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METHOD DEVELOPMENT AND VALIDATION FOR SIMULTANEOUS ESTIMATION OF EMTRICITABINE, TENOFOVIR AF, DOLUTEGRAVIR IN ITS BULK AND PHARMACEUTICAL DOSAGE FORM BY RP-HPLC METHOD

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ABSTRACT

A short selective, precise, accurate and sensitive for the estimation of Emtricitabine, Tenofovir AF and Dolutegravir was done by RP-HPLC. During the stress study, the degradation products were well-resolved from its impurities and the mass balances were found to be satisfactory in all the stress conditions, thus proving the stability-indicating capability of the method. The assay of Emtricitabine, Tenofovir AF and Dolutegravir was performed with tablets and the % assay was found to be 99.93 and 100.92 and 100.03 which shows that the method is useful for routine analysis. The linearity of Emtricitabine, Tenofovir AF and Dolutegravir was found to be linear with a correlation coefficient of 0.999 and 0.999 and 0.999 which shows that the method is capable of producing good sensitivity. The acceptance criteria of precision is RSD should be not more than 2.0% and the method show precision 0.8 and 0.3 and 0.8 for Emtricitabine, Tenofovir AF and Dolutegravir which shows that the method is precise.

KEYWORDS: Emtricitabine, Tenofovir AF and Dolutegravir, Validation, stability indicating method, degradation products.

INTRODUCTION

Emtricitabine, 4-amino-5-fluoro-1-[(2R,5S) -2-(hydroxymethyl)- 1,3-oxathiolan-5-yl] -1,2-dihydropyrimidin-2-one, is an anti viral, anti infective agent. Emtricitabine works by inhibiting reverse transcriptase, the enzyme that copies HIV RNA into new viral DNA. Emtricitabine is a synthetic nucleoside analogue of cytidine. It is phosphorylated by cellular enzymes to form Emtricitabine 5'-triphosphate, which is responsible for the inhibition of HIV-1 reverse transcriptase. It competes with the natural substrate deoxycytidine 5'-triphosphate and incorporates into nascent viral DNA, resulting in early chain termination. 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. By inhibiting HIV-1 reverse transcriptase, Emtricitabine can help to lower the amount of HIV, or "viral load", in a patient's body and can indirectly increase the number of immune system cells (called T cells or CD4+ T-cells). Both of these changes are associated with healthier immune systems and decreased likelihood of serious illness.

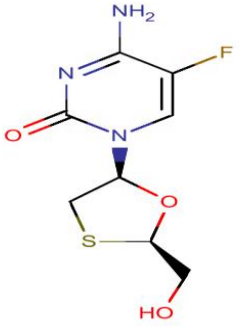

Fig. 1: Chemical structure of Emtricitabine, 4-amino-5-fluoro-1-[(2R,5S)-2-(hydroxymethyl)-1,3-oxathiolan-5-yl]-1,2-dihydropyrimidin-2-one.

Tenofovir, ({[(2R)-1-(6-amino-9H-purin-9-yl) propan-2-yl]oxy} methyl) phosphonic acid is an anti infective, anti viral agent.

Tenofovir inhibits the activity of HIV reverse transcriptase by competing with the natural substrate deoxyadenosine 5'-triphosphate and, after incorporation into DNA, by DNA chain termination. Specifically, the drugs are analogues of the naturally occurring deoxynucleotides needed to synthesize the viral DNA and they compete with the natural deoxynucleotides for

incorporation into the growing viral DNA chain. However, unlike the natural deoxynucleotides substrates, NRTIs and NTRTIs (nucleoside/tide reverse transcriptase inhibitors) lack a 3'-hydroxyl group on the deoxyribose moiety. As a result, following incorporation of an NRTI or an NtRTI, the next incoming deoxynucleotide cannot form the next 5'-3' phosphodiester bond needed to extend the DNA chain. Thus, when an NRTI or NtRTI is incorporated, viral DNA synthesis is halted, a process known as chain termination. All NRTIs and NtRTIs are classified as competitive substrate inhibitors.

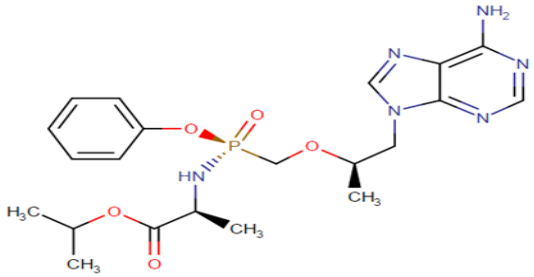

Fig. 2: Chemical structure of Tenofovir, ({[(2R)-1-(6-amino-9H-purin-9-yl) propan-2-yl] oxy} methyl) phosphonic acid.

Dolutegravir, (3S,7R)-N-[(2,4-difluorophenyl)methyl]-11-hydroxy-7-methyl-9,12-dioxo-4-oxa-1,8-diazatricyclo [8.4.0. 0 ^ {3,8}]tetradeca-10,13-diene-13-carboxamide is an anti viral agent. Dolutegravir is an HIV-1 antiviral agent. It inhibits HIV integrase by binding to the active site and blocking the strand transfer step of retroviral DNA integration in the host cell. The strand transfer step is essential in the HIV replication cycle and results in the inhibition of viral activity. Dolutegravir has a mean EC50 value of 0.5 nM (0.21 ng/mL) to 2.1 nM (0.85 ng/mL) in peripheral blood mononuclear cells (PBMCs) and MT-4 cells.

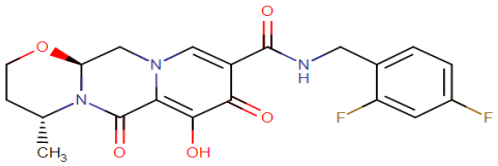

Fig. 3: Chemical structure of Dolutegravir, (3S,7R)-N-[(2,4-difluorophenyl)methyl]-11-hydroxy-7-methyl-9,12-dioxo-4-oxa-1,8-diazatricyclo[8.4.0.0^{3,8}] tetradeca -10,13-diene-13-carboxamide.

A detailed literature revealed that several analytical methods have been reported. In our present knowledge, there is no method reported for the estimation forced degradation studies.

As per the stringent regulatory requirements recommended by the ICH and regulatory agencies, it is mandatory and important to identify and structurally characterize any impurity formed during production and stability testing, exceeding the identification threshold. Various analytical instruments and advanced hyphenated techniques are routinely used to carry out the impurity profile study.

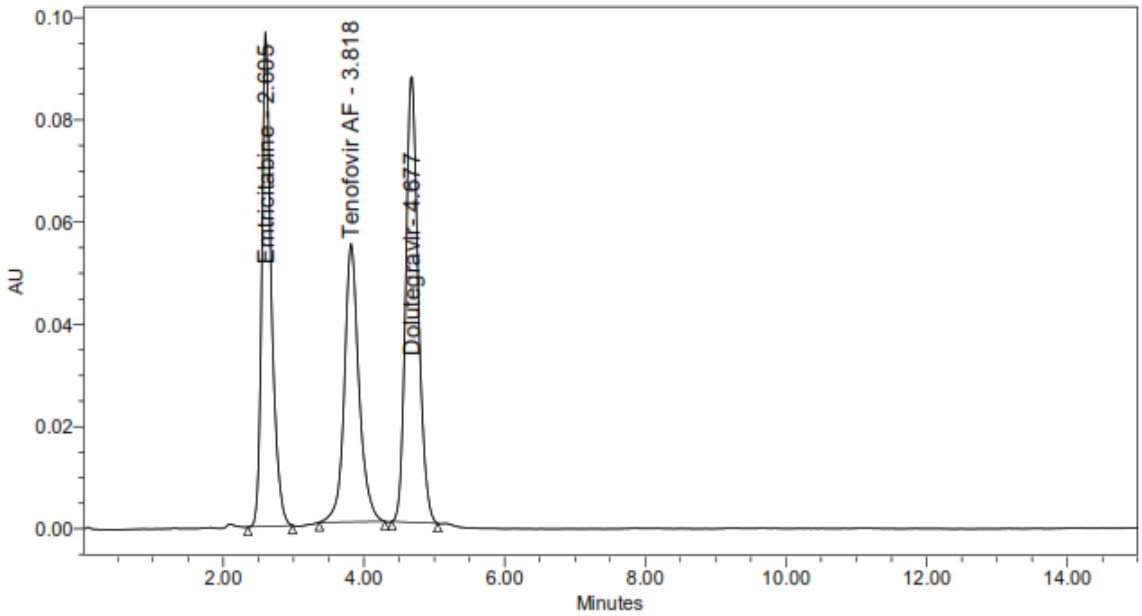

Fig. 4: Standard Chromatogram of Emtricitabine, Tenofovir and Dolutegravir.

Experimental

Optimized chromatographic conditions

Instrument used : Waters HPLC with auto sampler and PDA detector.
Temperature : Ambient

Column	:	Inertsil ODS C_{18} (4.6 x 250mm, 5µm)
Buffer	:	Phosphate buffer pH 3.5
P^H	:	3.5
Mobile phase	:	40% buffer: 60% Acetonitrile
Flow rate	:	1 ml per min
Wavelength	:	281 nm
Injection volume	:	20 µl
Run time	:	15 min.

Preparation Of Buffer And Mobile Phase

Preparation of 0.025M Phosphate buffer

3.4g of potassium dihydrogen ortho phosphate was weighed and taken in a 1000ml volumetric flask and adjust the P^H with Diluted NaOH upto 3,finally the solution was filtered by using 0.45 Micron membrane filter ,sonicate it for 10 mins.

Preparation of mobile phase

Accurately measured 850 ml (85%) of above buffer and 150 ml of Acetonitrile HPLC (15%) were mixed and degassed in an ultrasonic water bath for 10 minutes and then filtered through 0.45 µ filter under vacuum filtration.

Diluent Preparation: The Mobile phase was used as the diluent.

Preparation of the Emtricitabine, Tenofovir Af And Dolutegravir Standard & Sample Solution

Standard Solution Preparation

Accurately weigh and transfer 100mg of Emtricitabine, 12.5mg of Tenofovir AF and 25mg of Dolutegravir working standard into a 100 ml clean dry volumetric flask add small amount of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution).

Further pipette 3 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluent.

Sample Solution Preparation

Accurately weigh 10 tablets crush in mortor and pestle and transfer equivalent to 100mg of Emtricitabine, 12.5mg of Tenofovir AF and 25mg of Dolutegravir in (marketed formulation=177.1 mg of tablet Powder) sample into a 100mL clean dry volumetric flask add small amount of Diluent and sonicate it up to 30 mins to dissolve it completely and make volume up to the mark with the same solvent. Then it is Filtered through 0.45 micron Injection filter. (Stock solution).

Further pipette 3 ml of Emtricitabine, Tenofovir AF and Dolutegravir from the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent.

Procedure

Inject 20 µL of the standard, sample into the chromatographic system and measure the areas for Emtricitabine, Tenofovir AF and Dolutegravir peaks and calculate the %Assay by using the formulae.

RESULTS AND DISCUSSIONS

Validation

The analytical method was validated with respect to parameters such as linearity, precision, specificity and accuracy, limit of detection (LOD), limit of quantitation (LOQ) and robustness in compliance with ICH guidelines.

Table 1: Results of system suitability parameters.

S. No.	Name	RT(min)	Area (µV sec)	Height (µV)	USP resolution	USP tailing	USP plate count
1	Emtricitabine	2.605	910536	95390		1.37	3925.26
2	Tenofovir AF	3.818	749206	54411	4.08	1.13	4822.14
3	Dolutegravir	4.677	1078666	87396	2.92	1.18	3360.73

Table 2: Linearity Results: (for Emtricitabine).

S. No	Linearity Level	Concentration	Area
1	I	0	0
2	II	100	312084
3	III	200	611065
4	IV	300	920625
5	V	400	1206884
6	VI	500	1478014
Correlation Coefficient			0.999

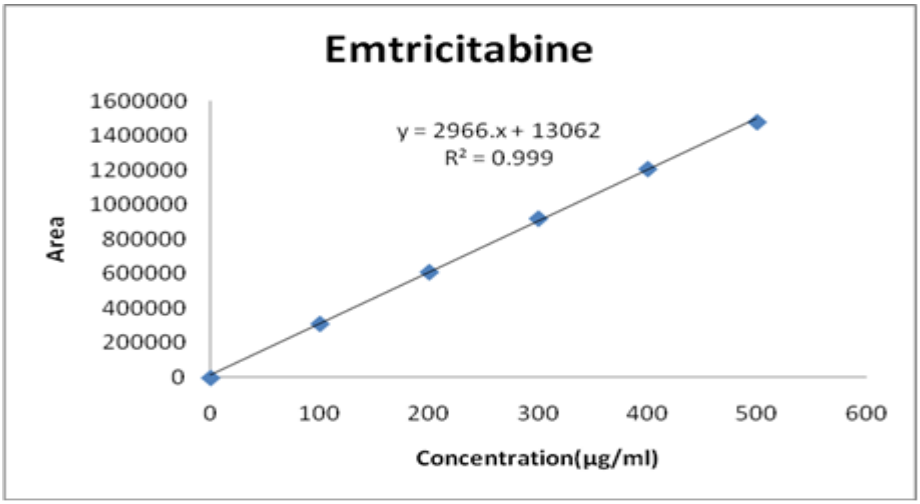

Fig. 5: Calibration graph for Emtricitabine.

Table 3: Linearity Results: (for Tenofovir AF).

S. No	Linearity Level	Concentration	Area
1	I	0	0
2	II	12.5	238656
3	III	25	520646
4	IV	37.5	760227
5	V	50	989211
6	VI	62.5	1249044
Correlation Coefficient			0.999

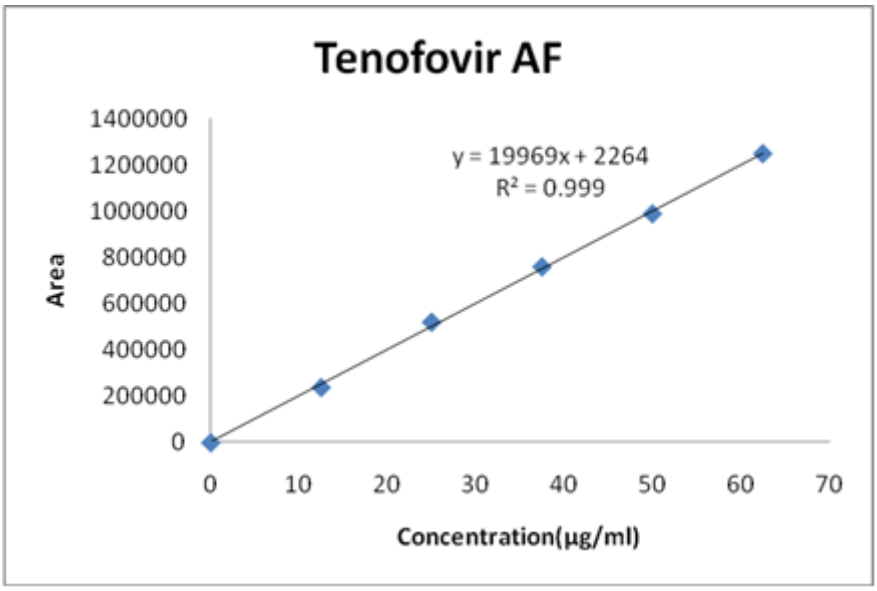

Fig. 6: Calibration graph for Tenofovir AF.

Table 4: Linearity Results: (for Dolutegravir).

S. No	Linearity Level	Concentration	Area
1	I	0	0
2	II	25	369708
3	III	50	726458
4	IV	75	1097778
5	V	100	1422354
6	VI	125	1753625
Correlation Coefficient			0.999

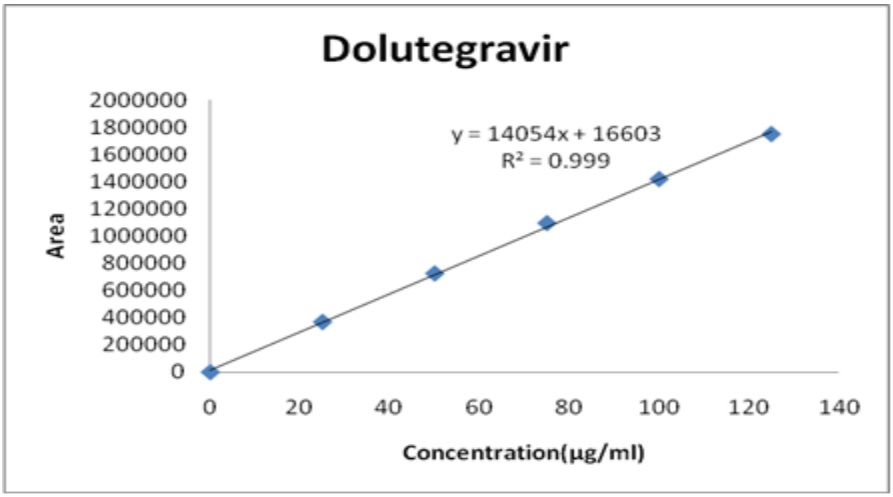

Fig. 7: Calibration graph for Dolutegravir.

Table 5: Results of Precision
The results are summarized for Emtricitabine, Tenofovir AF and Dolutegravir.

Injection	Emtricitabine	Tenofovir AF	Dolutegravir
Injection-1	968448	735275	1103822
Injection-2	958282	730510	1112642
Injection-3	961820	730667	1103264
Injection-4	956833	732869	1108829
Injection-5	947579	730284	1109231
Injection-6	969544	739543	1110379
Average	960417.7	733191.3	1108027.8
Standard Deviation	8148.4	3655.2	3722.7
%RSD	0.8	0.5	0.3

Table 6: Accuracy (recovery) data for Emtricitabine.

%Concentration (at specification Level)	Area	Amount Added (mg)	Amount Found (mg)	% Recovery	Mean Recovery
50%	455260	50	49.69	99.39	99.95
100%	915297	100	99.94	99.94	
150%	1378039	150	150.79	100.53	

Table 7: Accuracy (recovery) data for Tenofovir AF.

%Concentration (at specification Level)	Area	Amount Added (mg)	Amount Found (mg)	% Recovery	Mean Recovery
50%	375837	6.25	6.23	99.63	99.86
100%	755547	12.5	12.43	99.47	
150%	1139549	18.75	18.84	100.48	

Table 8: Accuracy (recovery) data for Dolutegravir.

%Concentration (at specification Level)	Area	Amount Added (mg)	Amount Found (mg)	% Recovery	Mean Recovery
50%	544386	12.5	12.57	100.56	100.27
100%	1090340	25	25.01	100.04	
150%	1631406	37.5	37.58	100.22	

Degradation

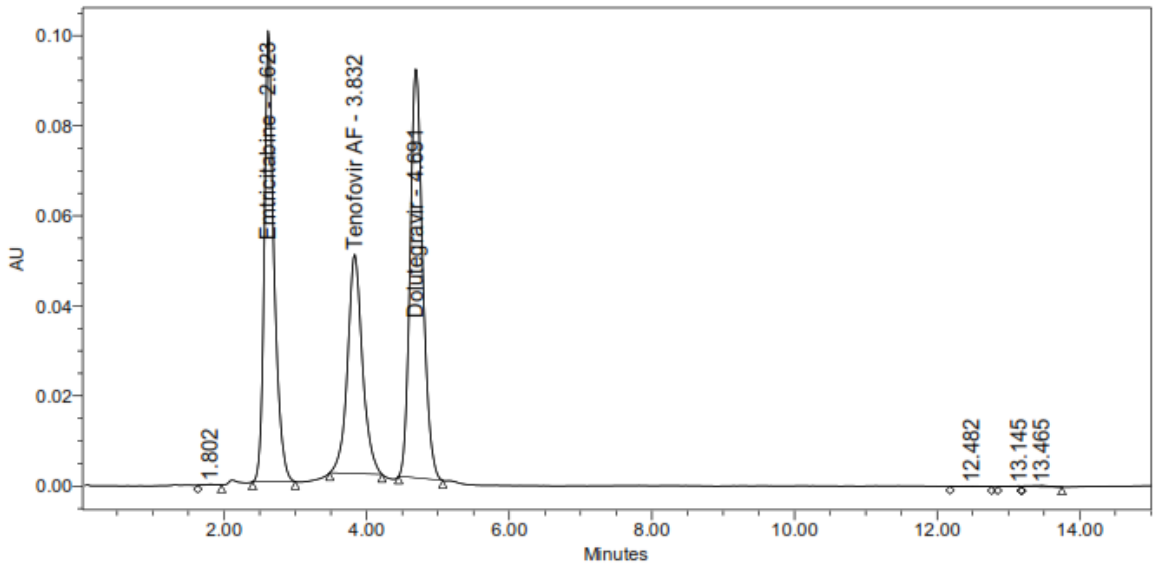

Figure 8: Chromatogram showing acid degradation.

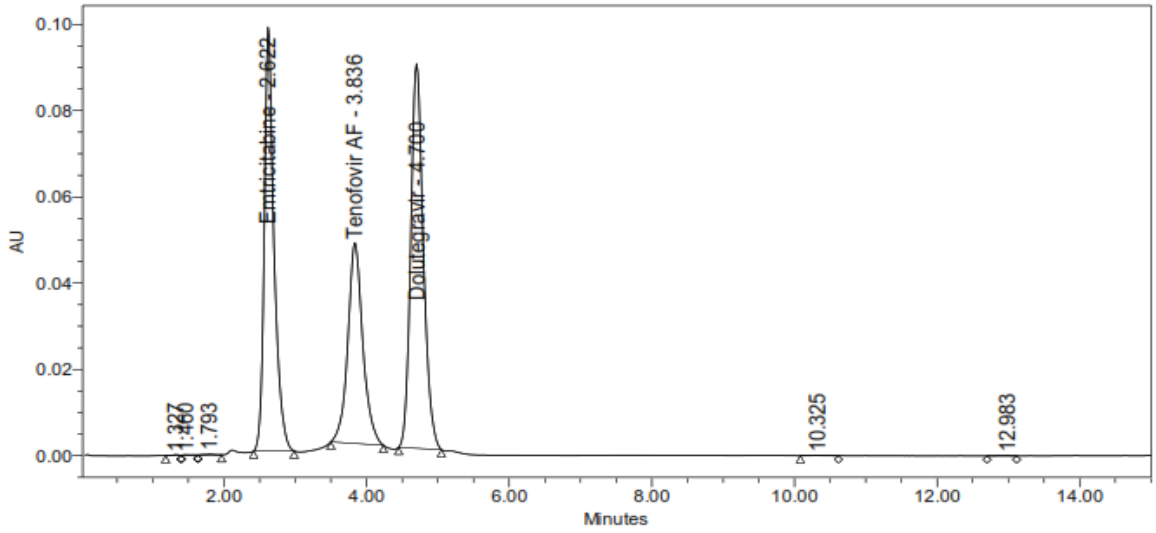

Figure 9: Chromatogram showing Base degradation.

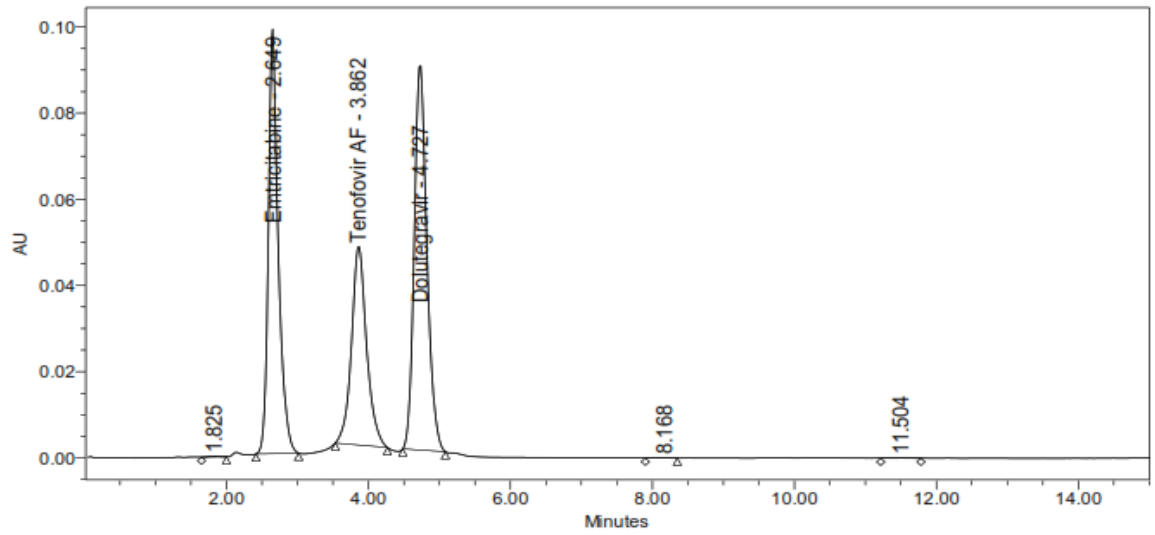

Figure 10: Chromatogram showing Peroxide degradation.

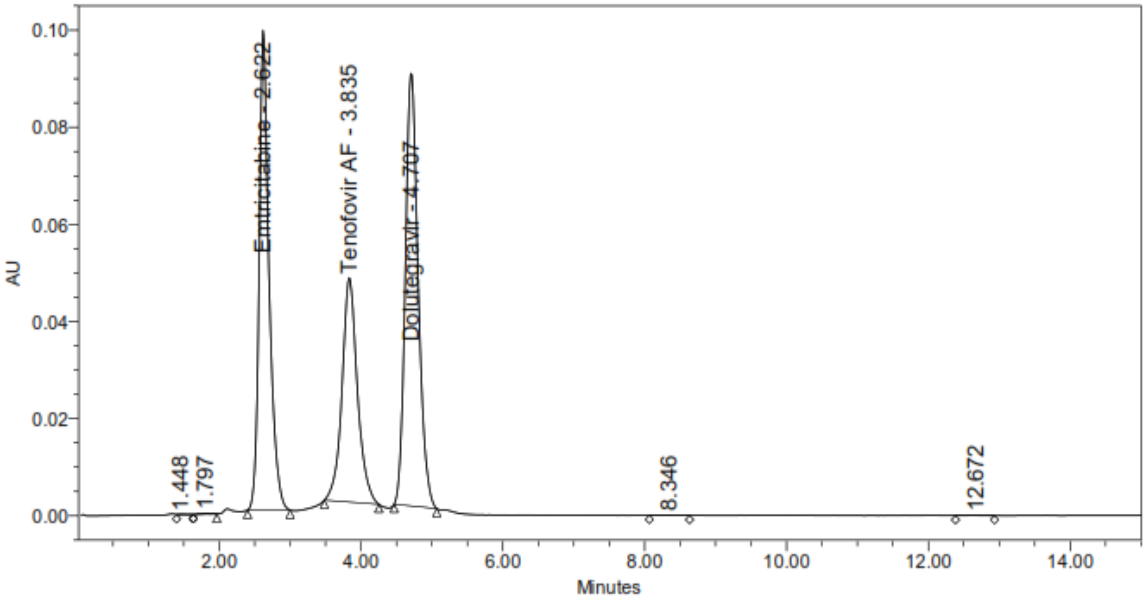

Figure 11: Chromatogram showing Thermal degradation.

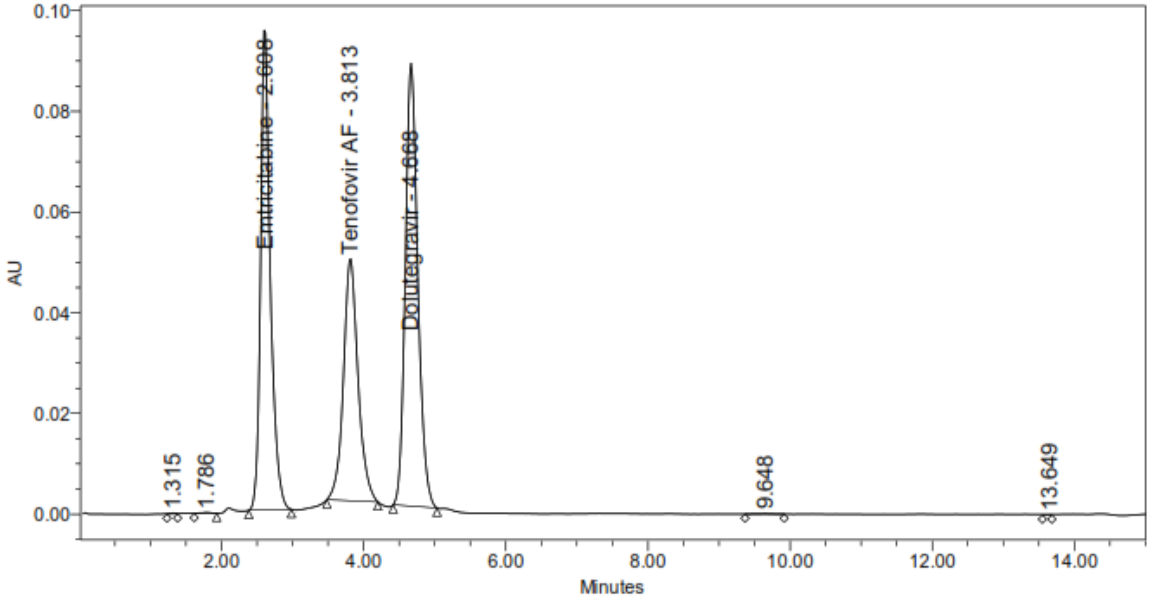

Figure 12: Chromatogram showing Photo degradation.

Table 9: % Degradation results.

	Emtricitabine		Tenofovir AF		Dolutegravir	
	Area	**% Degradation**	**Area**	**% Degradation**	**Area**	**% Degradation**
Standard	912303	NA	754473	NA	1082565	NA
Acid	882563	3.26	735622	2.50	964622	10.89
Base	897865	1.58	746565	1.05	974452	9.99
Peroxide	877382	3.83	715326	5.19	968643	10.52
Thermal	896677	1.71	725442	3.85	993645	8.21
Photo	870864	4.54	711078	5.75	978563	9.61

Table 10: Results of Assay for Emtricitabine and Tenofovir AF and Dolutegravir.

	Label Claim (mg)	% Assay
Emtricitabine	200	99.93
Tenofovir AF	25	100.92
Dolutegravir	50	100.03

SUMMARY AND CONCLUSION

The estimation of Emtricitabine, Tenofovir AF and Dolutegravir was done by RP-HPLC.

The assay of Emtricitabine, Tenofovir AF and Dolutegravir was performed with tablets and the % assay was found to be 99.93 and 100.92 and 100.03 which shows that the method is useful for routine analysis.

The linearity of Emtricitabine, Tenofovir AF and Dolutegravir was found to be linear with a correlation coefficient of 0.999 and 0.999 and 0.999 which shows that the method is capable of producing good sensitivity.

The acceptance criteria of precision is RSD should be not more than 2.0% and the method show precision 0.8 and 0.3 and 0.8 for Emtricitabine, Tenofovir AF and Dolutegravir which shows that the method is precise.

The acceptance criteria of intermediate precision is RSD should be not more than 2.0% and the method show precision 0.8 and 0.5 and 0.3 for Emtricitabine, Tenofovir AF and Dolutegravir which shows that the method is repeatable when performed in different days also.

The accuracy limit is the percentage recovery should be in the range of 97.0% - 103.0%. The total recovery was found to be 99.95% and 99.86% and 100.27 for Emtricitabine, Tenofovir AF and Dolutegravir. The validation of developed method shows that the accuracy is well within the limit, which shows that the method is capable of showing good accuracy and reproducibility.

The acceptance criteria for LOD and LOQ is 3 and 10. The LOD and LOQ for Emtricitabine was found to be 0.48 and 1.61 and LOD and LOQ for Tenofovir AF was found to be 0.11 and 0.36 and LOD and LOQ for Dolutegravir was found to be 0.13 and 0.44

The robustness limit for mobile phase variation and flow rate variation are well within the limit, which shows that the method is having good system suitability and precision under given set of conditions.

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