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RP-UPLC METHOD DEVELOPMENT AND VALIDATION FOR SIMULTANEOUS ESTIMATION AND FORCED DEGRADATION STUDIES OF ELVITEGRAVIR, COBICISTAT, EMTRICITABINE AND TENOFOVIR DISOPROXIL FUMARATE IN SOLID DOSAGE FORM

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Keywords:

Elvitegravir, Cobicistat, Emtricitabine, Tenofovir Disoproxil Fumarate, UPLC

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ABSTRACT: A stability indicating method was developed and validated for simultaneous estimation of Elvitegravir, Cobicistat, Emtricitabine, and Tenofovir Disoproxil Fumarate (Tenofovir D F) in solid dosage form using RP-UPLC method. Waters Acquity UPLC system with Column Endoversilo C18 (50 × 2.1 nm, 1.8 µm) having PDA detector at 252 nm wavelength was used. Mobile phase having a mixture of 700 ml of Acetonitrile and 300 ml 0.1% Ortho Phosphoric Acid (OPA) in the ratio 70:30 v/v was used. The flow rate was set to 0.3ml/min. The retention time was obtained at 0.59 min for Elvitegravir, 2.51 min for Cobicistat, 1.48 min for Emtricitabine, and 0.73 min for Tenofovir Disoproxil Fumarate respectively with a total run time of 4 min. The linearity was calculated with correlation coefficients (r^2=0.999), which were found to be within limits. The percentage recoveries of Elvitegravir, Cobicistat, Emtricitabine, and Tenofovir Disoproxil Fumarate were within the acceptance criteria. The drugs were found to be stable at forced degradation conditions, and the net degradation was found to be within limits. The developed method can be used for the quality control of the combination in the pharmaceutical dosage form.

INTRODUCTION: HIV (Human Immuno-deficiency Virus) [1] is a retrovirus that gradually attacks the immune system, which protects the human body against illness. This virus multiplies in T-helper cell (CD4) and gradually depletes them. HIV is mainly found in blood, semen, vaginal and anal fluids, and breast milk of the infected patients. However, it cannot be transmitted through sweat, saliva, or urine. Currently, there is no proper cure for HIV, but with early diagnosis and effective Antiretroviral (ARV) treatment, people with HIV can live a long and normal, healthy life. Therefore, it is important to take correct treatment having a combination of drugs.

Elvitegravir [2-3] is an HIV-1 integrase strand transfer inhibitor (INSTI). Integrase is 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.

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Elvitegravir does not inhibit human topoisomerases I or II. Its chemical name is 6-(3-Chloro-2-fluoro-benzyl)-1-[(2S)-1-hydroxy-3-methylbutan-2-yl]- 7-methoxy- 4- oxo-1, 4dihydroquinoline-3-carboxylic acid with molecular formula of $C_{23}H_{23}ClFNO_5$. Cobisistat [4] is a mechanism-based inhibitor of cytochrome P450 3A (CYP3A) isoforms. Cobisistat does not have any anti-HIV activity on its own. Its chemical name 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-yl)butanoyl] amino}- 1, 6-diphenyl-hexan -2-yl]carbamate with molecular formula of $C_{40}H_{53}N_7O_5S_2$.

Emtricitabine [5] works by inhibiting reverse transcriptase, the enzyme that copies HIV RNA into new viral DNA. Emtricitabine is a synthetic nucleoside analog of cytidine. It is phosphorylated by cellular enzymes to form Emtricitabine 5'-triphosphate, which is responsible for the inhibition of HIV-1 reverse transcriptase. Therefore, Emtricitabine inhibits the activity of HIV-1 reverse transcriptase (RT) both by competing with the natural substrate Deoxycytidine 5'-triphosphate and by its incorporation into viral DNA. Its chemical name is 5-fluoro-1-[(2R, 5S)-2-(hydroxymethyl)-1, 3-oxathiolan-5-yl] cytosine with molecular formula of $C_8H_{10}FN_3O_3S$.

Tenofovir Disoproxil Fumarate [6-7] is a nucleotide reverse transcriptase inhibitor (NRTI) and a novel ester prodrug of the antiretroviral Tenofovir. TAF is converted *in-vivo* to Tenofovir, an acyclic nucleoside phosphonate (nucleotide) analog of adenosine 5′-monophosphate. Tenofovir mimics normal DNA building blocks but is lacking a 3'-OH molecule required for phosphodiester bond linkage required for DNA elongation, Tenofovir causes early chain termination and prevents proviral DNA transcription. Its chemical name is ({[(2R)-1-(6-amino- 9H- purin- 9- yl)propan-2-yl]oxy}methyl) phosphonic acid with molecular formula of $C_9H_{14}N_5O_4P$.

Few HPLC and UPLC methods have been described in the literature for the determination of Elvitegravir, Cobicistat, Emtricitabine, and Tenofovir Disoproxil Fumarate in combined forms, but there was no official method for its simultaneous estimation. The developed method was found superior in certain respects, such as RT and Accuracy. The proposed method aimed to develop and validate a stability indicating a method for the estimation of Elvitegravir, Cobicistat, Emtricitabine, and Tenofovir Disoproxil Fumarate in solid dosage form by UPLC method as per ICH guidelines. The Chemical structures of the drugs were shown in **Fig. 1**.

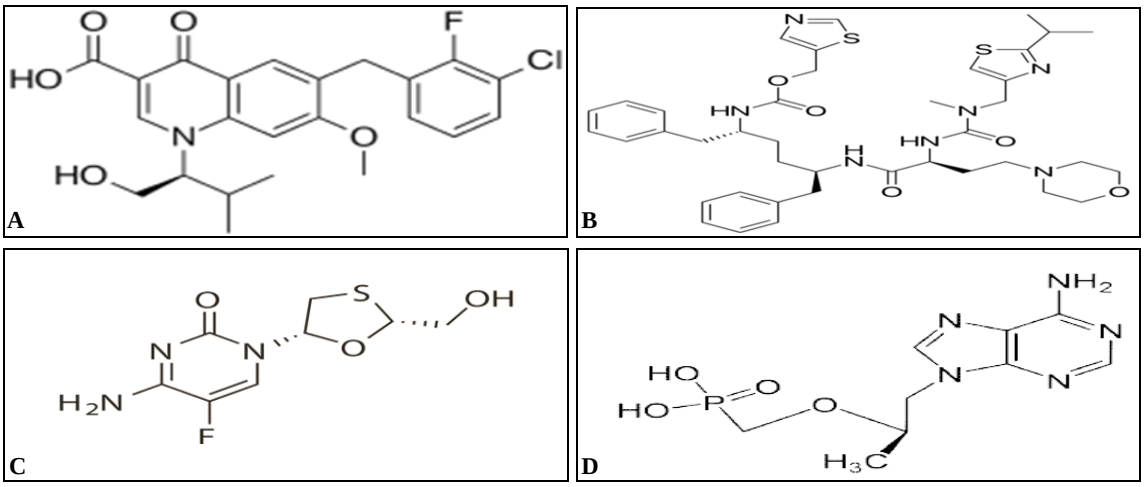

FIG. 1: CHEMICAL STRUCTURE OF (A) ELVITEGRAVIR, (B) COBISISTAT, (C) EMTRICITABINE, AND (D) TENOFOVIR DISOPROXIL FUMARATE

MATERIAL AND METHOD:

Chemicals and Reagents: Elvitegravir, Cobicistat, Emtricitabine, and Tenofovir Disoproxil Fumarate were obtained from Hetero Pharma Ltd., Hyderabad, India. The Stribild tablet manufactured by Gilead Sciences, Inc. was obtained from a local pharmacy in Hyderabad. Ortho Phosphoric Acid was obtained from FINER chemical LTD. Water and Methanol were obtained from LICHROSOLV (MERCK). Acetonitrile was obtained from MOLYCHEM.

Instruments and Chromatographic Conditions: Waters acquity UPLC equipped PDA detector controlled by Empower 2 software was used with Column Endoversilo C18 (50 × 2.1 nm, 1.8 µm) having PDA detector. Mobile phase having a mixture of 700ml of Acetonitrile and 300 ml 0.1% Ortho Phosphoric Acid (OPA) in the ratio 70:30 v/v was used. All Weighing were done on Micro Balance model Afcoset ER-200A. PH meter manufactured by Adwa - AD 1020 was used. Hot Air Oven manufactured by Thermo Lab was used. Ultrasonic bath of Labman was used. Rotary Evaporator manufactured by Buchi from Switzerland was used.

Preparation of Mobile Phase, Standard and Sample Solutions: A gradient mobile phase was prepared by mixing 700 ml of Acetonitrile and 300 ml of 0.1% Ortho Phosphoric Acid were mixed in the ratio 70:30 v/v and sonicated for 10 min for the removal of air bubbles and filtered through 0.45 µm filter under vacuum filtration. Accurately weigh and transfer 75 mg of Elvitegravir, 75 mg of Cobicistat, 100 mg of Emtricitabine and 150 mg of Tenofovir Disoproxil Fumarate 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 10 ml volumetric flask and dilute up to the mark with Diluent and used as a stock solution.

Accurately weighed and finely powdered 20 tablets of Stribild and transferred an amount of the powder equivalent to one tablet and transfer it to 100 ml volumetric flask and make up the volume with mobile phase and sonicated for 20 min and diluted to the volume with the mobile phase, filtered through 0.22 µm filter and used as the sample solution.

Method Validation: [8] The RP-UPLC method was validated according to ICH guidelines for validation of analytical procedures for different validation parameters. The method was validated for its specificity, linearity, accuracy, precision, robustness, ruggedness, LOD and LOQ.

Forced Degradation Studies: Forced degradation studies of the fixed dose combination of the drug were carried out by treating the sample under tress conditions like acid and base hydrolysis, oxidation, photolytic and thermal degradation, and resultant degradation products was investigated. These study help to know the stability characteristics of the drug and the possible degradation products.

Preparation of Solution for Degradation Studies: Accurately weigh and transfer 75 mg of Elvitegravir, 75 mg of Cobicistat, 100 mg of Emtricitabine and 150 mg of Tenofovir Disoproxil Fumarate 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 10 ml volumetric flask and dilute up to the mark with Diluent.

Preliminary Study: In the preliminary examination, observations were made about sample stability, including exposure of solid state samples to heat and light and exposure of solutions to various pH and oxidative conditions. The preliminary study can also be used to aid in the development of an analytical method.

Acid Degradation Condition: Pipette 2 ml of the above solution into a 10ml volumetric flask and 3 ml of 0.1N HCl was added. Then, the volumetric flask was kept at 60 ºC for 6 h and then neutralized with 0.1 N NaOH and makeup to 10ml with Diluent. Filter the solution with 0.22 microns syringe filters and placed in vials. Using mobile phase finally the volume was made up to the mark, and the percentage of degradation was calculated.

Alkali Degradation Condition: Pipette 2 ml of the above solution into a 10 ml volumetric flask into a 10ml volumetric flask and add 3 ml of 0.1N NaOH was added in 10 ml of volumetric flask. Then, the volumetric flask was kept at 60 ºC for 6 h and then neutralized with 0.1N HCl and makeup to 10 ml with Diluent. Filter the solution with 0.22 microns syringe filters and placed in vials. Finally, volume was made up to the mark with the mobile phase, and the percentage of degradation was calculated.

Thermal Induced Degradation Condition: Elvitegravir, Cobicistat, Emtricitabine, and Tenofovir Disoproxil Fumarate sample was taken in Petri dish and kept in a hot air oven at 110 ºC for

24 h. Then the sample was taken and diluted with diluents and injected into UPLC and percentage of degradation was calculated.

Photolytic Degradation Condition: A 5 ml aliquot of the above stock solution was exposed to sunlight for about 6 h, and then the sample diluted with 5 ml of mobile phase and the percentage of degradation was calculated.

Oxidative Degradation Condition: Pipette 2 ml above stock solution 2 into a 10 ml volumetric flask solution into a 10ml volumetric flask 1 ml of 3% w/v of hydrogen peroxide added in 10 ml of volumetric flask and the volume was made up to the mark with Diluent. The volumetric flask was then kept at room temperature for 15 min.

Filter the solution with 0.45 microns syringe filters and place in vials and percentage of degradation was calculated.

Optimization of Chromatographic Conditions: Waters, acuity (UPLC) consisting pump, autosampler, auto-injector and Photodiode array detector, thermostatic column compartment connected with an Endoversilo C_{18} (50 × 2.1 nm), 1.8 µm were determined at 252 nm. The mobile phase is 700ml of Acetonitrile, and 300 ml of 0.1% Ortho Phosphoric Acid were mixed in the ratio 70:30 v/v. The contents of the mobile phase were filtered before it was used through 0.22 µm membrane filter for 15 min and pumped from the respective solvent reservoirs to the column at a flow rate of 0.3 ml/min. The column temperature was maintained at 25 ºC and Run time 4 min. The injection volume of a sample was 4 µL, as shown in **Fig. 2.** Inject 20 µL of the above solution into the chromatographic system and measure the areas for Elvitegravir, Cobicistat, Emtricitabine, and Tenofovir Disoproxil Fumarate peaks and calculate the % Assay as shown in **Fig. 3.**

RESULTS AND DISCUSSION:

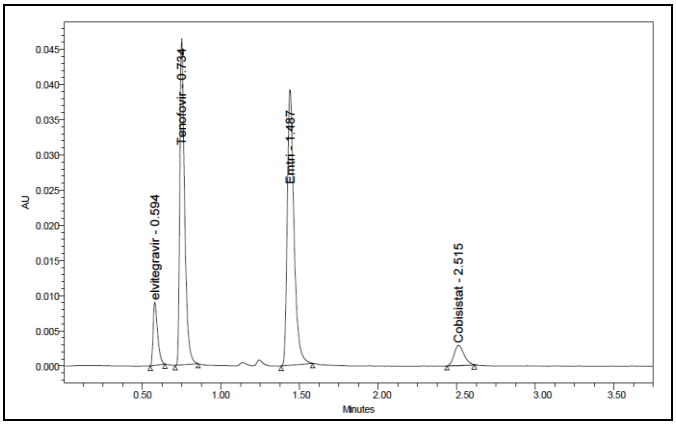

FIG. 2: OPTIMISED CHROMATOGRAM

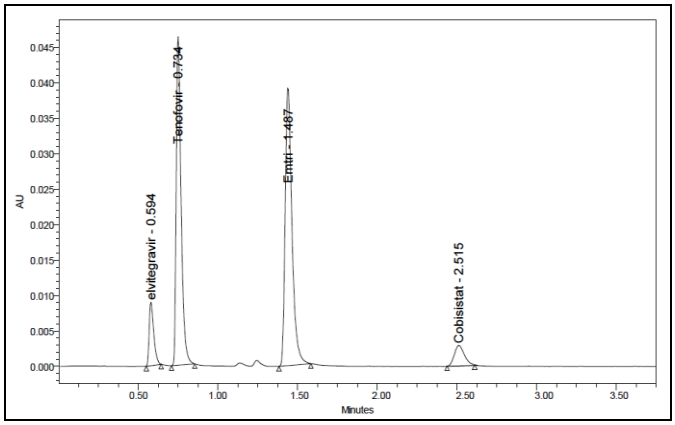

FIG. 3: ASSAY CHROMATOGRAM

System Suitability: To ascertain its effectiveness 10 µL of a freshly prepared standard solution containing 75 mg of Elvitegravir, 75 mg of Cobicistat, 100 mg of Emtricitabine and 150 mg of Tenofovir Disoproxil Fumarate was injected 6 times and System suitability results were calculated. The results obtained are shown in **Table 1.**

TABLE 1: SYSTEM SUITABILITY RESULTS

Parameter	Elvitegravir	Cobisistat	Emtricitabine	Tenofovir D F
Peak Area	0.7	0.7	0.3	0.3
Retention Time	0.59	2.21	1.48	0.73
Tailing Factor	1.21	1.19	1.29	1.19
Plate Count	3212	4582	5732	2852
Resolution	4.25	12.23	10.48	3.19

Specificity: A study to establish the interference of blank and placebo was conducted. The analysis was performed on placebo preparation described previously in triplicate equivalent to about the weight of placebo in a portion of test preparation as per the test method are shown in **Fig. 4-7** clearly show the ability of the method the presence of other excipients.

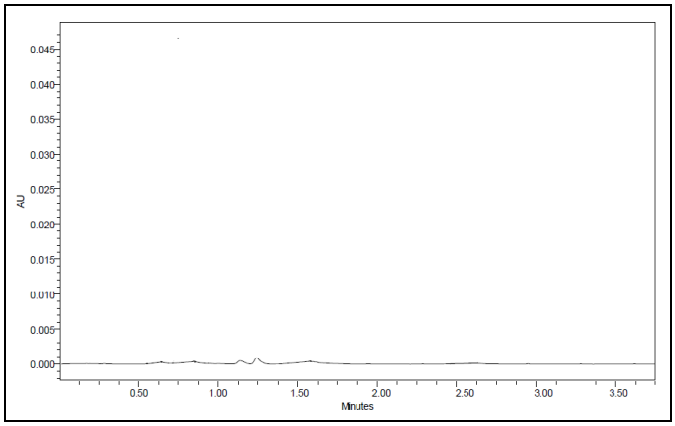

FIG. 4: BLANK CHROMATOGRAM

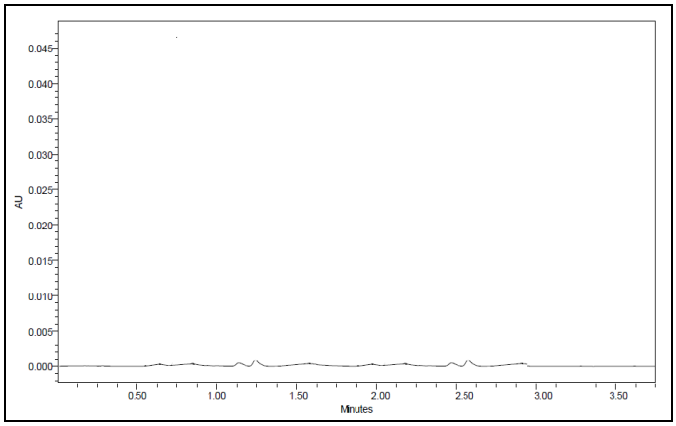

FIG. 5: PLACEBO CHROMATOGRAM

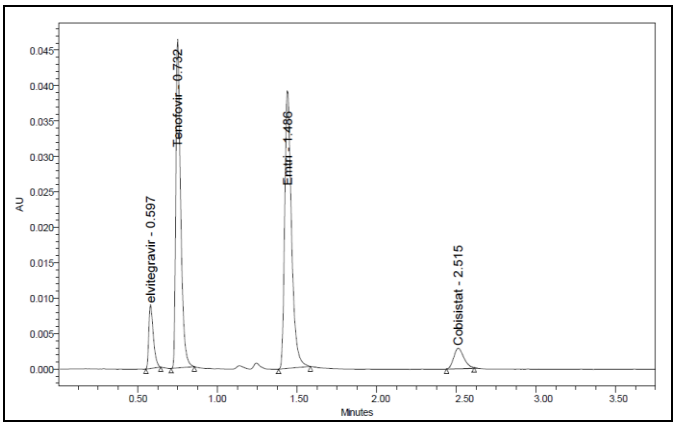

FIG. 6: SPECIFICITY STANDARD

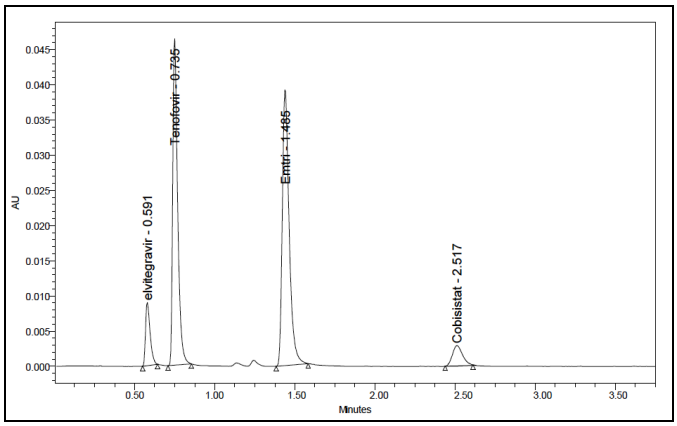

FIG. 7: SPECIFICITY SAMPLE

Linearity and Range: Inject each level into the chromatographic system and measure the peak area. Plot a graph of peak area versus concentration and calculate the correlation coefficient. Correlation coefficient should be not less than 0.999.

The linearity was calculated by measuring different concentrations like 75-225% for Elvitegravir, 75-225% for Cobicistat, 100-300% for Emtricitabine, and 150-450% for Tenofovir Disoproxil Fumarate and was shown in **Table 2** and **Fig. 8-11.**

TABLE 2: LINEARITY DATA

Elvitegravir		Cobisistat		Emtricitabine		Tenofovir D F	
Conc (µg/ml)	Peak Area	Conc (µg/ml)	Peak Area	Conc (µg/ml)	Peak Area	Conc (µg/ml)	Peak Area
75	31158	75	19268	100	200028	150	174283
112.5	62501	112.5	37340	150	412844	225	346309
150	98431	150	55298	200	616857	300	534616
187.5	126883	187.5	74833	250	842986	375	738393
225	159437	225	92106	300	1052774	450	910971

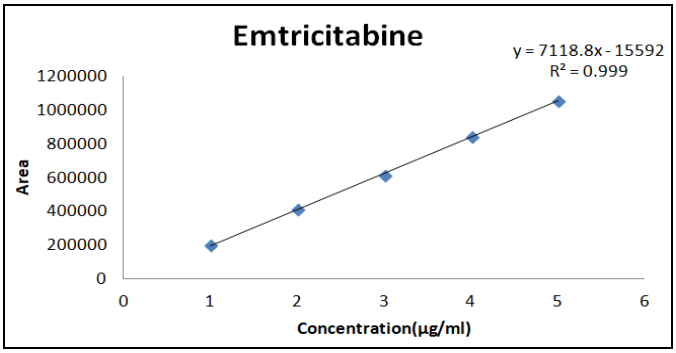

FIG. 8: LINEARITY GRAPH OF EMTRICITABINE

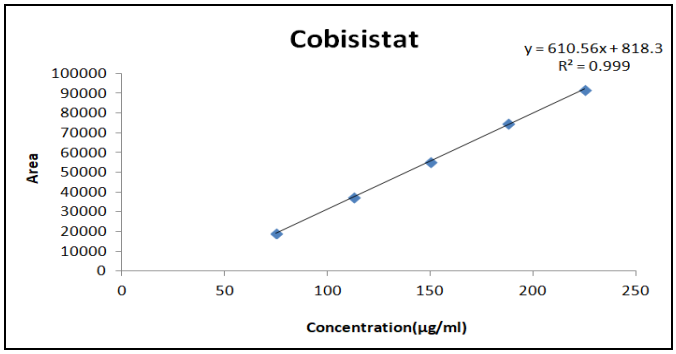

FIG. 9: LINEARITY GRAPH OF COBISISTAT

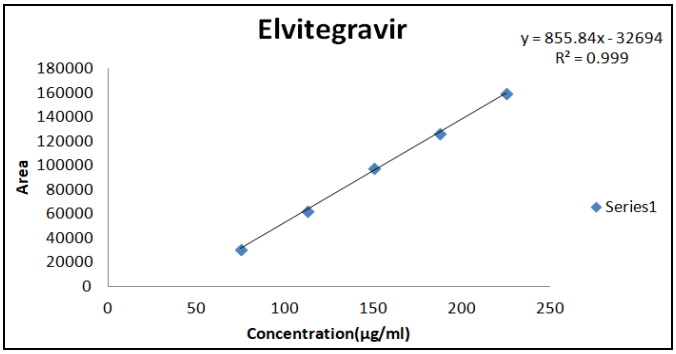

FIG. 10: LINEARITY GRAPH OF ELVITEGRAVIR

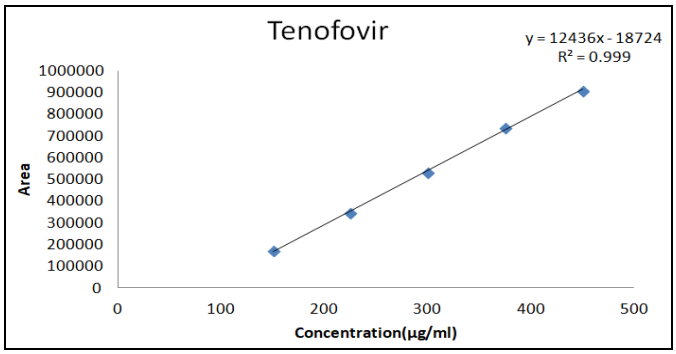

FIG. 11: LINEARITY GRAPH OF TENOFOVIR D F

Precision: The standard solution was injected six times and measured the area for all six injections in HPLC. The % RSD for the area of six standard injections results should not be more than 2%, as shown in **Table 3.**

TABLE 3: PRECISION RESULTS

Injection	Elvitegravir	Cobisistat	Emtricitabine	Tenofovir D F
Average	63154	37333	414939	342315
SD	630.3	240.4	3188.5	2027.7
%RSD	1	0.6	0.8	0.6

Ruggedness: To evaluate the intermediate precision (also known as Ruggedness) of the method, Precision was performed on a different day. The standard solutions prepared in the precision were injected on the other day, for six times and measured the area for all six injections in HPLC. The % RSD for the area of six standard injections results should not be more than 2%, as shown in **Table 4.**

TABLE 4: RUGGEDNESS RESULTS

Injection	Elvitegravir	Cobicistat	Emtricitabine	Tenofovir D F
Average	62593.8	37431.0	416809.2	346397.5
SD	259.1	199.8	2009.7	1529.5
%RSD	0.4	0.5	0.5	0.4

Method Precision: To evaluate the method precision, six individual samples solutions were prepared and calculate the % of Assay, as shown in **Table 5.** The % RSD for the area of six standard injections results should not be more than 2%.

TABLE 5: METHOD PRECISION RESULTS

Injection	Elvitegravir	Cobisistat	Emtricitabine	Tenofovir D F
Average	99.83	99.84	100.55	100.52
SD	0.32	0.39	0.21	0.15
%RSD	0.32	0.39	0.21	0.15

Accuracy:

For Preparation of 50% Solution: Accurately weigh and transfer 37.5 mg of Elvitegravir, 37.5 mg of Cobicistat, 50 mg of Emtricitabine and 75 mg of Tenofovir Disoproxil Fumarate 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. Further pipette 2 ml of the above stock solutions into a 10ml volumetric flask and dilute with diluent.

For Preparation of 100% Solution: Accurately weigh and transfer 75 mg of Elvitegravir, 75 mg of Cobicistat, 100 mg of Emtricitabine and 150 mg of Tenofovir Disoproxil Fumarate 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. Further pipette 2 ml of the above stock solutions into a 10 ml volumetric flask and dilute with diluent.

For Preparation of 150% Solution: Accurately weigh and transfer 112.5 ppm of Elvitegravir, 112.5 ppm of Cobicistat, 150 ppm of Emtricitabine and 225 ppm of Tenofovir Disoproxil Fumarate 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. Further, pipette 2 ml of the above stock solutions into a 10 ml volumetric flask and dilute up to the mark with diluent, as shown in **Table 6.**

TABLE 6: ACCURACY RESULTS

Drug	% Conc.	Amount (mg)		% Recovery	Mean Recovery
		Added	Found		
Elvitegravir	50%	37.5	37.98	101.28	100.11
	100%	75	74.65	99.53	
	150%	112.5	111.97	99.53	
Cobisistat	50%	37.5	37.69	100.52	100.08
	100%	75	74.74	99.65	
	150%	112.5	112.58	100.07	
Emtricitabine	50%	50	50.24	100.49	100.64
	100%	100	100.50	100.50	
	150%	150	151.40	100.93	
Tenofovir D F	50%	75	75.15	100.20	100.26
	100%	150	151.45	100.97	
	150%	225	224.11	99.60	

Robustness:

a) The Flow Rate was Varied at 0.27 ml/min to 0.33 ml/min: Standard solution 112.5 ppm of Elvitegravir, 112.5 ppm of Cobicistat, 150 ppm of Emtricitabine & 225 ppm of Tenofovir Disoproxil Fumarate were prepared and analyzed using the varied flow rates along with method flow rate ± 10% as shown in **Table 7.**

TABLE 7: SYSTEM SUITABILITY RESULTS FOR CHANGE IN FLOW

Drug	Flow Rate	System Suitability Results in USP		
		Plate Count	Tailing	Resolution
Elvitegravir	0.27	2984.61	1.26	-
	0.3	3122.89	1.34	-
	0.33	3885.39	1.38	-
Cobisistat	0.27	4854.78	1.22	12.32
	0.3	4461.23	1.13	12.25
	0.33	4854.78	1.22	12.32
Emtricitabine	0.27	5778.93	1.33	10.46
	0.3	5852.32	1.37	10.49
	0.33	4559.17	1.37	10.24
Tenofovir D F	0.27	2736.95	1.28	3.13
	0.3	2952.03	1.29	3.13
	0.33	2693.92	1.31	3.08

b) The Mobile Phase was Varied at 63% to 77%: Standard solution 112.5 ppm of Elvitegravir, 112.5 ppm of Cobicistat, 150 ppm of Emtricitabine & 225 ppm of Tenofovir Disoproxil Fumarate were prepared and analyzed using the varied Mobile phase ±10 as shown in **Table 8.**

TABLE 8: SYSTEM SUITABILITY RESULTS FOR CHANGE IN MOBILE PHASE

Drug	Change	System Suitability Results in USP		
		Plate Count	Tailing	Resolution
Elvitegravir	10% less	3787.75	1.43	-
	Actual	3122.89	1.34	-
	10% more	2151.78	1.49	-
Cobisistat	10% less	4761.86	1.17	11.70
	Actual	4461.23	1.13	12.25
	10% more	4652.61	1.24	6.14
Emtricitabine	10% less	4144.50	1.24	15.76
	Actual	5852.32	1.37	10.49
	10% more	4764.64	1.41	6.57
Tenofovir D F	10% less	3672.51	1.48	4.04
	Actual	2952.03	1.29	3.13
	10% more	2787.43	1.50	2.14

Forced Stability Studies: In the present study Forced degradation studies were carried out to develop stability profile for the fixed-dose combination of Elvitegravir, Cobicistat, Emtricitabine, and Tenofovir Disoproxil Fumarate and ensure the effective separation of drugs from degradation products. Degradation was observed by the generation of different peaks with different retention time with respective original peaks of the drug. The percentage assay of degradation was calculated from the peak area obtained in degradation conditions, and it was compared with assay of nondegraded conditions. The stability of an analytical method was determined by forced degradation studies, in which the stability of the method was carried out by performing Acid stress study, Base stress study, Peroxide stress study, Water stress study, UV light exposure study, and Dry heat stress study. The net degradation was found to be within limits. The results and chromatograms were summarized in **Table 9** and **Fig. 12-16**.

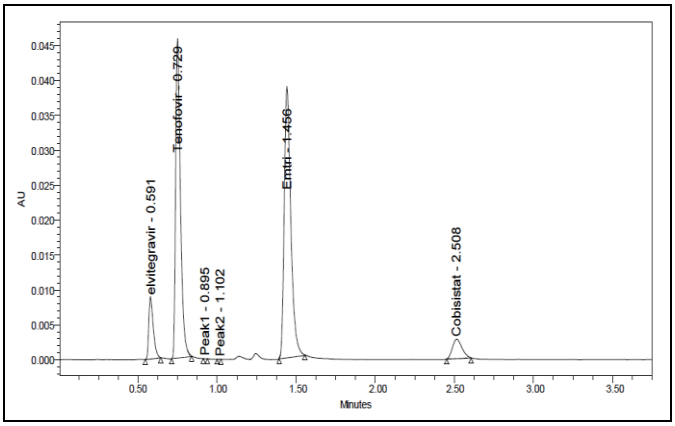

FIG. 12: ACID DEGRADATION

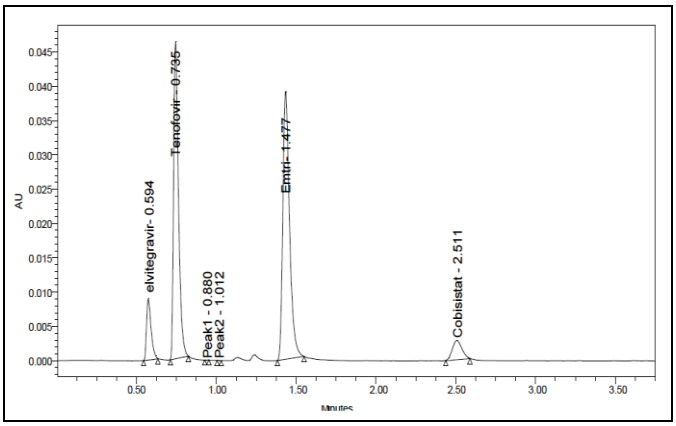

FIG.13: ALKALI DEGRADATION

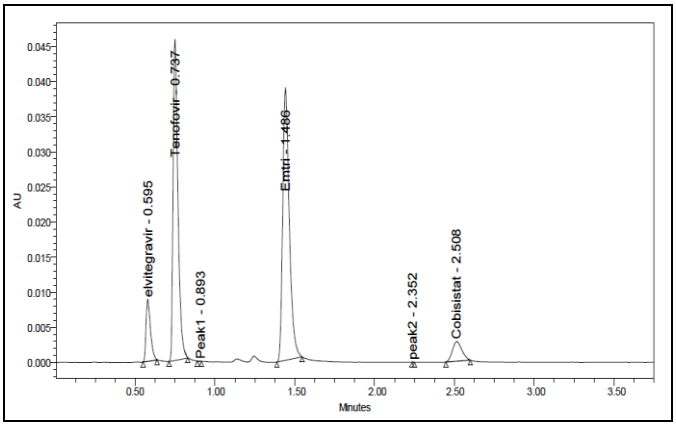

FIG. 14: THERMAL DEGRADATION

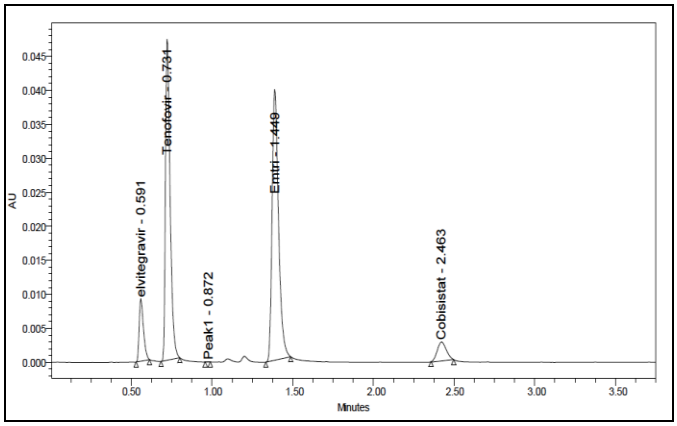

FIG.13: PHOTOLYTIC DEGRADATION

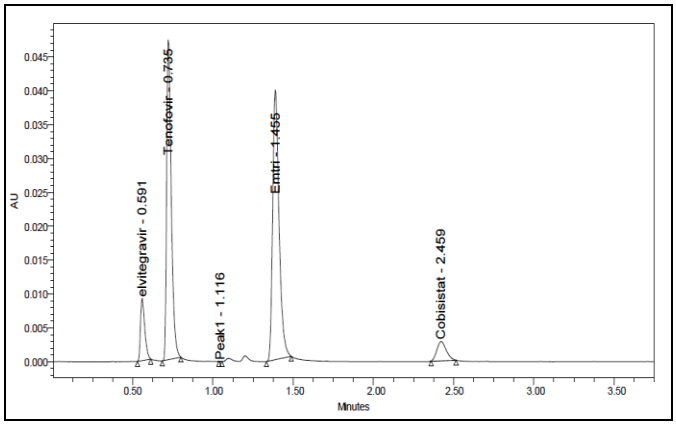

FIG. 16: OXIDATIVE DEGRADATION

TABLE 9: STABILITY STUDIES RESULTS

Condition	Elvitegravir		Cobicistat		Emtricitabine		Tenofovir DF	
	Area	% D	Area	% D	Area	% D	Area	% D
Control	62572	-	37550	-	412362	-	342646	-
Acid	60297	3.64	35645	5.08	393095	4.67	331313	3.31
Base	60635	3.10	35835	4.57	399441	3.13	329952	3.70
Thermal	60221	3.76	35432	5.64	393431	4.59	325462	5.02
Photolytic	60121	3.92	35488	5.49	380461	7.74	325902	4.89
Peroxide	60293	3.64	35209	6.24	396871	3.76	316915	7.51

CONCLUSION: A specific, accurate stability indicating method was developed for the simultaneous estimation of Elvitegravir, Cobicistat, Emtricitabine, and Tenofovir Disoproxil Fumarate in their combined dosage form using RP-UPLC and validated as per the ICH guidelines. The method was validated by using various validation parameters, and the method was found to be linear, precise, accurate, specific, and robust. The retention times were found to be 0.59 min for Elvitegravir, 2.21 min for Cobicistat, 1.48 min for Emtricitabine, and 0.73 min for Tenofovir Disoproxil Fumarate with a total run time of 4 min. From the degradation, studies conducted, it was concluded that Elvitegravir, Cobicistat, Emtricitabine, and Tenofovir Disoproxil Fumarate were more stable at acid, base, peroxide, thermal, UV, and oxidative stability conditions.

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