ruggedness data for Emtricitabine and Tenofovir Disoproxil Fumarate.

Ruggedness Data for Emtricitabine								
Laboratory-1 (Assay %)-UPLC-1					Laboratory-2 (Assay %)-UPLC-2			
	Analyst-1		Analyst-2		Analyst-1		Analyst-2	
Conc. (µg/mL)	Day-1	Day-2	Day-1	Day-2	Day-1	Day-2	Day-1	Day-2
60	99.90	100.04	99.95	100.18	99.90	100.05	100.10	100.25
60	100.20	99.69	99.73	99.85	99.83	100.13	100.41	99.96
60	99.71	99.96	100.10	100.23	99.82	100.24	100.01	99.97
60	99.89	99.48	99.62	99.44	100.04	99.90	100.13	100.13
60	99.51	99.49	99.77	99.50	100.05	100.26	100.23	100.19
60	99.58	99.51	99.53	99.55	99.98	99.85	100.25	99.82
Average	99.80	99.70	99.78	99.79	99.94	100.07	100.19	100.05
SD	0.25	0.25	0.21	0.35	0.10	0.17	0.14	0.17
RSD %	0.25	0.25	0.21	0.35	0.10	0.17	0.14	0.16
Intermediate precision within-laboratories variations (n=24)								
Laboratory-1 (Assay %)-UPLC-1					Laboratory-2 (Assay %)-UPLC-2			
Average	99.77				**Average**	100.06		
SD	0.27				**SD**	0.14		
RSD %	0.27				**RSD %**	0.14		
Reproducibility between laboratories (n=48) (Assay %)								
Average	99.92							
SD	0.21							
RSD %	0.21							

Ruggedness Data for Tenofovir Disoproxil Fumarate								
Laboratory-1 (Assay %)-UPLC-1					Laboratory-2 (Assay %)-UPLC-2			
	Analyst-1		Analyst-2		Analyst-1		Analyst-2	
Conc. (µg/mL)	Day-1	Day-2	Day-1	Day-2	Day-1	Day-2	Day-1	Day-2
90	100.33	99.88	99.96	99.61	100.28	100.06	100.09	99.65
90	99.76	99.92	99.61	99.78	99.96	99.65	100.06	100.04

90	99.78	99.70	99.94	99.80	99.98	99.29	100.05	99.72
90	100.21	99.78	99.95	99.86	100.14	99.72	99.76	99.89
90	99.16	99.99	99.69	99.92	100.06	99.37	100.05	100.14
90	99.65	99.75	99.74	99.54	99.98	100.41	100.15	99.69
Average	99.82	99.84	99.82	99.75	100.07	99.75	100.03	99.85
SD	0.42	0.11	0.15	0.15	0.12	0.42	0.14	0.20
RSD %	0.42	0.11	0.15	0.15	0.12	0.42	0.14	0.20
Intermediate precision within-laboratories variations (n=24)								
Laboratory-1 (Assay %)-UPLC-1					Laboratory-2 (Assay %)-UPLC-2			
Average	99.81				**Average**	99.92		
SD	0.21				**SD**	0.22		
RSD %	0.21				**RSD %**	0.22		
Reproducibility between laboratories (n=48) (Assay %)								
Average	99.86							
SD	0.21							
RSD %	0.21							

Table 8. Ruggedness data for Cobicistat and Elvitegravir.

Ruggedness Data for Cobicistat								
Laboratory-1 (Assay %)-UPLC-1					Laboratory-2 (Assay %)-UPLC-2			
	Analyst-1		Analyst-2		Analyst-1		Analyst-2	
Conc. (µg/mL)	Day-1	Day-2	Day-1	Day-2	Day-1	Day-2	Day-1	Day-2
45	99.43	99.82	99.88	100.18	100.23	100.06	100.14	99.86
45	100.30	100.27	100.15	100.15	100.24	100.27	100.01	99.75
45	99.89	100.07	99.96	99.54	100.12	99.54	100.06	100.04
45	99.69	100.11	99.95	100.11	99.95	99.74	100.08	100.11
45	99.27	100.07	100.24	99.75	99.86	100.07	99.43	99.89
45	100.22	99.98	100.03	99.60	99.89	99.92	100.23	100.23
Average	99.80	100.05	100.03	99.89	100.05	99.93	99.99	99.98
SD	0.42	0.15	0.14	0.29	0.17	0.26	0.28	0.18
RSD %	0.42	0.15	0.14	0.29	0.17	0.26	0.28	0.18
Intermediate precision within-laboratories variations (n=24)								
Laboratory-1 (Assay %)-UPLC-1					Laboratory-2 (Assay %)-UPLC-2			
Average	99.94				**Average**	99.99		
SD	0.25				**SD**	0.22		
RSD %	0.25				**RSD %**	0.22		
Reproducibility between laboratories (n=48) (Assay %)								
Average	99.97							
SD	0.24							
RSD %	0.24							

Ruggedness Data for Elvitegravir								
Laboratory-1 (Assay %)-UPLC-1					Laboratory-2 (Assay %)-UPLC-2			
	Analyst-1		Analyst-2		Analyst-1		Analyst-2	
Conc. (µg/mL)	Day-1	Day-2	Day-1	Day-2	Day-1	Day-2	Day-1	Day-2
45	99.55	99.76	99.75	99.82	99.81	99.95	99.26	99.86
45	99.99	99.79	99.76	99.78	99.76	99.47	99.93	100.06
45	100.08	100.01	100.12	100.01	100.05	99.84	100.04	100.04
45	99.33	99.37	99.71	99.37	99.34	99.81	100.16	100.13
45	100.82	99.57	99.68	99.57	99.75	100.02	100.02	100.32
45	99.02	100.10	100.13	100.10	100.11	99.17	100.18	100.06
Average	99.80	99.77	99.86	99.78	99.80	99.71	99.93	100.08
SD	0.64	0.27	0.21	0.27	0.28	0.33	0.34	0.15
RSD %	0.64	0.27	0.21	0.27	0.28	0.33	0.34	0.15
Intermediate precision within-laboratories variations (n=24)								
Laboratory-1 (Assay %)-UPLC-1					Laboratory-2 (Assay %)-UPLC-2			
Average	99.80				**Average**	99.88		
SD	0.35				**SD**	0.27		
RSD %	0.35				**RSD %**	0.27		
Reproducibility between laboratories (n=48) (Assay %)								
Average	99.84							
SD	0.31							
RSD %	0.31							

3.2.6 Limit of Detection (LOD) and Limit of Quantification (LOQ)

Limit of Detection (LOD) and Limit of Quantification (LOQ) were calculated as 3.3×SD/S and 10×SD/S respectively as per ICH guidelines, Where SD is the standard deviation of the response (Y-intercept) and S is the slope of the calibration curve. The LOD is the smallest concentration of the analyte that gives a measurable response (signal to noise ratio of 3). The LOD of Emtricitabine, Tenofovir Disoproxil Fumarate,

Cobicistat and Elvitegravir was calculated and shown in Table 9. The LOQ is the smallest concentration of the analyte which gives response that can be accurately quantified (signal to noise ratio of 10). The LOQ of Emtricitabine, Tenofovir Disoproxil Fumarate, Cobicistat and Elvitegravir was calculated and shown in Table 9.

Table 9. Summary of validation parameter for Emtricitabine, Tenofovir Disoproxil Fumarate, Cobicistat and Elvitegravir.

Parameters	**UPLC method**			
	Emtricitabine		Tenofovir Disoproxil Fumarate	
Linearity range (µg/mL)	20-100		30-150	
Slope	11455		3093	
Intercept	9282		38.52	
Correlation coefficient	0.999		0.999	
LOD (µg/mL)	0.14		0.45	
LOQ (µg/mL)	0.44		1.36	
Method Precision (RSD %, n=6)	0.18		0.64	
System precision (RSD %, n=6)	0.52		0.91	
	Lab-1	Lab-2	Lab-1	Lab-2
Ruggedness (RSD %, n=24)	0.27	0.14	0.21	0.22
Reproducibility (RSD %, n=48)	0.21		0.21	
Accuracy %	99.55-99.96		100.04-100.07	
Robustness (RSD %, n=6)	Less Flow rate	More Flow rate	Less Flow rate	More Flow rate
	0.21	0.16	0.55	0.23
	Less Organic phase	More Organic phase	Less Organic phase	More Organic phase
	0.02	0.09	0.07	0.11
	UPLC method			
Parameters	Cobicistat		Elvitegravir	
Linearity range (µg/mL)	15-75		15-75	
Slope	4020		14719	
Intercept	178.6		3146	
Correlation coefficient	0.999		0.999	
LOD (µg/mL)	0.37		0.25	
LOQ (µg/mL)	1.12		0.76	
Method Precision (RSD %, n=6)	0.41		0.79	
System precision (RSD %, n=6)	0.85		0.98	
	Lab-1	Lab-2	Lab-1	Lab-2
Ruggedness (RSD %, n=24)	0.25	0.22	0.35	0.27
Reproducibility (RSD %, n=48)	0.24		0.31	
Accuracy %	99.86-100.09		99.95-100.19	
Robustness (RSD %, n=6)	Less Flow rate	More Flow rate	Less Flow rate	More Flow rate
	0.83	0.34	0.84	0.79
	Less Organic phase	More Organic phase	Less Organic phase	More Organic phase
	0.08	0.34	0.03	0.23

3.2.7 Robustness

As part of the Robustness, deliberate change in the flow rate and mobile phase proportion was made to evaluate the impact on the method. The results reveal that the method is robust. The results are summarized in Table 10 and 11.

Table 10. Summary of robustness (Change in flow rate) for Emtricitabine, Tenofovir Disoproxil Fumarate, Cobicistat and Elvitegravir.

Drug	Change in Flow rate (mL/min)	Retention Time (mins)	Change in flow Rate (0.23 mL/min to 0.27 mL/min)				
			Average peak area (n=6)	SD	RSD %	USP Plate Count	Asymmetry
Emtricitabine	0.23	0.923	723555	1505.66	0.21	2077	1.36
	0.25	0.904	698743	1932.22	0.28	2117	1.41
	0.27	0.902	698270	1122.639	0.16	2584	1.39
Tenofovir Disoproxil Fumarate	0.23	1.282	290212	1587.16	0.55	3239	1.4
	0.25	1.240	284295	961.2957	0.34	3261	1.46
	0.27	1.236	283224	638.9368	0.23	3220	1.45
Cobicistat	0.23	2.806	188119	1568.46	0.83	7552	1.01
	0.25	2.615	184005	1313.224	0.71	7064	1.02
	0.27	2.602	183594	619.0691	0.34	7245	1.02
Elvitegravir	0.23	4.141	688068	5760.38	0.84	9205	0.99
	0.25	3.801	680457	2867.54	0.42	8757	0.98
	0.27	3.786	680833	5347.351	0.79	8660	1.01

Table 11. Summary of robustness (Change in mobile phase) for Emtricitabine, Tenofovir Disoproxil Fumarate, Cobicistat and Elvitegravir.

Drug	Change in Mobile Phase	Retention Time (mins)	Change in mobile phase (0.01 M Ammonium acetate buffer (pH adjusted to 7.5 with Ammonium hydroxide) and Acetonitrile) (50:50 v/v to 40:60 v/v)				
			Average peak area (n=6)	SD	RSD %	USP Plate Count	Asymmetry
Emtricitabine	10% less Organic (50:50 v/v)	0.919	713300	169.9478	0.02	2458	1.34
	Actual (45:55 v/v)	0.904	698743	1932.22	0.28	2117	1.41
	10% more Organic (40:60 v/v)	0.911	712761	628.535	0.09	2103	1.4
Tenofovir Disoproxil Fumarate	10% less Organic (50:50 v/v)	1.321	285477	198.5115	0.07	3265	1.42
	Actual (45:55 v/v)	1.240	284295	961.2957	0.34	3261	1.46
	10% more Organic (40:60 v/v)	1.224	287664	325.323	0.11	3159	1.44
Cobicistat	10% less Organic (50:50 v/v)	3.204	188705	154.8974	0.08	7469	0.99
	Actual (45:55 v/v)	2.615	184005	1313.224	0.71	7064	1.02
	10% more Organic (40:60 v/v)	2.439	187682	643.8105	0.34	7078	1.05
Elvitegravir	10% less Organic (50:50 v/v)	4.733	675634	212.3052	0.03	8827	0.97
	Actual (45:55 v/v)	3.801	680457	2867.54	0.42	8757	0.98
	10% more Organic (40:60 v/v)	3.596	686369	1609.987	0.23	9034	1.01

3.2.8 Stability of solution

The RSD % of the assay of Emtricitabine, Tenofovir Disoproxil Fumarate, Cobicistat and Elvitegravir from the solution stability and mobile phase stability experiments was within 2%. The results of the solution and mobile phase stability experiments confirm that the sample solutions and mobile phase used during the assays were stable upto 48 hours at room temperature was calculated and shown in Table 12.

Table 12. Summary of solution stability-effect of P^H of mobile phase (0.01 M Ammonium acetate buffer and Acetonitrile (45:55, v/v) (P^H adjusted to 7.5 with Ammonium hydroxide) for Emtricitabine, Tenofovir Disoproxil Fumarate, Cobicistat and Elvitegravir for 48 hours at room temperature.

	Solution stability for Emtricitabine					
S.No.	Concentration (µg/mL)	Retention time (min)	Peak Area	Assay %	USP Plate Count	Asymmetry
1	60	0.922	713796	99.84	2113	1.41
2	60	0.927	715905	100.14	2108	1.4
3	60	0.919	712443	99.65	2117	1.39
4	60	0.918	713735	99.84	2114	1.41
5	60	0.920	711003	99.45	2116	1.4
6	60	0.921	711485	99.52	2114	1.41
Average		0.921	713061.2	99.74	2114	1.403333
SD		0.003189	1799.105	0.2517	3.141125	0.008165
RSD %		0.35	0.25	0.25	0.15	0.58
Solution stability for Tenofovir Disoproxil Fumarate						
S.No.	Concentration (µg/mL)	Retention time (min)	Peak Area	Assay %	USP Plate Count	Asymmetry
1	90	1.284	289957	100.29	3261	1.41
2	90	1.291	288321	99.73	3278	1.41
3	90	1.282	288393	99.75	3234	1.4
4	90	1.279	289629	100.18	3283	1.42
5	90	1.283	286603	99.13	3265	1.43
6	90	1.284	287797	99.54	3269	1.41
Average		1.284	288450	99.77	3265	1.413333
SD		0.003971	1226.279	0.4241	17.23949	0.010328
RSD %		0.31	0.43	0.43	0.53	0.73
Solution stability for Cobicistat						
S.No.	Concentration (µg/mL)	Retention time (min)	Peak Area	Assay %	USP Plate Count	Asymmetry
1	45	2.835	186307	99.43	7064	1.02
2	45	2.839	187952	100.30	7218	1.03
3	45	2.823	187181	99.89	7223	1.02
4	45	2.822	186797	99.69	7115	1.02
5	45	2.831	186013	99.27	7110	1.01
6	45	2.834	187804	100.22	7213	1.03

Average		2.831	187009	99.80	7157.167	1.021667
SD		0.006831	784.9359	0.4189	69.04322	0.007528
RSD %		0.24	0.42	0.42	0.96	0.74
Solution stability for Elvitegravir						
S.No.	Concentration (µg/mL)	Retention time (min)	Peak Area	Assay %	USP Plate Count	Asymmetry
1	45	4.166	677081	99.70	8757	0.98
2	45	4.175	679055	99.99	8528	0.99
3	45	4.150	679669	100.08	8738	0.99
4	45	4.150	674577	99.33	8629	0.99
5	45	4.166	684694	100.82	8739	0.99
6	45	4.168	672451	99.02	8751	0.98
Average		4.163	677921.2	99.82	8690.333	0.986667
SD		0.010232	4290.078	0.6317	92.60598	0.005164
RSD %		0.25	0.63	0.63	1.06	0.52

3.2.9 Forced degradation studies

3.2.9.1 Acid Degradation Studies

To 1 mL of stock solution of Emtricitabine, Tenofovir Disoproxil Fumarate, Cobicistat and Elvitegravir, 1 mL of 2 N Hydrochloric acid was added and refluxed for 30 mins at 60^0C. The resultant solution was diluted to obtain 60 µg/mL of Emtricitabine, 90 µg/mL of Tenofovir Disoproxil Fumarate, 45 µg/mL of Cobicistat and 45 µg/mL of Elvitegravir solution and 20 µL solutions were injected into the UPLC system and the chromatogram were recorded to assess the stability of sample was shown in figure 8 and purity plot of acid degradation for Emtricitabine, Tenofovir Disoproxil Fumarate, Cobicistat and Elvitegravir was shown in figure 9.

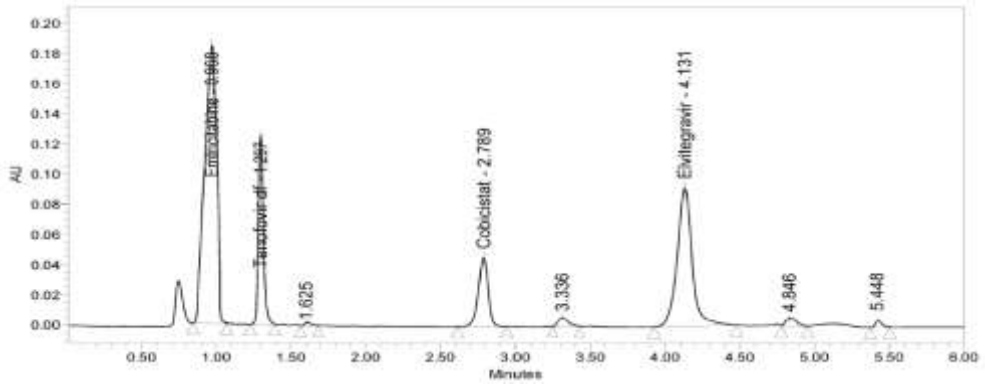
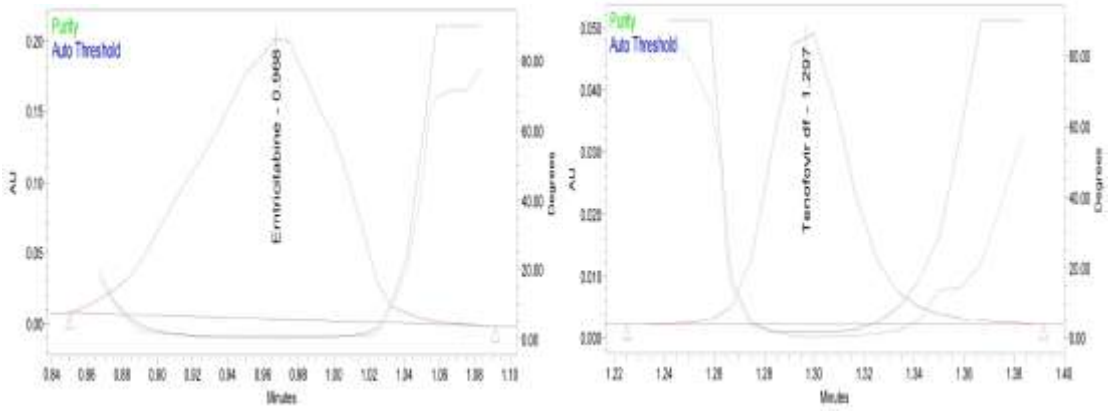
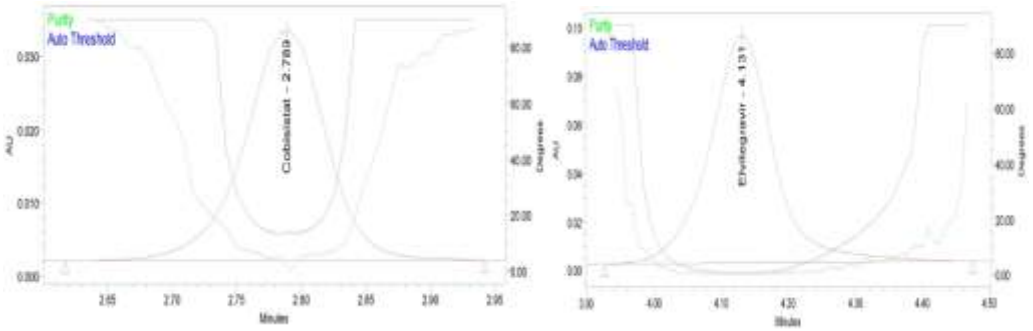


Figure 8: Chromatogram of acid hydrolysis for Emtricitabine, Tenofovir Disoproxil Fumarate, Cobicistat and Elvitegravir.



(A) (B)



(C) **(D)**

Figure 9: Purity plot of acid hydrolysis for (A) Emtricitabine (B) Tenofovir Disoproxil Fumarate (C) Cobicistat (D) Elvitegravir.

3.2.9.2 Alkali Degradation Studies

To 1 mL of stock solution of Emtricitabine, Tenofovir Disoproxil Fumarate, Cobicistat and Elvitegravir, 1 mL of 2 N sodium hydroxide was added and refluxed for 30 mins at 60^0C. The resultant solution was diluted to obtain 60 µg/mL of Emtricitabine, 90 µg/mL of Tenofovir Disoproxil Fumarate, 45 µg/mL of Cobicistat and 45 µg/mL of Elvitegravir solution and 20 µL solutions were injected into the UPLC system and the chromatogram were recorded to assess the stability of sample was shown in figure 10 and purity plot of alkali degradation for Emtricitabine, Tenofovir Disoproxil Fumarate, Cobicistat and Elvitegravir was shown in figure 11.

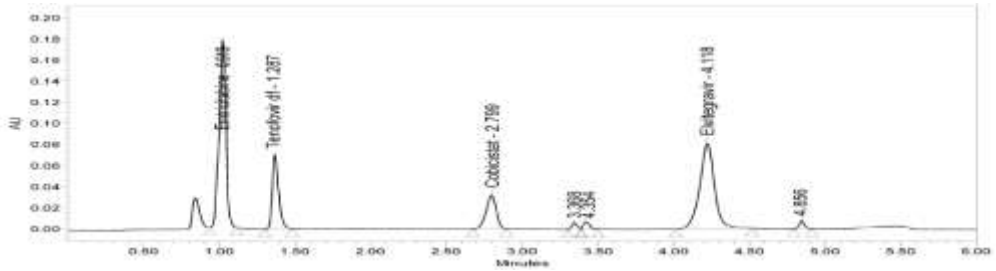
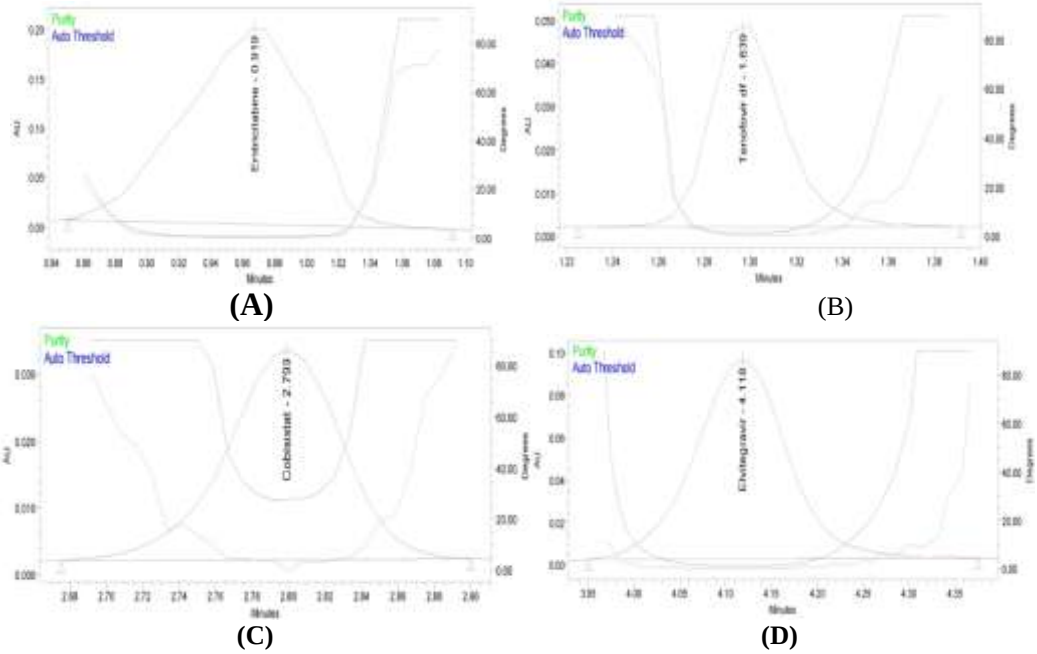


Figure 10: Chromatogram of alkali hydrolysis for Emtricitabine, Tenofovir Disoproxil Fumarate, Cobicistat and Elvitegravir.



(A) (B)

(C) **(D)**

Figure 11: Purity plot of alkali degradation for (A) Emtricitabine (B) Tenofovir Disoproxil Fumarate (C) Cobicistat (D) Elvitegravir.

3.2.9.3 Oxidative degradation Studies

To 1 mL of stock solution of Emtricitabine, Tenofovir Disoproxil Fumarate, Cobicistat and Elvitegravir, 1 mL of 3 % Hydrogen peroxide (H_2O_2) was added and the solution was kept for 30 mins at 60^0C. For UPLC study, the resultant solution was diluted to obtain 60 µg/mL of Emtricitabine, 90 µg/mL of Tenofovir Disoproxil Fumarate, 45 µg/mL of Cobicistat and 45 µg/mL of Elvitegravir solution and 20 µL solutions were injected into the UPLC system and the chromatogram were recorded to assess the stability of sample was shown in figure 12 and purity plot of oxidative degradation for Emtricitabine, Tenofovir Disoproxil Fumarate, Cobicistat and Elvitegravir was shown in figure 13.

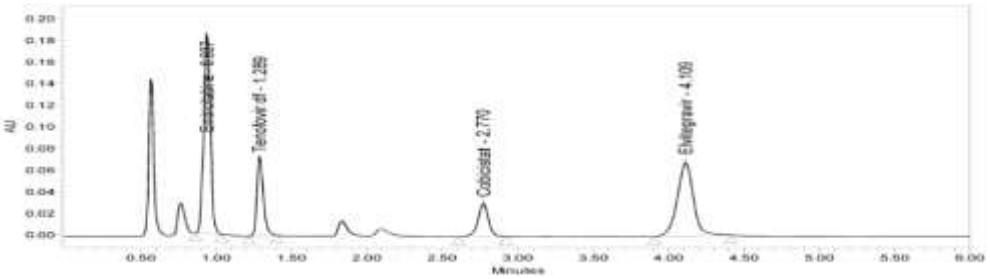
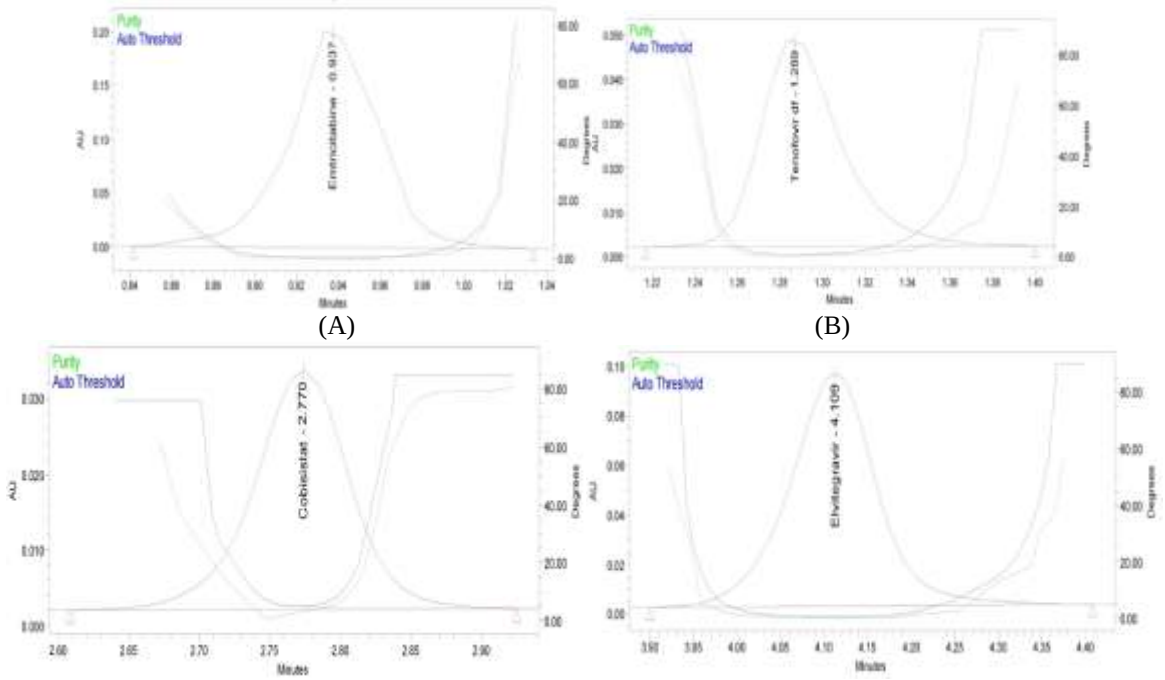


Figure 12: Chromatogram of oxidative degradation for Emtricitabine, Tenofovir Disoproxil Fumarate, Cobicistat and Elvitegravir.



(A) (B)

(C) (D)

Figure 13: Purity plot of oxidative degradation for (A) Emtricitabine (B) Tenofovir Disoproxil Fumarate (C) Cobicistat (D) Elvitegravir.

3.2.9.4 Photolytic degradation studies

The photochemical stability of the drug was also studied by exposing the drug solution to UV light by keeping the beaker in UV Chamber for 7 days or 200 Watt hours/m^2 in photo stability chamber. For UPLC study, the resultant solution was diluted to obtain 60 µg/mL of Emtricitabine, 90 µg/mL of Tenofovir Disoproxil Fumarate, 45 µg/mL of Cobicistat and 45 µg/mL of Elvitegravir solution and 20 µL solutions were injected into the UPLC system and the chromatogram were recorded to assess the stability of sample was shown in figure 14 and purity plot of photolytic degradation for Emtricitabine, Tenofovir Disoproxil Fumarate, Cobicistat and Elvitegravir was shown in figure 15.

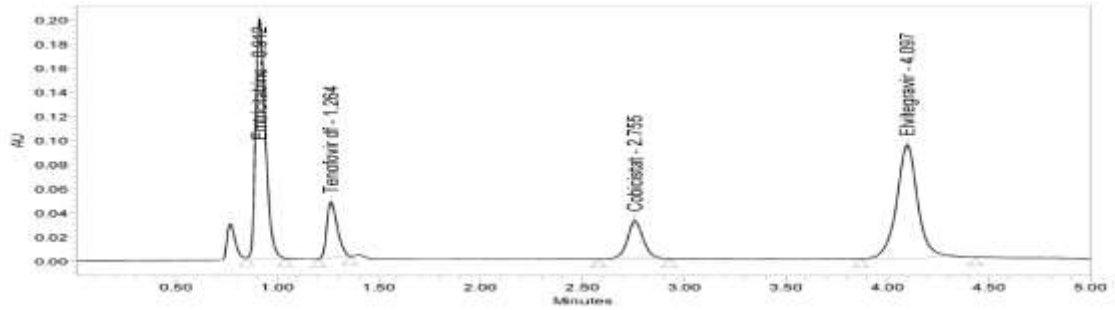
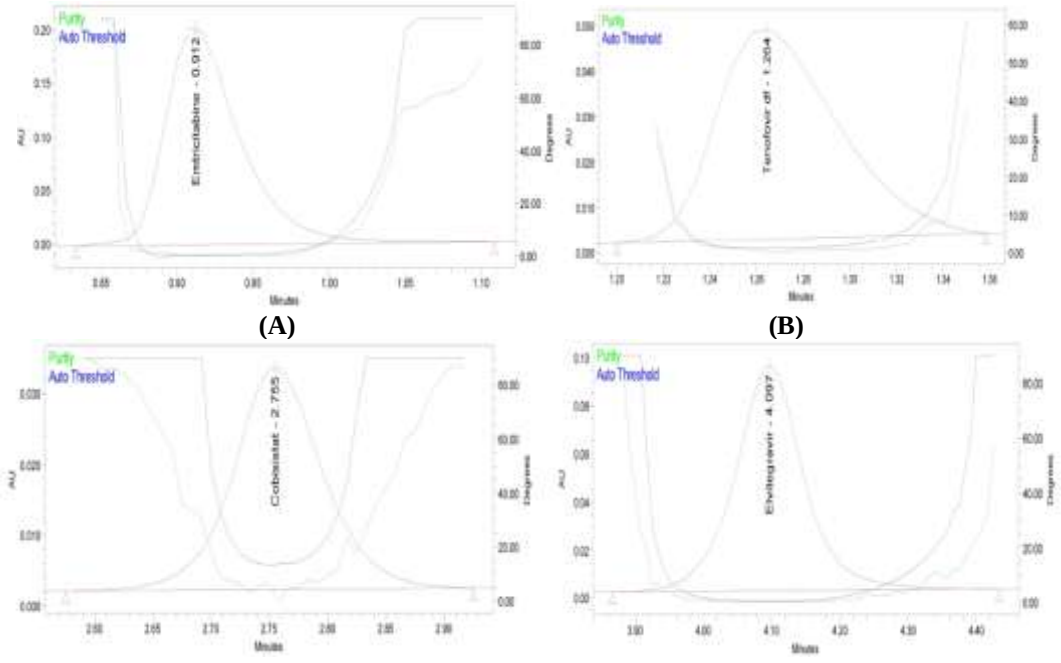


Figure 14: Chromatogram of photolytic degradation for Emtricitabine, Tenofovir Disoproxil Fumarate, Cobicistat and Elvitegravir.



(A) **(B)**

(C) **(D)**

Figure 15: Purity plot of photolytic degradation for (A) Emtricitabine (B) Tenofovir Disoproxil Fumarate (C) Cobicistat (D) Elvitegravir.

3.2.9.5 Thermal Degradation Studies

The standard drug solution was placed in an oven at 105^0C for 6 hrs to study dry heat degradation. For UPLC study, the resultant solution was diluted to 60 µg/mL of Emtricitabine, 90 µg/mL of Tenofovir Disoproxil Fumarate, 45 µg/mL of Cobicistat and 45 µg/mL of Elvitegravir solution and 20 µL solutions were injected into the UPLC system and the chromatogram were recorded to assess the stability of sample was shown in figure 16 and purity plot of thermal degradation for Emtricitabine, Tenofovir Disoproxil Fumarate, Cobicistat and Elvitegravir was shown in figure 17.

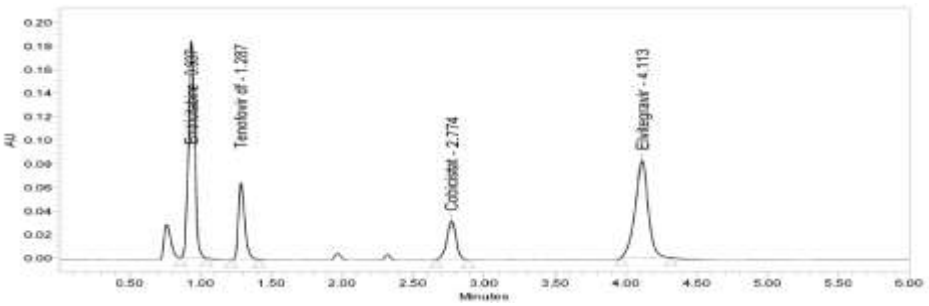


Figure 16: Chromatogram of thermal degradation for Emtricitabine, Tenofovir Disoproxil Fumarate, Cobicistat and Elvitegravir.

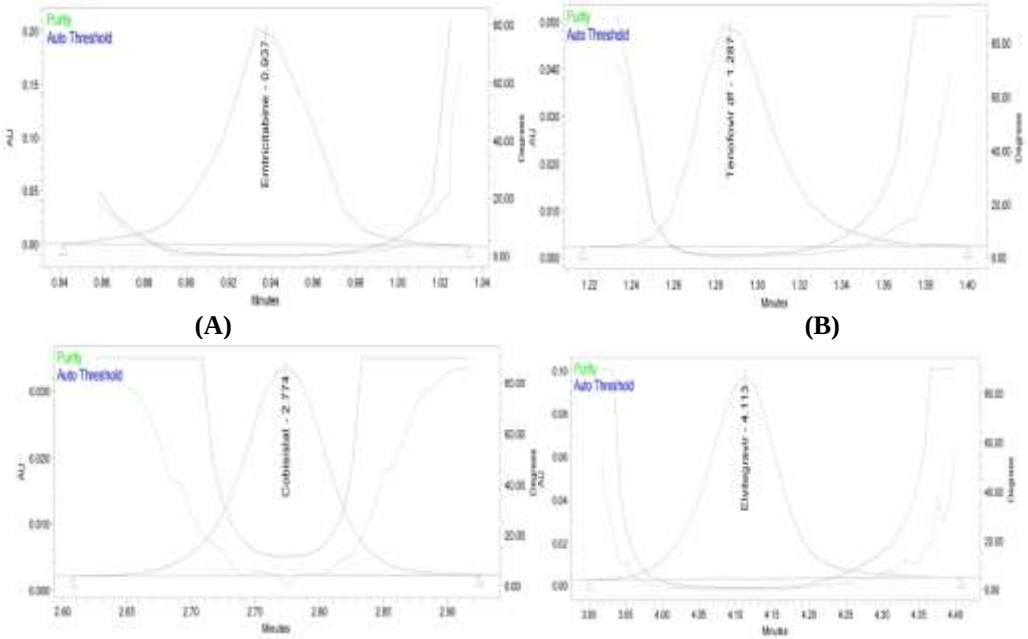


Figure 17: Purity plot of thermal degradation for (A) Emtricitabine (B) Tenofovir Disoproxil Fumarate (C) Cobicistat (D) Elvitegravir.

III. Discussion

This method was intended for rapid estimation of Emtricitabine, Tenofovir Disoproxil Fumarate, Cobicistat and Elvitegravir in bulk and pharmaceutical dosage form. Good separation of the chromatographic peaks was observed and no interfering peaks are found. A number of commercially available UPLC columns and various mobile phases were evaluated for its chromatographic behavior of Emtricitabine, Tenofovir Disoproxil Fumarate, Cobicistat and Elvitegravir. The best response were obtained with ACQUITY UPLC BEH C_{18} (100 mm×2.1 mm, 1.7 µm particle size) column, Waters ACQUITY UPLC system with PDA detector and mobile phase contained a mixture of 0.01 M Ammonium acetate buffer (pH adjusted to 7.5 with ammonium hydroxide) and Acetonitrile (45:55, v/v) was delivered at a flow rate of 0.25 mL/min. Quantification was achieved with PDA detection at 268 nm based on peak area. The retention time of Emtricitabine, Tenofovir Disoproxil Fumarate, Cobicistat and Elvitegravir was 0.904 min, 1.240 min, 2.615 min and 3.801 min with resolution of 4.05, 13.02 and 8.27 respectively. Linearity was established for Emtricitabine, Tenofovir Disoproxil Fumarate, Cobicistat and Elvitegravir in the range of 20-100 µg/mL for Emtricitabine, 30-150 µg/mL for Tenofovir Disoproxil Fumarate, 15-75 µg/mL for Cobicistat and 15-75 µg/mL for Elvitegravir with correlation coefficients (r^2=0.999) and the percentage recoveries were between 99.55-99.96 %, 100.04-100.07 %, 99.86-100.09 %, and 99.95-100.19 % for Emtricitabine, Tenofovir Disoproxil Fumarate, Cobicistat and Elvitegravir respectively, which indicate accuracy of the proposed method. The RSD % values of accuracy for Emtricitabine, Tenofovir Disoproxil Fumarate, Cobicistat and Elvitegravir were found to be < 2 %. The RSD % values of method precision are 0.18 %, 0.64 %, 0.41 % and 0.79 % for Emtricitabine, Tenofovir Disoproxil Fumarate, Cobicistat and Elvitegravir respectively and RSD % values of system precision are 0.52 %, 0.91 %, 0.85 % and 0.98 % for Emtricitabine, Tenofovir Disoproxil Fumarate, Cobicistat and Elvitegravir. The RSD % values of reproducibility are 0.21 %, 0.21 %, 0.24 % and 0.31 % for Emtricitabine, Tenofovir Disoproxil Fumarate, Cobicistat and Elvitegravir respectively, reveal that the proposed method is precise. LOD values for Emtricitabine, Tenofovir Disoproxil Fumarate, Cobicistat and Elvitegravir were found to be 0.14 µg/mL, 0.45 µg/mL, 0.37 µg/mL and 0.25 µg/mL respectively and LOQ values for Emtricitabine, Tenofovir Disoproxil Fumarate, Cobicistat and Elvitegravir were found to be 0.44 µg/mL, 1.36 µg/mL, 1.12 µg/mL and 0.76 µg/mL respectively. The RSD % values of robustness studies were found to be < 2% reveal that the method is robust enough. These data show that the proposed method is accurate and precise for the determination of Emtricitabine, Tenofovir Disoproxil Fumarate, Cobicistat and Elvitegravir in its bulk and pharmaceutical dosage form.

IV. Conclusion

The present RP-UPLC-DAD method for simultaneous estimation of Emtricitabine, Tenofovir Disoproxil Fumarate, Cobicistat and Elvitegravir in their combine dosage form was established and validated as

per the ICH guidelines. Linearity was achieved for Emtricitabine, Tenofovir Disoproxil Fumarate, Cobicistat and Elvitegravir in the range of 20-100 µg/mL for Emtricitabine, 30-150 µg/mL for Tenofovir Disoproxil Fumarate, 15-75 µg/mL for Cobicistat and 15-75 µg/mL for Elvitegravir with correlation coefficients (r^2=0.999). The percentage recoveries of Emtricitabine, Tenofovir Disoproxil Fumarate, Cobicistat and Elvitegravir were achieved in the range of 98-102 % 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 forced degradation studies were performed by using HCl, NaOH, H_2O_2, thermal, UV radiation. Emtricitabine are more sensitive towards alkaline hydrolysis degradation condition, Tenofovir Disoproxil Fumarate is more sensitive towards oxidative degradation condition, Cobicistat are more sensitive towards alkaline hydrolysis degradation condition and Elvitegravir are more sensitive towards acidic hydrolysis degradation condition which was shown in Table 13 and 14. No interference from any components of pharmaceutical dosage form or degradation products was observed and the method has been successfully used to perform long term and accelerated stability studies of Emtricitabine, Tenofovir Disoproxil Fumarate, Cobicistat and Elvitegravir formulations. Hence it can be used for the hyphenated instrumental analysis of Emtricitabine, Tenofovir Disoproxil Fumarate, Cobicistat and Elvitegravir in their bulk and combine dosage form.

Table 13. Forced degradation data of Emtricitabine and Tenofovir Disoproxil Fumarate in different degradation conditions.

Forced degradation data of Emtricitabine						
Degradation condition	Retention time (mins)	Area	Purity Angle	Purity Threshold	USP Plate Count	Asymmetry
Acid hydrolysis	0.968	648514	0.682	0.878	2502	0.86
Alkaline hydrolysis	0.919	641573	0.526	0.869	2802	1.75
Oxidative degradation	0.937	652632	0.325	0.627	2425	0.92
Photolytic degradation	0.912	693862	0.452	0.581	2693	1.41
Thermal degradation	0.937	662559	0.384	0.531	2035	0.95
Forced degradation data of Tenofovir Disoproxil Fumarate						
Degradation condition	Retention time(mins)	Area	Purity Angle	Purity Threshold	USP Plate Count	Asymmetry
Acid hydrolysis	1.297	160580	2.162	2.335	7015	1.25
Alkaline hydrolysis	1.639	155941	2.023	2.289	9381	1.11
Oxidative degradation	1.289	150625	0.869	1.436	8052	1.31
Photolytic degradation	1.264	168874	1.599	1.807	2594	1.31
Thermal degradation	1.287	152634	1.121	1.509	5068	1.35
Degradation condition	Drug Recovered (%)			Drug Decomposed (%)		
	Emtricitabine	Tenofovir Disoproxil Fumarate		Emtricitabine	Tenofovir Disoproxil Fumarate	
Standard	100	100		100	100	
Acid hydrolysis	93.32	94.94		6.68	5.06	
Alkaline hydrolysis	92.32	92.20		7.68	7.80	
Oxidative degradation	93.91	89.06		6.09	10.94	
Photolytic degradation	99.84	99.85		0.16	0.15	
Thermal degradation	95.34	90.25		4.66	9.75	

Table 14. Forced degradation data of Cobicistat and Elvitegravir in different degradation conditions.

Forced degradation data of Cobicistat						
Degradation condition	Retention time (mins)	Area	Purity Angle	Purity Threshold	USP Plate Count	Asymmetry
Acid hydrolysis	2.789	167349	9.408	24.659	9683	0.92
Alkaline hydrolysis	2.799	156770	7.162	42.522	9210	0.91
Oxidative degradation	2.770	161546	1.062	14.691	5127	0.94
Photolytic degradation	2.755	170975	11.539	22.568	6148	1.04
Thermal degradation	2.774	165013	8.988	19.732	8148	0.94
Forced degradation data of Elvitegravir						
Degradation condition	Retention time (mins)	Area	Purity Angle	Purity Threshold	USP Plate Count	Asymmetry

Acid hydrolysis	4.131	615800	0.384	1.302	9990	1.05
Alkaline hydrolysis	4.118	638835	0.328	1.620	8091	1.0
Oxidative degradation	4.109	645281	0.348	0.792	2152	0.92
Photolytic degradation	4.097	676997	0.248	0.639	8334	1.03
Thermal degradation	4.113	658773	0.319	0.781	8552	0.97

Degradation condition	Drug Recovered (%)		Drug Decomposed (%)	
	Cobicistat	Elvitegravir	Cobicistat	Elvitegravir
Standard	100	100	100	100
Acid hydrolysis	97.76	90.79	2.24	9.21
Alkaline hydrolysis	91.58	94.19	8.42	5.81
Oxidative degradation	94.37	95.14	5.63	4.86
Photolytic degradation	99.88	99.81	0.12	0.19
Thermal degradation	96.40	97.12	3.60	2.88

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