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NEW ANALYTICAL METHOD VALIDATION REPORT AND FORCED DEGRADATION STUDIES FOR ASSAY OF ELVITEGRAVIR, TENOFOVIR, EMTRICITABINE AND COBICISTAT BY RP-UPLC

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Abstract:

The main aim of the present work is to develop and validate a simple, specific, efficient, accurate, and precise stability-indicating rapid reversed phase ultra performance liquid chromatographic method is developed for the simultaneous determination of Elvitegravir, Tenofovir, Emtricitabine, and Cobicistat in its bulk and pharmaceutical combined dosage form with forced degradation studies. The four compounds is separated on a reversed phase Endoversil C18(50 x 2.1mm,1.8µm particle size) column, waters ACQUITY UPLC system with PDA detector and a mobile phase composed of 0.1% OPA: acetonitrile (70:30, v/v), pH 3.0 adjusted with o-phosphoric acid. The flow rate is set to 0.3ml/min with respones measured at 252nm. The retention time of Elvitegravir, Tenofovir, Emtricitabine, and Cobicistat is found to be 0.594min, 0.734m i n , 0.487min, 2.515min with resolution of 3.19, 10.49, 12.25 respectively. Linearity is established in the range of 75-225µg/ml for Elvitegravir, 150-450µg/m for Tenofovir, 100-300µg/m for Emtricitabine and 75-225µg/ml for Cobicistat with correlation coefficients ($r^2$0.999). The percentage recoveries is between 99.53-10.28%, 99.60-100.97%, 100.49-100.93%, 99.65-100.52%, for Elvitegravir, Tenofovir, Emtricitabine, and Cobicistat.

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

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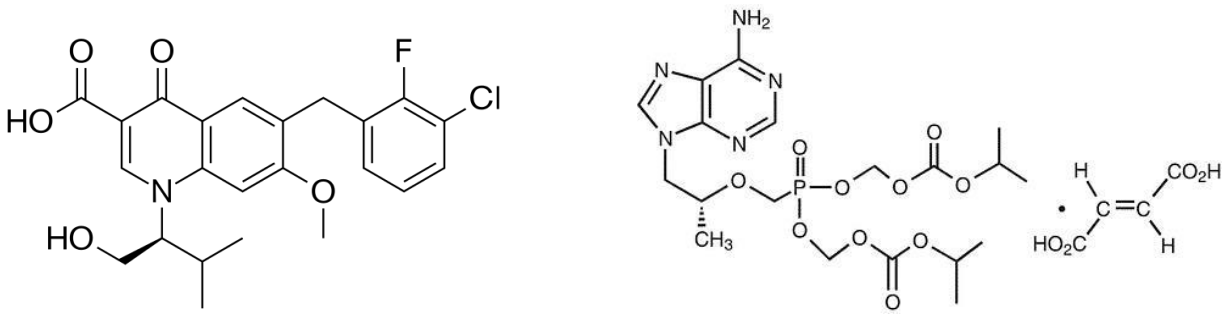
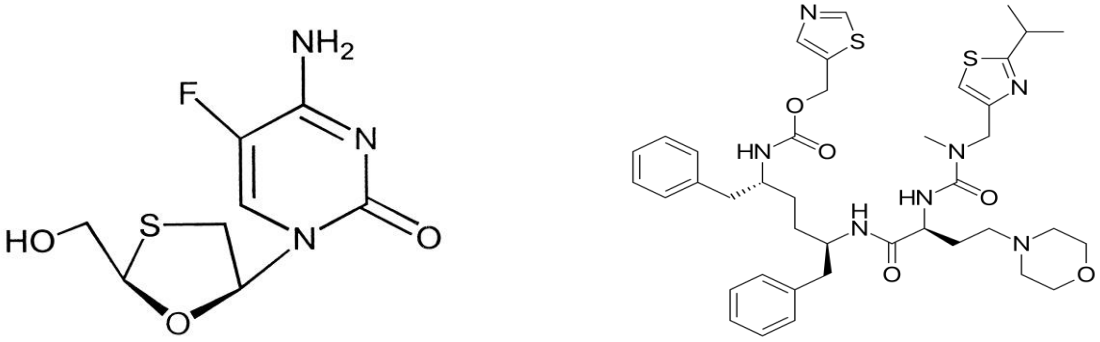
INTRODUCTION:

Anti retroviral Therapy was intended to eradication, treatment, and prevention for the viral suppression in human immuno deficiency virus treatment. Virus infection was the challenges were continuously and constantly met by the infected HIV persons. Number of new drug molecules that have been developed for the effective treatment of human immuno deficiency virus(HIV) infection and other viral infections. One of the potent life saving combinational essential drugs which include, Elvitegravir, Tenofovir, Emtricitabine, and Cobicistat combined dosage form is used for the treatment of HIV-1infection in adult patients [1-3]. 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 **Fig. 1**. 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 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 topoisomers I or II .

The literature survey revealed that there are very few HPLC analytical methods and sepectroscopic methods available for the determination of Elvitegravir, Tenofovir, Emtricitabine, and Cobicistat in pure and combined pharmaceutical dosage forms [4-9]. Some reports have described bioanalytical methods and chromatographic methods for detection of these [10-12]. However, no method is reported for simultaneous estimation of Elvitegravir, Tenofovir, Emtricitabine, and Cobicistat in combined pharmaceutical dosage form by Reversed Phase Ultra Performance Liquid Chromatography (UPLC) with forced degradation studies [13-16]. No Chromatographic method has been reported for the quantification of these four drugs combination in any of the matrices. These method was successfully validated according to the International Conference on Harmonization,(ICH) guidelines [17-19].



(A) **(B)**



(C) **(D)**

Fig.1: Chemical Structure of (A) Elvitegravir (B) Tenofovir (C) Emtricitabine (D) Cobicistat

MATERIALS AND METHOD:

Elvitegravir, Tenofovir, Emtricitabine, and Cobicistat of pharmaceutical grade as samples by Pharma Train, Hyderabad, India, and Methanol and water of UPLC grade. Acetonitrile used of UPLC grade. Elvitegravir, Tenofovir, Emtricitabine, and Cobicistat was procured from local market and used for analysis of marketed formulation. In addition, an electronic balance Afcoset ER-200A, a pH meter(Adwa-AD 1020), a sonicator (Spectra Lab, model UCB 40), a hot air oven (Labhosp), i s used in this study.

Chromatographic Conditions:

Waters, Acquity(UPLC) consisting pump, auto sampler, auto injector, VWD and Photo diode array detector, thermostatic column compartment connected with an Endoversilo C18(50 x 2.1nm), 1.8 µm were determined at 252nm. The UPLC analysis was performed on reversed-phase Ultra-performance liquid chromatographic system with gradient elution mode using a mobile phase: A- 0.1% OPA in Acetonitrile, B- 0.1% OPA in Mill-Q water. The contents of the mobile phase were filtered, before it was used through 0.22 µm membrane filter for 15mints and pumped from the respective solvent reservoirs to the column at a flow rate of 0.3ml/min. The column temperature was maintained at 25ºC and Run time 4mins. The injection volume of a sample was 4 µl. The chromatograpghic conditions were tabulated in **Table 1**.

Preparation of mobile phase: Mobile phase; A- 0.1% OPA in Acetonitrile, B- 0.1% OPA in Mill-Q water (70:30% v/v).

Table 1: Mobile Phase Gradient Table

Time (min)	Mobile phase A (%v/v)	Mobile phase B (%v/v)
0	10	90
2	30	70
3	90	10
5	15	85
6	60	40
10	20	80

Preparation of standard solution: Stock solutions were prepared by weighing 75 mg of Elvitegravir, 150 mg of Tenofovir, 100 mg of Emtricitabine, and 75 mg of Cobicistat. The weighed drugs were transferred to 100ml volumetric flasks and add about 70ml of diluent and sonicate to dissolve it completely and make volume upto the mark with the same solvent. Further pipette 2ml of the above stock solutions into 10ml volumetric flask and dilute up to the mark with diluent.

Preparation of test solution: Accurately weigh 10 tablets crush in mortor and pestle and transfer 75 mg of Elvitegravir, 150 mg of Tenofovir, 100 mg of Emtricitabine and 75 mg of Cobisistat working standard into a 100 ml clean dry volumetric flask add about 70 ml of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 2 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluent.

Selection of wavelength:

UV spectrum of Elvitegravir, Tenofovir, Emtricitabine and Cobisistat diluents is recorded at wavelength of 252nm because all the drugs show good absorbance at this wavelength.

Estimation of pharmaceutical formulation:

Accurately weigh 10 tablets crush in mortor and pestle and transfer 75 mg of Elvitegravir, 150 mg of Tenofovir, 100 mg of Emtricitabine and 75 mg of Cobisistat working standard into a 100 ml clean dry volumetric flask add about 70 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 2 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluent.

Method Validation:

The method is validated for the parameters like accuracy, linearity, precision, detection limit, quantification limit and robustness. The accuracy of the method was determined by calculating percentage recovery of Elvitegravir, Tenofovir, Emtricitabine, and Cobicistat.

Forced degradation studies:

Forced degradation study is performed to evaluate the stability of the developed methods using the stress conditions like exposure of sample solution to acid (0.1N HCl), base (0.1N NaOH), peroxide(H_2O_2) , photolytic(UV), and Thermal condition(Heat).

RESULTS AND DISCUSSION:

In order to achieve good separation between all the four components different buffer pH conditions is maintained and different propotions of solvents like methanol, acetonitrile and water tested binary and tertiary, the pH 3 adjusted with orthophosphoric acid with a flow rate of 0.3 ml/min and measured at wavelength 252nm for Elvitegravir, Tenofovir, Emtricitabine, and Cobicistat. Blank and optimized conditions were shown in **Fig. 2-5**. System suitability is an integral part of the method validation to evaluate the parameters like tailing factor, standard deviation (%RSD) for replicate injections. The results are presented in **Table 2**.

Table 2: System Suitability Results

Parameter	Results				Required limits
	Elvitegravir	Tenofovir	Emtricitabine	Cobicistat	
RSD of peak area	0.7	0.3	0.3	0.7	<2.0 for n≥6
RSD of retention time	0.23	0.25	0.26	0.23	<1.0 for n≥6
USP Tailing factor (T)	1.34	1.29	1.37	1.13	T<2
USP Plate Count (N)	3122	2952	5852	4461	>2000
USP Resolution (R)	-	3.19	10.49	12.25	R>2

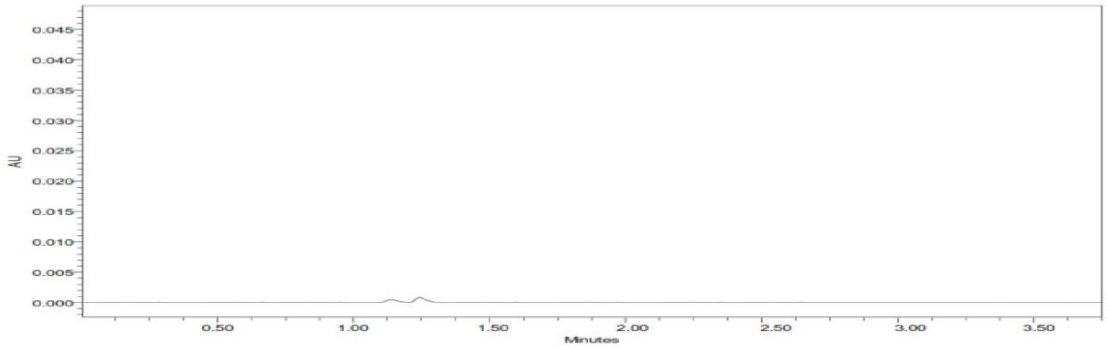


Fig. 2: Blank Chromatogram

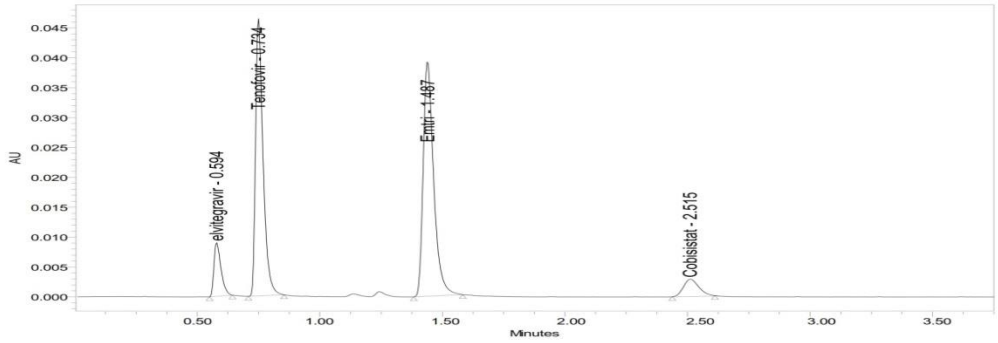


Fig. 3: Optimized Chromatogram of Standard Solution

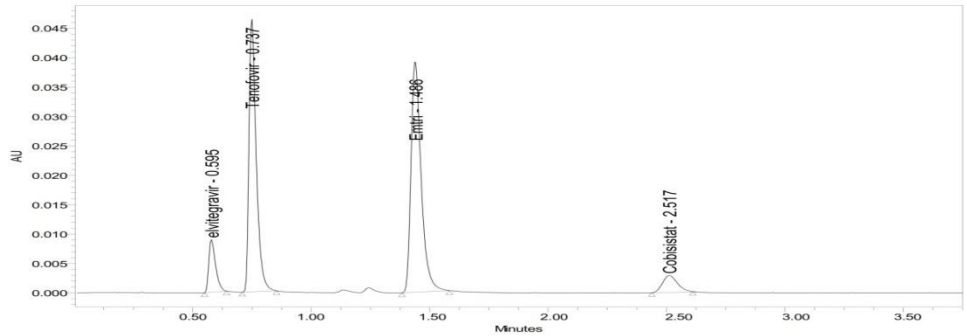


Fig. 4: Chromatogram of Level 1

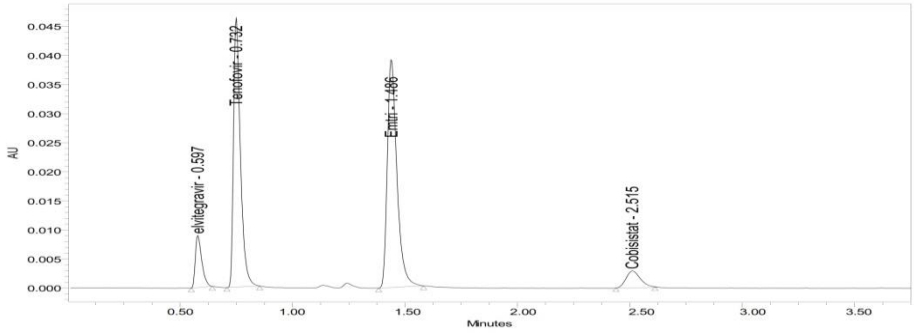


Fig. 5: Chromatogram of Level 2

In the blank chromatograms there are no peaks observed at the retention times of Elvitegravir, Tenofovir, Emtricitabine, and Cobicistat, and also the degradation studies showed that there is no interference with degradants that shows the method is specific **(fig. 2-4).**

Linearity and Range:

The linearity was calculated by measuring different concentrations like 75-225% for Elvitegravir, 150-450% for Tenofovir, 100-300% for Emtricitabine, and 75-225% for Cobicistat. The calibration curve was constructed by plotting concentration of standard solutions against mean peak areas and the regression equation was computed. The summary of the parameters were shown in **Table 3** and **Fig. 6, 7, 8, 9.**

Fig.6,7,8,9 : Linearity (calibration) curve of Elvitegravir, Tenofovir, Emtricitabine, and Cobicistat

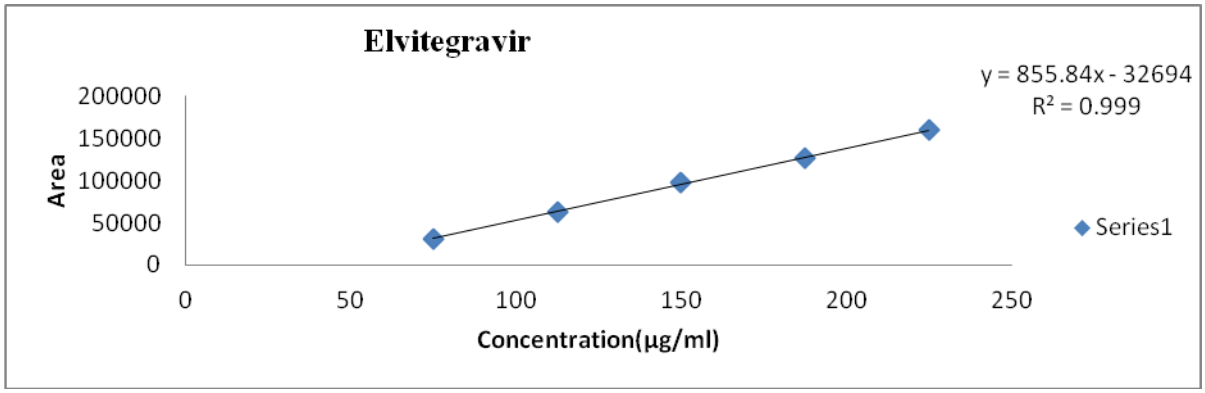


Fig.6: Linearity graph of Elvitegravir

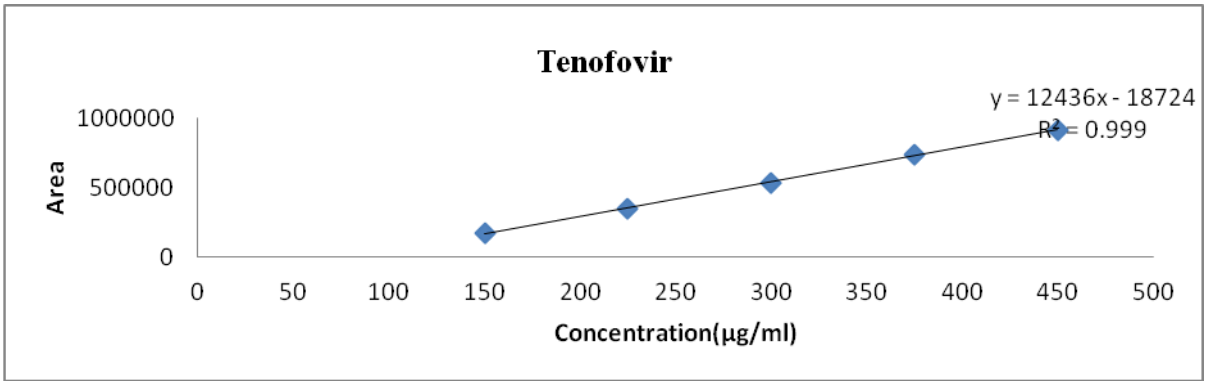


Fig.7 : Linearity graph of Tenofovir

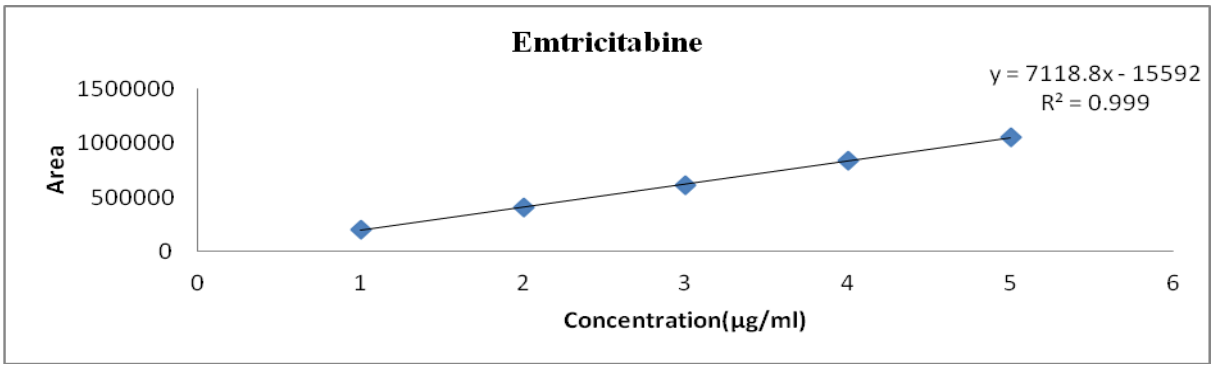


Fig.8: Linearity graph of Emtricitabine

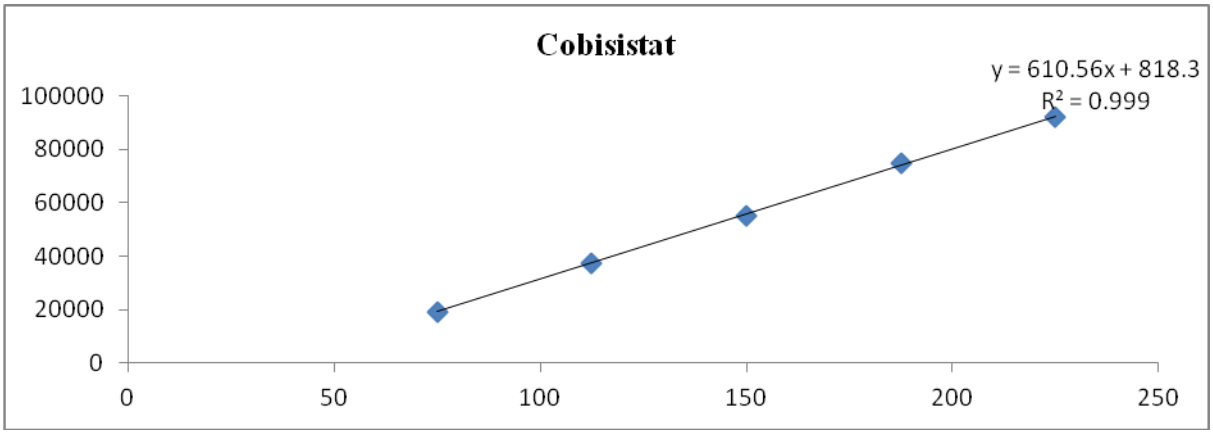


Fig.9: Linearity graph of Cobicistat

Table 3: Regression Equation Parameters

Parameter	Results			
	Elvitegravir	Tenofovir	Emtricitabine	Cobicistat
Linearity range (µg/ml)	75.225	150-450	100-300	75-225
Correlation co-efficient	0.999	0.999	0.999	0.999
Slope	855.19	2455.62	4263.73	485.58
Y-intercept	855.8x-32694	12436x-18724	7118.8x-15592	610.56x+818.3

Precision:

The precision was evaluated at three levels, repeatability, reproducibility and intermediate precision each level of precision was investigated by six replicate injections of 100% concentrations of Elvitegravir, Tenofovir, Emtricitabine, and Cobicistat. The result of precision was expressed as %RSD and was tabulated in **Table 4** .

Table 4: Precision Studies

Parameter	Results			
	Elvitegravir	Tenofovir	Emtricitabine	Cobicistat
Repeatability				
Mean % RSD of retention time	0.32	0.27	0.10	0.22
Mean % RSD of peak area	1	0.6	0.8	0.6
Mean % RSD of assay	99.95	100.25	99.80	100.27
Reproducibility/intraday precision				
Mean % RSD of retention time	0.28	0.25	0.17	0.19
Mean % RSD of peak area	0.4	0.1	0.2	0.3
Mean % RSD of assay	99.98	100.31	99.85	100.29
intermediate precision				
Mean % RSD of retention time	0.22	0.18	3.13	0.23
Mean % RSD of peak area	0.4	0.4	0.5	0.5
Mean % RSD of assay	99.98	100.33	99.88	100.35

Accuracy:

To determine the accuracy of the proposed method, a recovery study is conducted at three different Levels 50%, 100%, 150%. The results are tabulated in **Table 5.**

Table 5: Accuracy data

Paramater	Amount added(mg)	Amount recovered(mg)	% of recovery	Mean % of recovery
Elvitegravir				
50% level	37.5	37.98	101.28	
100% level	75	74.65	99.53	100.11
150% level	112.5	111.97	99.53	
Tenofovir				
50% level	75	75.15	100.20	
100% level	150	151.45	100.97	100.26
150% level	225	224.11	99.60	
Emtricitabine				
50% level	50	50.24	100.49	
100% level	100	100.50	100.50	100.64
150% level	150	151.40	100.93	
Cobicistat				
50% level	37.5	37.69	100.52	
100% level	75	74.74	99.65	
150% level	112.5	112.58	100.07	100.08

Limit of Detection(LOD) and Limit of Quantification(LOQ):
The LOD and LOQ values for Elvitegravir, Tenofovir, Emtricitabine, and Cobicistat were Tabulated in **Table 6.**

Table 6: LOD and LOQ data

Parameter	Elvitegravir	Tenofovir	Emtricitabine	Cobicistat
LOD(µg/ml)	2.98	1.35	1.00	3.00
LOQ(µg/ml)	9.98	4.7	3.54	10.02

Table 7: Assay Results of Marketed Tablets

Drug	Labeled amount(mg/tab)	Amount found(mg/tab)	% of assay
Elvitegravir	150	149.86	99.91
Tenofovir	300	300.69	100.23
Emtricitabine	200	199.52	99.76
Cobicistat	150	150.40	100.27

Robustness:
The robustness of the method was unaffected when small, deliberate changes like, flow change, mobile phase composition, column temperature were performed at 100% test concentration. The ruggedness of the proposed method studied under different columns, analyst, instrument, laboratories analysis of the same sample. The results are tabulated in **Table 7.**

The stability of the standard solution was tested at the intervals of 24 and 48 hr at room temperature. There were no significance changes observed in the system suitable parameters like theoretical plates, tailing factors, retention time and resolution. Hence, the standard solution is stable up to 48hr of room temperature.

The proposed method was applied for the analysis of Elvitegravir, Tenofovir, Emtricitabine, and Cobicistat in tablet dosage form, the results were found to be between 99.0 and 101.0% and the results were summarized in **Table 7**. Results of forced degradation were shown in **Table 8** and **Fig. 10,11,12,13,14** shows the chromatograms of forced degradation studies.

Table 8: Forced Degradation Study

Condition	Elvitegravir		Tenofovir		Emtricitabine		Cobicistat	
	% Assay	% Degradation	% Assay	% Degradation	% Assay	% Degradation	% Assay	% Degradation
Acid	96.36	3.64	96.69	3.31	95.33	4.67	94.92	5.08
Base	96.90	3.10	96.30	3.70	96.87	3.13	95.43	4.57
Peroxide	96.36	3.64	92.49	7.51	96.24	3.76	93.76	6.24
Thermal	96.24	3.76	94.98	5.02	95.41	4.59	94.36	5.64
Photolytic	96.08	3.92	95.11	4.89	92.26	7.74	94.51	5.49

Fig. 10,11,12,13,14 : Forced Degradation Chromatogram of Elvitegravir, Tenofovir, Emtricitabine, and Cobicistat

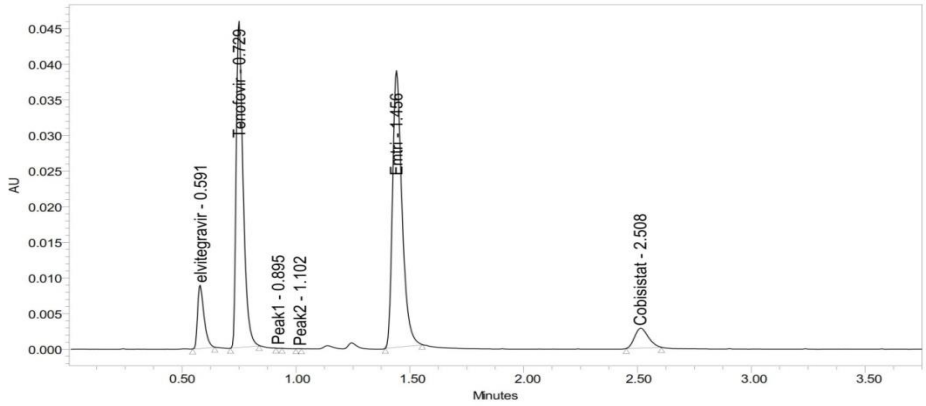


Fig. 10: Acid Degradation

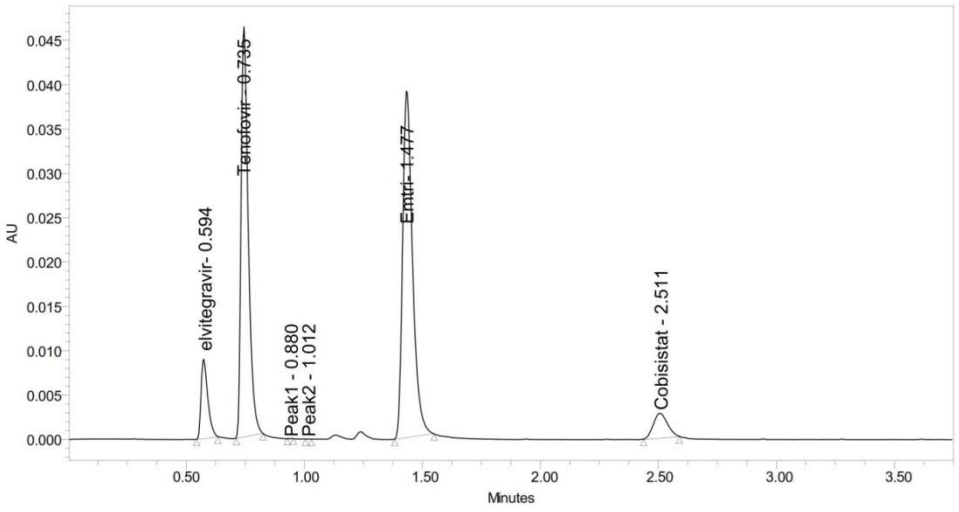


Fig. 11 : Alkali Degradation

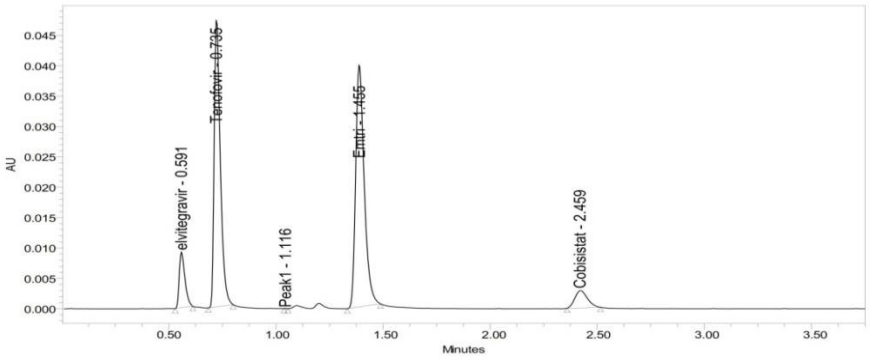


Fig. 12: Peroxide Degradation

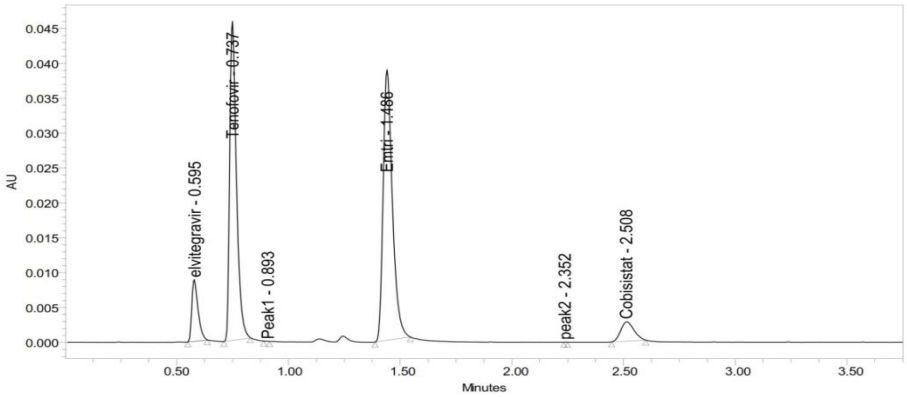


Fig. 13: Thermal Degradation

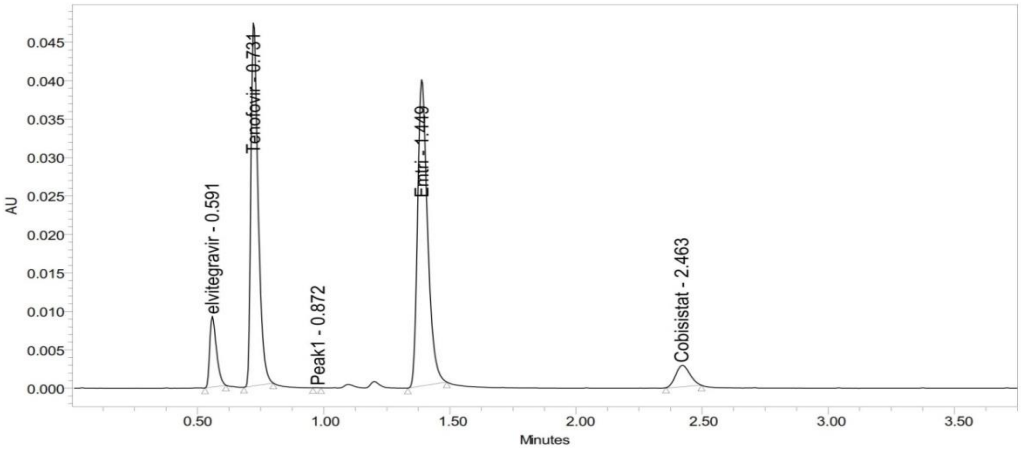


Fig. 14 : Photolytic Degradation

CONCLUSION:

The developed RP-HPLC method is accurate, precise, robust, sensitive and selective. And the method is cost effective and less time consuming. The Force degradation studies method was found to be simple, sensitive, selective, and suitable for determination of Elvitegravir, Tenofovir, Emtricitabine, and Cobicistat in presence of its degraded product. It can successfully applied for estimation of Elvitegravir, Tenofovir, Emtricitabine, and Cobicistat in its pharmaceutical dosage form and bio-pharmaceutical and bio-equivalence studies and in clinical pharmacokinetic studies in near future.

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