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NEW VALIDATED, OPTIMIZED AND FORCED DEGRADATION STUDY FOR THE SIMULTANEOUS ESTIMATION OF RILPIVIRINE, EMTRICITABINE, AND TENOFOVIR ALAFENAMIDE IN BULK AND PHARMACEUTICAL DOSAGE PREPARATIONS BY RP-HPLC

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ABSTRACT

A simple and selective RP-HPLC method is described for the simultaneous determination of Rilpivirine, Emtricitabine, and Tenofovir alafenamide dosage forms. Chromatographic separation was achieved on a Inertsil C_{18} Column(150x4.6 mm, 5 µmparticle size)using mobile phase consisting of a mixture of mixed 0.1N Phosphate buffer(pH: 4): Acetonitrile (40:60v/v), with detection of 275nm. Linearity was observed in the range 3-10 µg/ml for Rilpivirine (r^2 =0.996) 20-60 µg /ml for Emtricitabine (r^2 =0.9967), 30-70µg /ml for Tenofovir alafenamide (r^2 =0.995) for the amount of drugs estimated by the proposed methods was in good agreement with the label claim. The proposed methods were validated. The accuracy of the methods was assessed by recovery studies at three different levels. Recovery studies indicated that the absence of additives interference. Repeatability analysis of the method was found to be precise, showing %RSD less than 2.

KEYWORDS

Rilpivirine, Emtricitabine, and Tenofovir alafenamide, RP-HPLC, Relative standard deviation(RSD), Correlation coefficient (r^2), Simultaneous estimation and ICH.

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INTRODUCTION[1-5]

Rilpivirine is used to treat HIV-1 infections. It is a diarylpyrimidine nucleotides found in DNA. Highly active antiretroviral therapy (HAART) has brought new hope for treatment of HIV/AIDS by decreasing the morbidity and mortality. Flexible chemical structure of rilpivirine is less likely to develop resistance than other NNRTI's. It is chemically 4-{[4-({4-[(1E)-2-cyanoeth-1-en-1-yl]-2, 6

dimethylphenyl} amino) pyrimidin-2-yl] amino} benzonitrile.

Rilpivirine is an NNRTI which binds to reverse transcriptase which results in a block in RNA and DNA- dependent DNA polymerase activities. One such activity is HIV-1 replication. Because of the structure of rilpivirine is flexible around the aromatic rings, the molecule can have multiple conformations so that can bind to residues in the reverse transcriptase enzyme which have a lower mutation rate. Treatment of HIV-1 infections in treatment-naive patients with HIV-1 RNA ≤100,000 copies/mL in combination with at least 2 other antiretroviral agents. Highly active antiretroviral therapy also has improved the quality of life among the people who live with HIV/AIDS. The fixed dose combinations of RT inhibitors Rilpivirine, Emtricitabine, and Tenofovir alafenamideare effective in the therapy of human immunodeficiency virus infection.

Emtricitabine is used to treatment of HIV infection. Emtricitabine is an analogue of cytidine. Emtricitabine works by inhibiting reverse transcriptase enzyme that copies HIV RNA into new viral DNA.

Emtricitabine inhibits reverse transcriptase enzyme that copies HIV RNA into new viral DNA. Emtricitabine is a synthetic nucleoside analogue of cytidine. Emtricitabine 5'-triphosphate is formed from Emtricitabine by phosphorylation, which is responsible for the inhibition of HIV-1 reverse transcriptase. It competes with deoxycytidine 5'-triphosphate resulting in chain termination. Therefore emtricitabine inhibits the activity of HIV-1 reverse transcriptase (RT). By inhibiting HIV-1 reverse transcriptase, it helps to lower "viral load", in a patient's body and increase the number of immune system CD4+ T cells indirectly. Both these changes are associated with healthier immune systems and decreased likelihood of serious illness. Chemically it is 4-amino-5-fluoro-1-[(2R, 5S)-2-(hydroxymethyl)-1, 3-oxathiolan-5-yl]-1, 2-dihydropyrimidin-2-one.

Tenofovir alafenamide (a prodrug of tenofovir), marketed by Gilead Sciences under the trade name Vemlidy®, belongs to a class of antiretroviral drugs known as nucleotide analogue reverse transcriptase inhibitors (nRTIs), which block reverse transcriptase crucial to viral production in HIV-infected people. In vivo tenofovir alafenamide is converted to tenofovir, an acyclic nucleoside phosphonate (nucleotide) analog of adenosine 5'-monophosphate.Chemically it is ({[(2R)-1-(6-amino-9H-purin-9-yl) propan-2-yl] oxy} methyl) phosphonic acid. The chemical structures of Rilpivirine, Emitricitabine, and Tenofovir alafenamide were shown in Figure No.1.

The literature reveals various analytical methods for quantitative determination of Rilpivirine, Emtricitabine, and Tenofovir alafenamide individually and in combination with other drugs by HPLC. A spectrophotometric method was reported for the determination of Rilpivirine, Emtricitabine, and Tenofovir alafenamidein bulk and pharmaceutical dosage form[6-13]. These method was successfully validated according to the International Conference on Harmonization, (ICH) guidelines[14,15].

MATERIAL AND METHODS

All reagents were of HPLC grade unless specified. Methanol, Acetonitrile, were purchased from Merck science and technology co. Ltd (Mumbai). THF, HPLC water was analytical grade obtained from Rankem India Co.Ltd. Three lots of Rilpivirine, Emitricitabine, and Tenofovir alafenamide were supplied by Chandra labs Research Institute, Hyderabad. Odefsey tablet containing Rilpivirine 25mg Emitricitabine 200mg, and Tenofovir alafenamide 25mg was kindly supplied by Gilead sciences Inc.

Chromatographic Conditions[16,17]

The Chromatographic studies were performed on a WATERS2695 HPLC Column(Alliance) with an auto sampler and equipped with a 2996 series of PDA detector with a spectral band pass of 1.2nm.Components were detected using UV and that processing was achieved by Empower2 Software. Nicolet evolution 100 was used for ultraviolet spectrum scan. Ultrasonic bath (Labman), digital PH meter (Metsar) were used in the study.

The proposed method was carried out on a Inertisil C18 (150x4.6 ID) 5µm and the mobile phase consisted of 0.1N Phosphate buffer (pH: 4): Acetonitrile (40:60v/v) the column temperature was set 30ºC; the determine wavelength was set 275 nm the flow rate was 1ml/min in gradient elution and the Injection volume was 10µg/ml. The Chromatograms of the prepared standard stock solutions of Rilpivirine, Emitricitabine, and Tenofovir alafenamide were recorded under optimized chromataographic conditions (Figure No.3)

Diluent

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

Preparation of Mobile Phase

The mixture of 0.1N Phosphate buffer: Acetonitrile (40:60 v/v) was prepared. Filtered and degassed the mobile phase.

Preparation of Standard solution

System suitability solution was prepared by dissolving appropriate amount of Rilpivirine, Emitricitabine, and Tenofovir alafenamide in 0.1N Phosphate buffer (PH:4): Acetonitrile(40:60V/V) and adding right amount of standard store solution of Rilpivirine, Emitricitabine, and Tenofovir alafenamide to give a final concentration of 1.62µg/ml, 13µg/ml, 20µg/ml respectively. Sample solution was made by dissolving a suitable amount of Rilpivirine Emitricitabine, and Tenofovir alafenamide in Phosphate buffer PH: 4: Acetonitrile(40:60V/V) to give concentration of 1000µg/ml.

Method development

The UV Spectrum scan from 190 to 420 nm was carried out on Nicolet evolution 100 spectrophotometer for wavelength selection. The concentration of Rilpivirine, Emitricitabine, and Tenofovir alafenamide to give a concentration of 1.62µg/ml, 13µg/ml, 20µg/ml respectively in a solvent of 0.1N Phosphate buffer (PH: 4): Acetonitrile (40:60V/V). For selecting column chiral columns of OD52546 and SCDP 52546 Inertisil was choosen to separate Rilpivirine, Emitricitabine and Tenofovir alafenamide by injecting system suitability solution with the mobile phase at 1.0ml/min individually.

Various solvents including HPLC-Water, Acetonitrile, THF, Ammonium acetate, Methanol were used in different combinations to get good peaks resolutions and lesser run time. Different flow rates from 0.4 to 1 ml/min in gradient mode have been studied to achieve a good peak resolution. The column temperature was set at 25^0,30^0 and 35^0 C for optimizing according to its effect on peak resolutions and retention times of the drug samples.

Method Validation

The system suitability test was carried out to validate that whether Rilpivirine, Emitricitabine, and Tenofovir alafenamide could be separated using the proposed method by injecting system suitability solution. The repeatability was checked by measuring the peak areas of Rilpivirine, Emitricitabine, and Tenofovir alafenamide of successive injections of 5 individual system suitability solutions, the values of relative standard deviation (RSD) of 5 measure results were calculated for repeatability evaluation.

The Intermediate precision was tested through measuring the peak areas of Rilpivirine, Emitricitabine and Tenofovir alafenamide of successive injections of 5 individual system suitability solutions by different analyst on different dates, the values of RSD of 5 measuring results were computed to evaluate the intermediate precision evaluation.

The limit of detection (LOD) and LOQ for Rilpivirine, Emitricitabine and Tenofovir alafenamide were estimated as the amounts for which that the signal to noise ratios were 3:1 and 10:1 respectively, by injecting a series of diluents solutions of standard store solution of 3 analytes.

By quantitatively diluting the standard store solutions of Rilpivirine, Emitricitabine, and Tenofovir alafenamide a series of different concentrations solutions ranging from the LOQ to 200% of the permitted maximum level (not more than 0.1% in test sample of 1000µg/ml), i.e LOQ, 0.15% 0.1%, 0.2% for Rilpivirine, Emitricitabine, and Tenofovir alafenamide were prepared in triplicate to evaluate linearity, respectively. Peak areas and concentrations of the analytes were subjected to regression analysis to calculate the

calibration equation and the correlation coefficient(r).

Accuracy was evaluated by determination of recoveries of Rilpivirine, Emitricitabine, and Tenofovir alafenamide at four levels, respectively. Considering the linear interval of the validated method, the tests were performed at the concentrations of spiked LOQ 50, 100 and 150% of limit level to drug sample solutions (1000µg/ml), in five replicates, respectively. The recoveries were calculated using the formulae,

Recovery (%) = $M_{measured}$/ M_{Spiked}x100,

Where $M_{measured}$ was the weight of spiked compound measured using the developed method,

M_{Spiked} was the weight spiked in to sample solution.

By modifying flow rate from 0.4 to 1 ml/min. column temperature from 28^0 to 32^0 C, additives content from 0.09% to 0.1% and employing columns from different lots method robustness was tested. While a factor was investigating, the other chromatographic conditions were held constant.

The chromatographic resolution between Rilpivirine, Emitricitabine, and Tenofovir alafenamide were used to evaluate the method robustness.

The stability test of drug sample solutions were studied for the determination of test solution at 0, 1, 2, 4, 8, 12 hr to know the self half life period. The RSD of Rilpivirine, Emitricitabine, and Tenofovir alafenamide peak area was used to evaluate solution stability.

RESULTS AND DISCUSSION

Determination of Working Wavelength (λmax)

The UV spectrum scan result showed that Rilpivirine, Emitricitabine, and Tenofovir alafenamide had a larger absorbance at 275 nm. Because of Rilpivirine, Emitricitabine, and Tenofovir alafenamide quality criteria and some papers relative to determination of all selected 275nm as test wavelength, taking these reasons into consideration, the UV wavelength of 275nm was selected as test wavelength. In simultaneous estimation of three drugs isobestic wavelength is used. Isobestic point is the wavelength where the molar absorptivity is the same for two substances that are interconvertible. So this wavelength is used in simultaneous estimation to estimate three drugs accurately.

The wavelength of maximum absorption (λ_{max}) of the drug, 10 µg/ml solution of the drugs in methanol were scanned using UV-Visible spectrophotometer within the wavelength region of 200–400 nm against methanol as blank. The resulting spectra are shown in the Figure No.2 and the absorption curve shows characteristic absorption maxima for Rilpivirine, Emtricitabine, and Tenofovir alafenamide 275nm for the combination.

After several initial trails with mixtures of Methanol, Acetonitrile and Different buffer in various combinations and proportions, a trail with a mobile phase mixture of 0.1N Phosphate Buffer (PH:4): Acetonitrile (40:60 v/v) brought sharp and well resolved peaks. The chromatogram was shown in Figure No.3.

System Suitability

The Retention time of Rilpivirine, Emitricitabine, and Tenofovir alafenamide using Optimum conditions was 3.273min, 2.517min, and 6.697min respectively. For all of them, the peak symmetries were <1.5 and the theoretical plates numbers were >2000 and %RSD of areas of five standard injections of Rilpivirine, Emitricitabine, and Tenofovir alafenamide were less than 2. These values are within the acceptable range of United States Pharmacopoeia definition and the chromatographic conditions. The results obtained are shown in Table No.1

Linearity

Preparation of standard solution

Weigh accurately 1.62mg of Rilpivirine, 13mg of Emtricitabine and 20mg of Tenofovir alafenamide in 100 ml of volumetric flask and dissolve in 10ml of mobile phase and make up the volume with mobile phase From above stock solution 1.62µg/ml of Rilpivirine and 13µg/ml of Emtricitabine and 20µg/ml of Tenofovir alafenamide is prepared by diluting 5.3ml to 10ml with mobile phase. This solution is used for recording chromatogram.

Preparation of sample solution

5tablets (each tablet contains 25mg of Rilpivirine, 200mg of Emtricitabine and 300mg of Tenofovir

alafenamide) were weighed and taken into a mortar and crushed to fine powder and uniformly mixed. Weight equivalent to 34.62mg of Rilpivirine, Emtricitabine and Tenofovir alafenamide and dissolved in sufficient mobile phase. After that filtered the solution using 0.45-micron syringe filter and Sonicated for 5 min and dilute to 100ml with mobile phase. Further dilutions are prepared in 5 replicates of 1.62µg/ml of Rilpivirine, 13µg/ml of Emtricitabine and 20µg/ml of Tenofovir alafenamide was made by adding 5.3ml of stock solution to 10 ml of mobile phase. The results were shown in (Table No.2, 3, 4, 5, and Figure No.8, 9, 10).

PRECISION

Method precision (Repeatability)

Prepared sample preparations of Rilpivirine, Emtricitabine and Tenofovir alafenamide as per test method and injected 5 times in to the column. The precision of the instrument was checked by repeated injections and measurement of peak areas and retention times of solutions (n = 6) for, 1.62µg/ml of Rilpivirine, 13µg/ml of Emitricitabine and 20µg/ml of Tenofovir alafenamide without changing the parameter of the proposed chromatographic method as shown in the Table No.9

System Precision

The System precision of the proposed method was determined by analyzing the corresponding responses 3 different days over a period of 1 week for 1.62µg/ml of Rilpivirine, 13µg/ml of Emitricitabine and 20µg/ml of Tenofovir alafenamide. The result was reported in terms of relative standard deviation (% RSD) as shown in the Table No.10

Limit of detection and limit of quantification

The limit of detection (LOD) and limit of quantification (LOQ) were separately determined based on standard deviation of the y-intercept and the slope of the calibration curve. The results were tabulated in Table No.13.

Accuracy (Recovery study)

Accuracy of the method was determined by Recovery studies. To the formulation (pre analyzed sample), the reference standards of the drugs were added at the level of 50%, 100%, 150%. The recovery studies were carried out three times and the percentage recovery and percentage mean recovery were calculated for drug is shown in Table No.6, 7, 8. Accuracy of the method were carried out using recovery studies by addition of standard drug solution to pre-analyzed sample solution at three different levels 50%, 100%, 150%.

The accuracy of the method was determined by calculating the recoveries of Rilpivirine, Emitricitabine and Tenofovir alafenamide by the standard addition method. Known amounts of standard solutions of Rilpivirine, Emitricitabine and Tenofovir alafenamide were added at 10% concentration to pre quantified sample solutions (Figure No.4). The amount of Rilpivirine, Emitricitabine and Tenofovir alafenamide recovered was estimated by using the following formulae.

% Recovery = Amount found x 100
Amount added

Amount found (mcg/ml) = Mean test area x Standard concentration
Mean standard area

Specificity

In an assay, demonstration of specificity requires that it can be shown that the procedure is unaffected by the presence of impurities or excipients. This can be done by spiking the drug substance or product with different levels of impurities or excipients and demonstrating that the assay results are unaffected by the presence of these extraneous materials. There should be no interference of the diluents, placebo at retention time of drug substances as shown in Figure No.5, 6, 7.

Robustness

Robustness is the measure of a method remain unaffected by small, deliberate changes in method parameters like flow rate and detection wavelength on assay of the analyte of interest. Here the detection wavelength varied ±2nm and flow rate was varied ±0.2 ml/min. The results were shown in (Table No.11).

The robustness of the method were done using prepared solution as per test method and injected at different variable conditions like using different

conditions like Temperature and wavelength. System suitability parameters were compared with that of method precision.

FORCED DEGRADATION STUDIES

Preparation of stock solution

Accurately weighed 25 mg of Rilpivirine, 200mg of Emtricitabine, and 300mg of Tenofovir alafenamide into 50ml capacity standard volumetric flask. The contents in the flask was dissolved using methanol and sonicate it and diluted up to the mark with methanol[18,19]. The results were shown in (Table No.16, Figure No.11, 12, 13, 14, 15).

Acid degradation

Accurately 5.0 ml aliquot of above stock solution was transferred into a 50 ml round bottom flask, and 2.5 ml of 0.1N HCl was added. The flask was refluxed at 60^0C for 30 min using Buchi rota evaporator and then allowed to cool. Then neutralized with 0.1N NaOH solution. Using mobile phase finally volume was made up to the mark and percentage of degradation was calculated.

Alkali degradation

Accurately 5.0 ml aliquot of above stock solution was transferred into a 50 ml round bottom flask, and 2.5 ml of 0.1N NaOH was added. The flask was refluxed at 60^0C for 30 min using Buchi rota evaporator and then allowed to cool. Then neutralized with 0.1N HCl solution. Finally, volume was made up to the mark with mobile phase, and percentage of degradation was calculated.

Peroxide Condition

Accurately 5.0 ml aliquot of above stock solution was transferred into a 50 ml round bottom flask, and 3.0 ml of 3% H_2O_2 was added. The flask was kept at room temperature for 30 min then allowed to cool, Finally volume was made up to the mark with mobile phase, and percentage of degradation was calculated.

Thermal Condition

25 mg of Rilpivirine, 200mg of Emtricitabine, and 300mg of Tenofovir alafenamide were weighed accurately and transfer into four different Petri dishes and kept in a hot air oven for 8h at 105ºC.The samples were then placed in a desiccators till reaches the room temperature. From this Petri dishes accurately weighed 12.5 mg of Rilpivirine, 100mg of Emtricitabine, and 150mg of Tenofovir alafenamide into 50 ml capacity standard volumetric flasks. The content in the flasks was dissolved using methanol and diluted up to the mark with methanol. A 5 ml aliquot of each stock solution was transferred into 50ml volumetric flasks and diluted up to the mark using mobile phase.

Photolytic Condition

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

In RP HPLC method, the primary requirement for developing a method for analysis is that the using different solvents, buffers and columns to get better retention time, cost effective and time saving method than the previously developed methods. The Isobestic Point of Rilpivirine, Emitricitabine, and Tenofovir alafenamide were found to be 275nm by scanning in UV region. The chromatographic method was optimized with mobile phase consisting of0.1N Phosphate Buffer (PH: 4): Acetonitrile: (40:60V/V) and Inertsil columnC18 (150x 4.6mm and 5µm). All the validation parameters were studied at a the wavelength 275nm. Accuracy was determined by calculating the recovery (Table No.6, 7, 8) and the results were in acceptable range (limit 98-102%). The method was successfully used to determine the amount of Rilpivirine, Emitricitabine, and Tenofovir alafenamide present in the tablet. The Assay results obtained were in good agreement with the corresponding labeled amount (Table No.14). The method was linear in the concentration range of 3 to 10µg/ml for Rilpivirine, 20 to 60µg/ml for Emitricitabine, 30 to 70µg/ml for Tenofovir alafenamide (FigureNo.8, 9, 10 and Table No.3, 4, 5). Precision was calculated as repeatability and intra and inter day variations (% RSD) for the drug (Table No.9, 10). Robustness and ruggedness results were in acceptable range (Table No.11 and Table No.12). LOD, LOQ results were within the range (Table No.13). Summary of all validation parameters for method is given (TableNo.15). By observing the validation parameters, the method was found to be simple,

sensitive, accurate and precise. Hence the method can be employed for the routine analysis Rilpivirine, Emitricitabine, and Tenofovir alafenamidein Pharmaceutical and tablet dosage form. A new RP-HPLC method for the simultaneous estimation of Rilpivirine, Emitricitabine, and Tenofovir alafenamide in their combine dosage form was developed and validated as per the ICH guidelines. Linearity was observed in the range of 3-10µg/ml for Rilpivirine, 20-60µg/ml for Emitrictabine, and 30-70µg/ml for Tenofovir alafenamide with correlation coefficients (r2=0.999). The percentage recoveries of Rilpivirine, Emitrictabine, and Tenofovir alafenamide were in the range of 98.55-102.5% which was within the acceptance criteria. The results of forced degradation studies for the simultaneous estimation of Rilpivirine, Emitricitabine, and Tenofovir alafenamide were in limits (Table No.16, Figure No.11, 12, 13, 14, 15). The percentage RSD was NMT2% which proved the Precision of the developed method.

The developed method is simple, sensitive, rapid, linear, precise, rugged, accurate, specific and robust. The developed method was found superior in certain respects such as RT and Accuracy. The method was more economical when compared to reported method.

Table No.1: System Suitability of Rilpivirine, Emitricitabine, and Tenofovir alafenamide

S.No	Parameter	Rilpivirine	Emitricitabine	Tenofovir alafenamide
1	Peak area	1105605	1012865	1118501
2	Theoretical plates	6433	2862.66	6402.16
3	Retention time	3.273	2.517	6.697
4	Tailing factor	1.22	0.96	1.335

Table No.2: Various Linearity concentration data of solutions (µg /ml) for Rilpivirine, Emitricitabine, and Tenofovir alafenamide

S.No	Preparations	Volume from standard stock transferred in ml			Volume made up in ml (with mobile phase)	Concentration of solution(µg /ml)		
						Rilpivirine	Emtricitabine	Tenofovir alafenamide
1	Preparation 1	0.24	0.03	0.3	10	3	24	30
2	Preparation 2	0.32	0.04	0.4	10	4	32	40
3	Preparation 3	0.4	0.05	0.5	10	5	40	50
4	Preparation 4	0.48	0.06	0.6	10	6	48	60
5	Preparation 5	0.56	0.07	0.7	10	7	56	70

Table No.3: Linearity data of Rilpivirine

Level	Concentration mcg(µg /ml)	Peak Area	Satistical Analysis
1	3	659.236	Mean=1112.96 S.D=16.58 %RSD=1.42
2	4	919.393	
3	5	1086.050	
4	6	1348.518	
5	7	1552.332	

Table No.4: Linearity data of Emtricitabine

Level	Concentration mcg(µg /ml)	Peak Area	Statistical Analysis
1	24	631.586	Mean=1103.72 S.D=15.68 %RSD=1.35
2	32	907.713	
3	40	1091.004	
4	48	1339.312	
5	56	1549.123	

Table No.5: Linearity data of Tenofovir alafenamide

Level	Concentration mcg(µg /ml)	Peak Area	Statistical Analysis
1	30	1229.584	Mean=1800.03 S.D=24.74 %RSD=1.45
2	40	1482.509	
3	50	1750.266	
4	60	2124.626	
5	70	2413.579	

Recovery Results

Table No.6: Recovery results data for Rilpivirine

S.No	Recovery level	Accuracy of Rilpivirine			Statistical Analysis
		Amount added (µg/ml)	Amount found (µg/ml)	% Recovery	
1	50%	4	3.89	97.25	Mean=99.92(n=3) S.D=0.891 %RSD=1.091
		4	3.87	96.75	
		4	4.35	108.75	
2	100%	5	4.87	97.40	Mean=98.86 S.D=0.786 %RSD=0.894
		5	4.77	95.40	
		5	5.37	107.40	
3	150%	6	5.97	99.50	Mean=100.44 S.D=0.455 %RSD=0.45
		6	5.85	97.50	
		6	6.42	107.00	

Table No.7: Recovery results data for Emitricitabine

S.No	Recovery level	Accuracy of Emitricitabine			Statistical Analysis
		Amount added (µg/ml)	Amount found (µg/ml)	% Recovery	
1	50%	32	31.58	98.68	Mean=99.18 S.D=1.173 %RSD=1.18
		32	31.27	97.71	
		32	32.27	100.84	
2	100%	40	39.58	98.97	Mean=99.28 S.D=1.273 %RSD=1.28
		40	39.27	98.19	
		40	40.27	100.68	
3	150%	48	47.97	99.93	Mean=100.33 S.D=0.933 %RSD=0.92
		48	47.85	99.68	
		48	48.42	100.87	

Table No.8: Recovery results data for Tenofovir alafenamide

S.No	Recovery level	Accuracy of Tenofovir alafenamide			Statistical Analysis
		Amount added (µg/ml)	Amount found (µg/ml)	% Recovery	
1	50%	40	39.58	98.97	Mean=99.28 S.D=1.273 %RSD=1.28
		40	39.27	98.19	
		40	40.27	100.68	
2	100%	50	49.87	99.74	Mean=100.55 S.D=0.132 %RSD=0.162
		50	50.18	100.36	
		50	50.36	100.72	
3	150%	60	60.36	100.66	Mean=100.44 S.D=0.455 %RSD=0.45
		60	59.92	99.92	
		60	60.42	100.75	

Table No.9: Data results for Method precision of Rilpivirine, Emtricitabine, and Tenofovir alafenamide

Rilpivirine		
S.No	**Rt**	**Peak Area**
1	3.277	1094.372
2	3.270	1101.764
3	3.277	1073.595
4	3.273	1108.428
5	3.270	1079.236
6	3.273	1075.584
Mean	3.273	1088.830
SD	0.003	14.708
%RSD	0.10	1.35

Emtricitabine		
S.No	**Rt**	**Peak Area**
1	2.520	1087.803
2	2.517	1089.666
3	2.523	1067.836
4	2.520	1097.279
5	2.517	1059.014
6	2.517	1071.854
Mean	2.5190	1078.909
SD	0.0024	14.836
%RSD	0.10	1.38

Tenofovir alafenamide		
S.No.	Rt	Peak Area
1	6.707	1753.454
2	6.690	1774.741
3	6.707	1729.087
4	6.707	1774.492
5	6.693	1729.167
6	6.693	1727.320
Mean	6.700	1748.044
SD	0.008	22.745
%RSD	0.12	1.30

Table No.10: Data results for System precision of Rilpivirine, Emtricitabine, and Tenofovir alafenamide

System Precision								
Rilpivirine			**Emtricitabine**			**Tenofovir alafenamide**		
S.No	**Rt**	**Area**	**S.No**	**Rt**	**Area**	**S.No**	**Rt**	**Area**
1	3.277	1094.372	1	2.520	1087.803	1	6.707	1753.454
2	3.270	1101.764	2	2.517	1089.666	2	6.690	1774.741
3	3.277	1073.595	3	2.523	1067.836	3	6.707	1729.087
4	3.273	1108.428	4	2.520	1097.279	4	6.707	1774.492
5	3.270	1079.236	5	2.517	1059.014	5	6.693	1729.167
6	3.273	1075.584	6	2.517	1071.854	6	6.693	1727.320
Mean	3.273	1088.830	Mean	2.5190	1078.909	Mean	6.700	1748.044
SD	0.003	14.708	SD	0.002	14.836	SD	0.008	22.745
%RSD	0.10	1.35	%RSD	0.05	1.39	%RSD	0.12	1.50
%RSD NMT 2.00%								

Table No.11: Data results for Robustness study of Rilpivirine, Emtricitabine, and Tenofovir alafenamide

S.No	**Parameter**	**Rilpivirine**		**Emtricitabine**		**Tenofovir alafenamide**	
		Retention time(min)	**Tailing factor**	**Retention time(min)**	**Tailing factor**	**Retention time(min)**	**Tailing factor**
1	Flow 1.0ml/min 1.4ml/min	3.880 2.810	1.676 1.354	2.987 2.167	1.338 1.758	7.893 5.700	1.525 1.550
2	Wavelength 260nm 264nm	3.2603.240	1.310 1.310	2.513 2.490	1.704 1.769	6.617 6.627	1.600 1.565

Table No.12: Data results for Ruggedness study of Rilpivirine, Emtricitabine, and Tenofovir alafenamide

S.No	**Rilpivirine**	**%Assay**	**Emtricitabine**	**%Assay**	**Tenofovir alafenamide**	**%Assay**
1	Analyst 01	100.479884	Analyst 01	100.86	Analyst 01	100.723731
2	Analyst 02	100.51467	Analyst 02	99.97565	Analyst 02	99.1048846

Table No.13: Data results for LOD, LOQ study of Rilpivirine, Emtricitabine, and Tenofovir alafenamide

S.No	**Parameters**	**Rilpivirine**	**Emtricitabine**	**Tenofovir alafenamide**
1	Linearity range (µg/ml)	3-10	20-60	30-70
2	LOD (µg/ml)	0.241	0.89	0.251
3	LOQ (µg/ml)	0.72	2.707	2.511
4	Slope			
5	Correlation coefficient (r)	0.996	0.996	0.995

Table No.14: Data results for Assay Results

S.No	Rilpivirine			Emtricitabine		Tenofovir alafenamide	
		Standard Area	Sample Area	Standard Area	Sample Area	Standard Area	Sample Area
1	Injection-1	1094.978	1073.394	1077.431	1077.348	1775.284	1739.803
2	Injection-2	1094.085	1095.614	1082.305	1087.415	1760.260	1735.753
3	Injection-3	1085.067	1069.418	1079.334	1068.454	1748.738	1741.096
4	Injection-4	1090.770	1105.547	1088.743	1084.874	1732.934	1762.022
5	Injection-5	1081.967	1092.076	1075.047	1079.334	1741.886	1746.089
6	Average Area	1089.373	1087.21	1080.572	1079.485	1751.820	1744.953
7	Assay(%purity)	99.74		100.04		102.41	

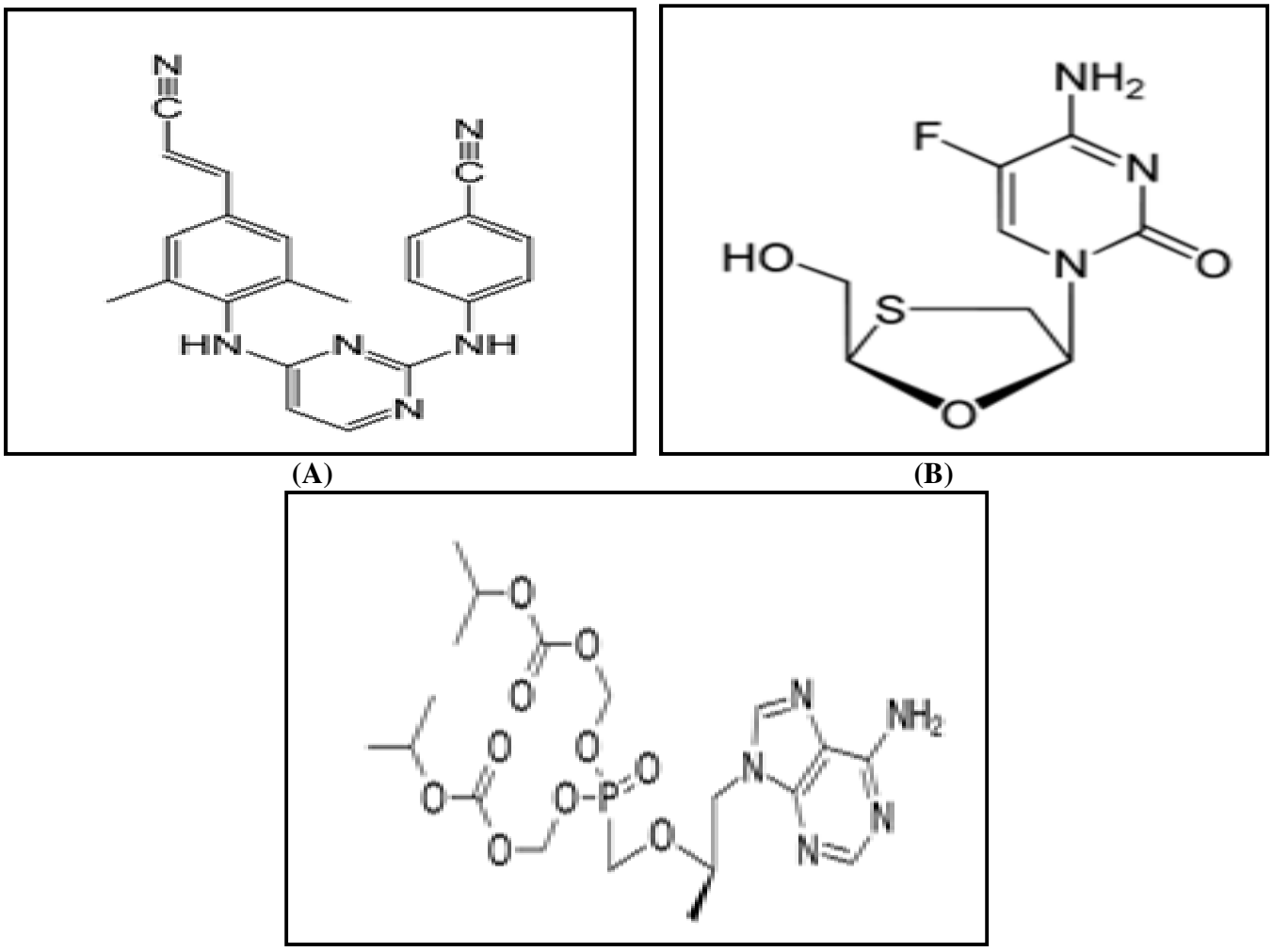
Table No.15: Data results for validated parameters of evaluated method

S.No	Parameter	Limit	Value Obtained
1	Accuracy (% Recovery)	98-102%	99.089 to 102.5% (Rilpivirine) 98.1 to 102.87% (Emtricitabine) 98.13 to 101.28% (Tenofovir alafenamide)
2	Linearity concentrations Range (µg/mL) Regression coefficient (R2 value)	NLT 0.99%	0.996 for Rilpivirine, 0.996 for Emtricitabine, 0.995 for Tenofovir alafenamide
3	Precision (% RSD) Method precision (Repeatability) (% RSD, n = 6)	NMT 2%	%RSD of Rilpivirine 1.35, Emtricitabine 1.38,1.30 for Tenofovir alafenamide
4	Intermediate Precision	NMT 2%	%RSD of Rilpivirine 1.35, Emtricitabine 1.38,1.30 for Tenofovir alafenamide
5	Robustness (% assay)	System suitability Criteria Shoed be within the limit	Complies
6	LOD (S/N)	NMT 3	Rilpivirine - 0.241, Emtricitabine 0.89, Tenofovir alafenamide 0.251
7	LOQ (S/N)	NMT 10 (LOQ>LOD)	Rilpivirine 0.72, Emtricitabine 2.707, Tenofovir alafenamide 2.511

SD= Standard deviation, % RSD = Relative standard deviation, LOD= Limit of detection, LOQ= Limit of quantification

Table No.16: Table Showing Forced degradation studies data

S.No	Stress Conditions	% Degradation		
		Rilpivirine	**Emtricitabine**	**Tenofovir alafenamide**
1	Acid degradation	6.04	6.25	6.70
2	Alkali degradation	5.19	5.08	5.76
3	Oxidative degradation	6.99	7.05	7.55
4	Thermal degradation	2.54	3.42	3.59
5	Photolytic degradation	1.16	1.85	2.32



(A) **(B)**

(C)

Figure No.1: The chemical structures of compounds
A. Rilpivirine; B. Emtricitabine; C. Tenofovir alafenamide

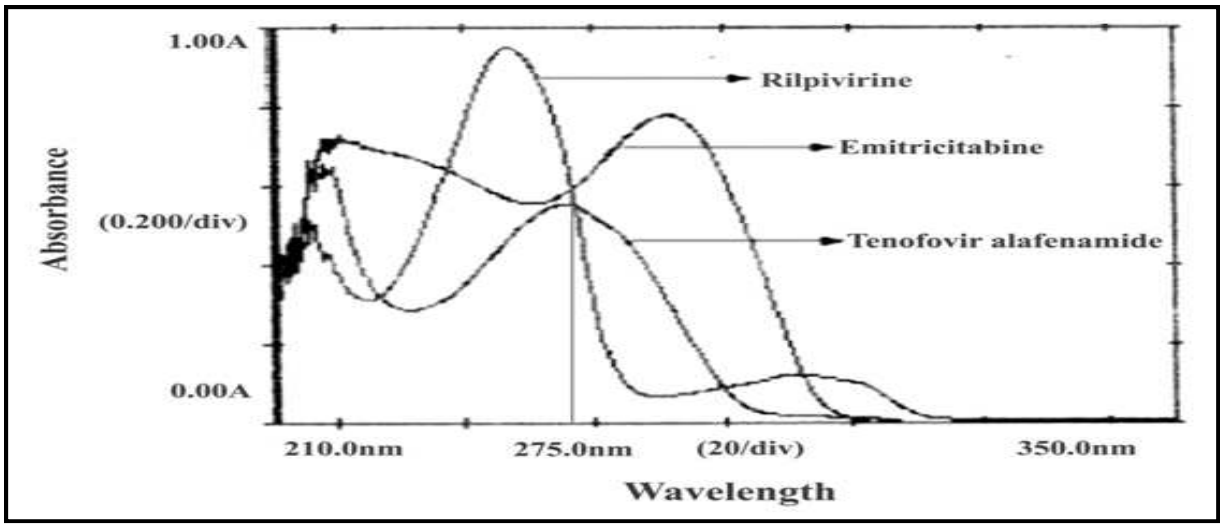


Figure No.2: UV-VIS spectrum shows the Isosbestic point was found to be 275nm for Rilpivirine, Emtricitabine, and Tenofovir alafenamide combination

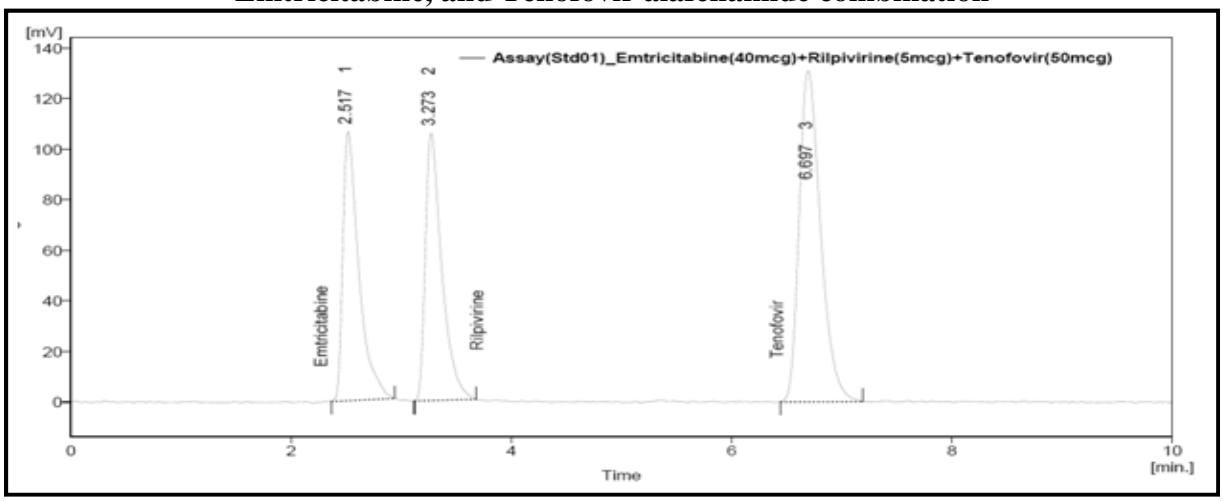


Figure No.3: Optimized Chromotagram of Emitricitabine, Rilpivirine and Tenofovir alafenamide

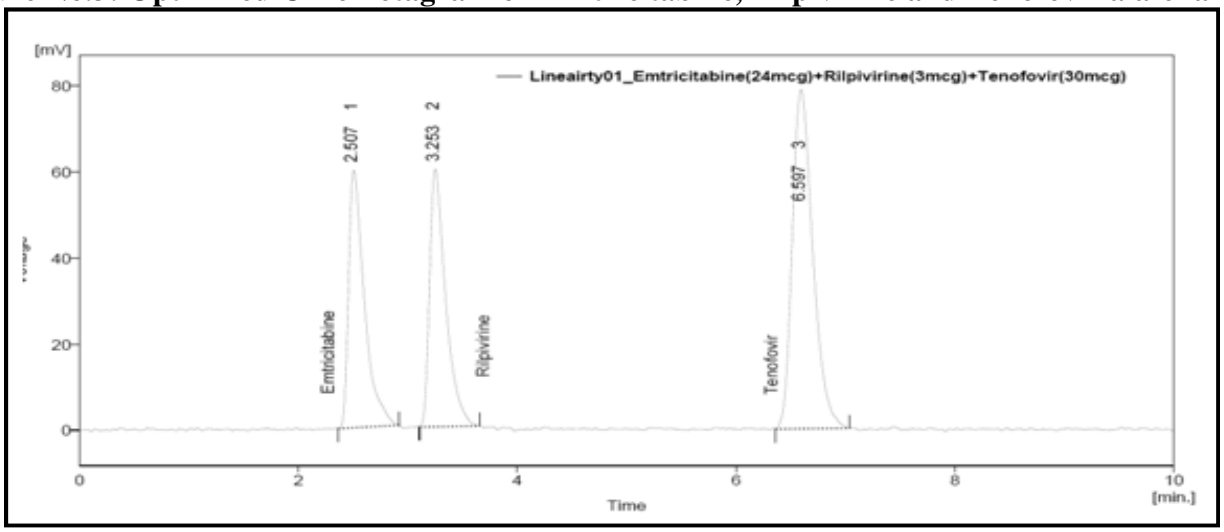


Figure No.4: Chromotogram of Level 1

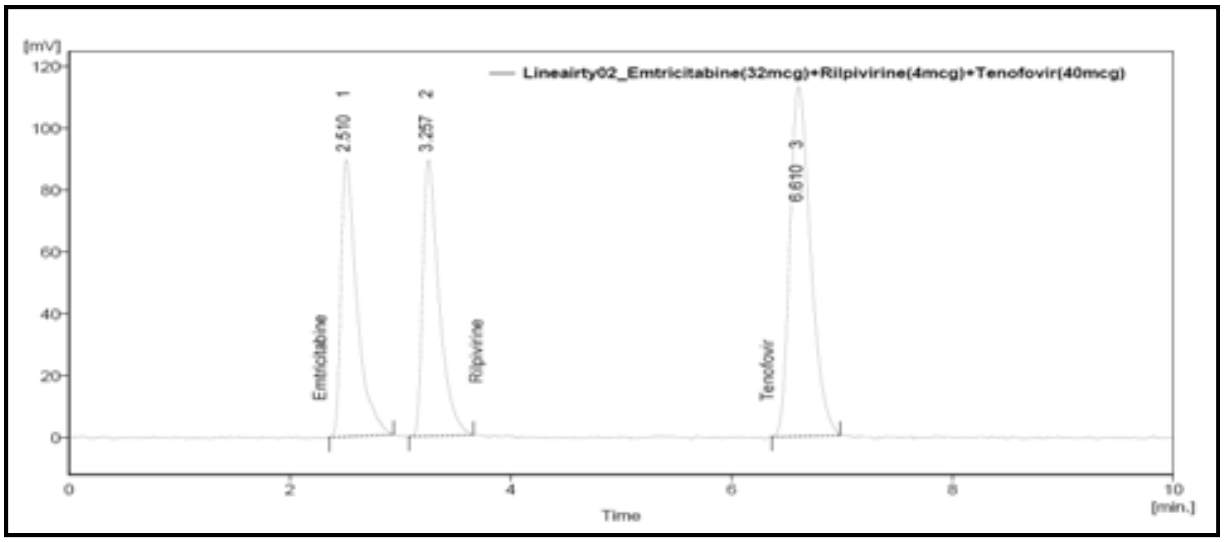


Figure No.5: Chromotogram of Level 2

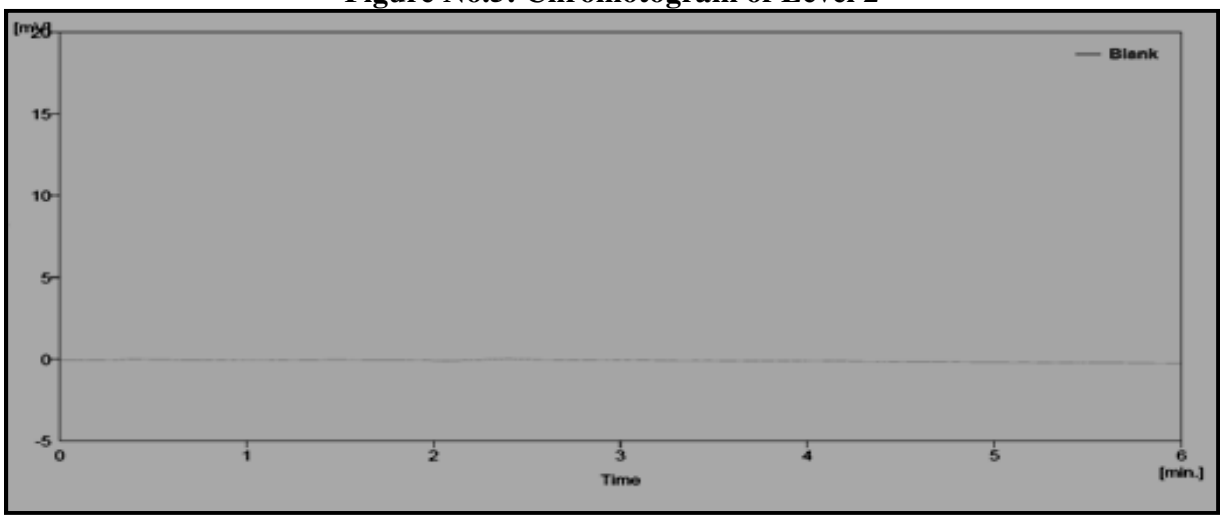


Figure No.6: Typical Blank Chromatogram for specificity

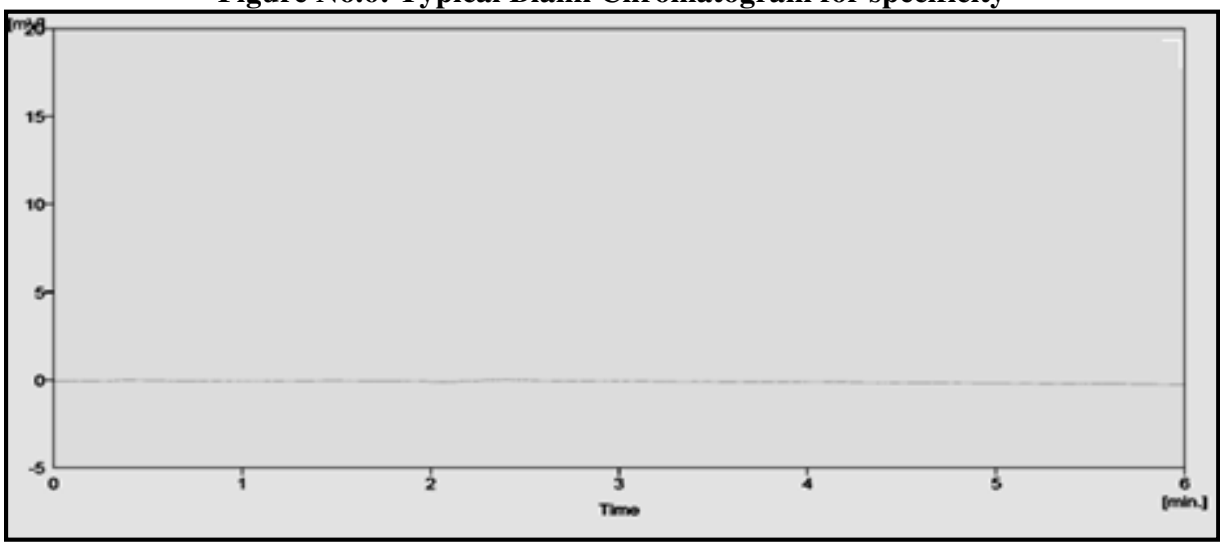


Figure No.7: Typical Chromatogram for Palcebo

Figure No.8, 9, 10: Linearity (calibration) curve of Rilpivirine, Emitricitabine, and Tenofovir alafenamide

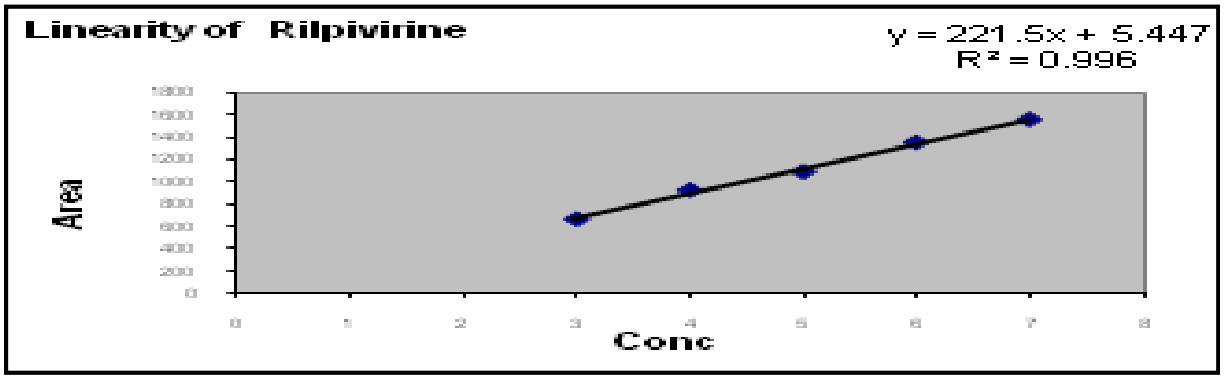


Figure No.8: Linearity graph of Rilpivirine

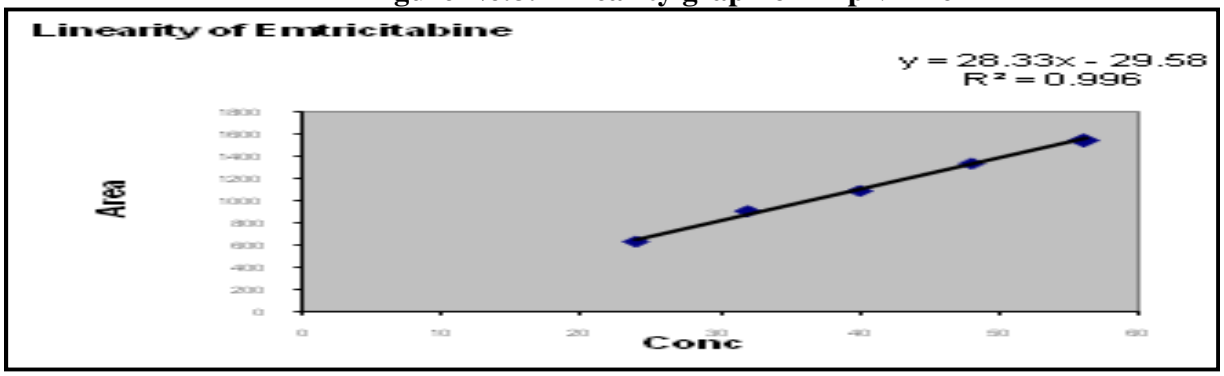


Figure No.9: Linearity graph of Emtricitabine

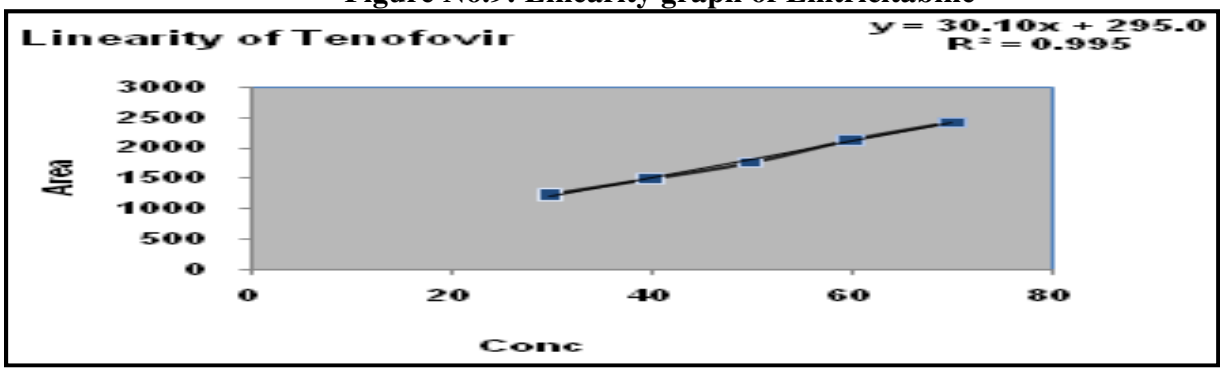


Figure No.10: Linearity graph of Tenofovir alafenamide

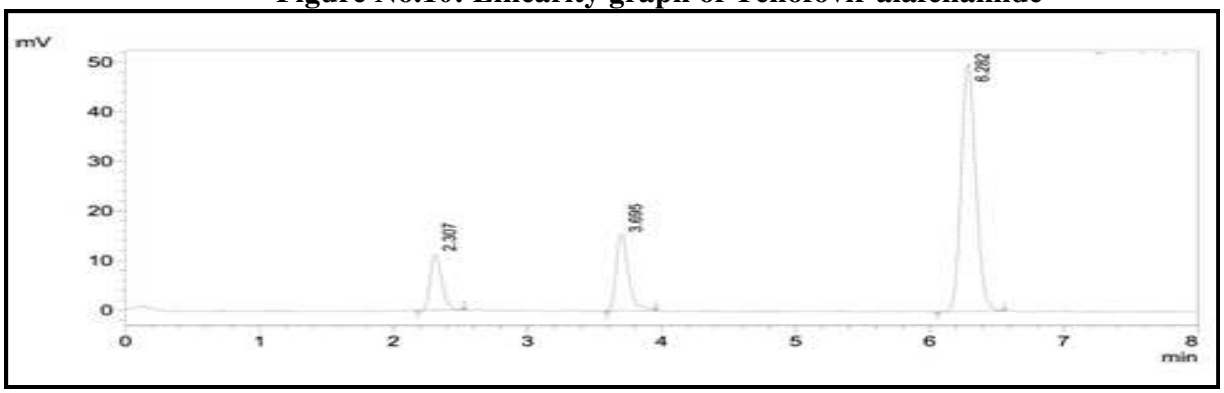


Figure No.11: Chromatogram Showing Acid degradation

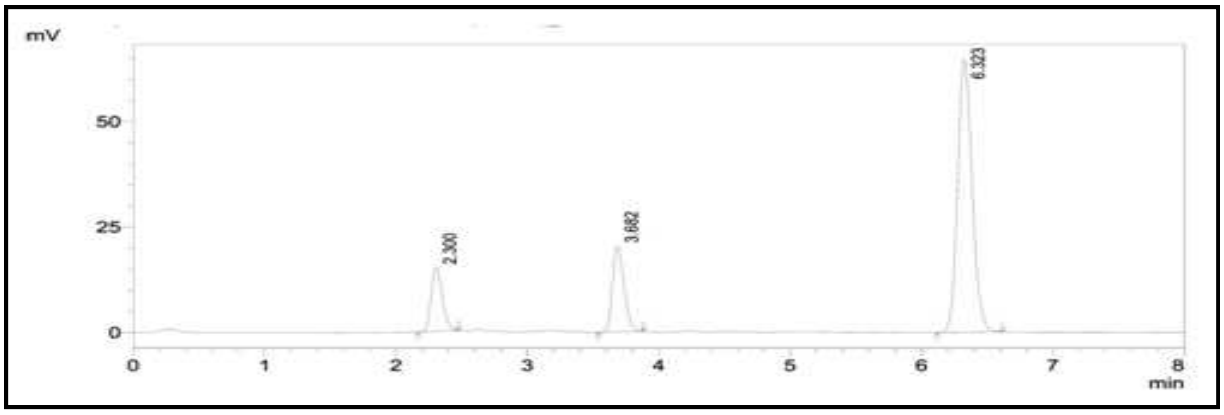


Figure No.12: Chromatogram Showing Alkali degradation

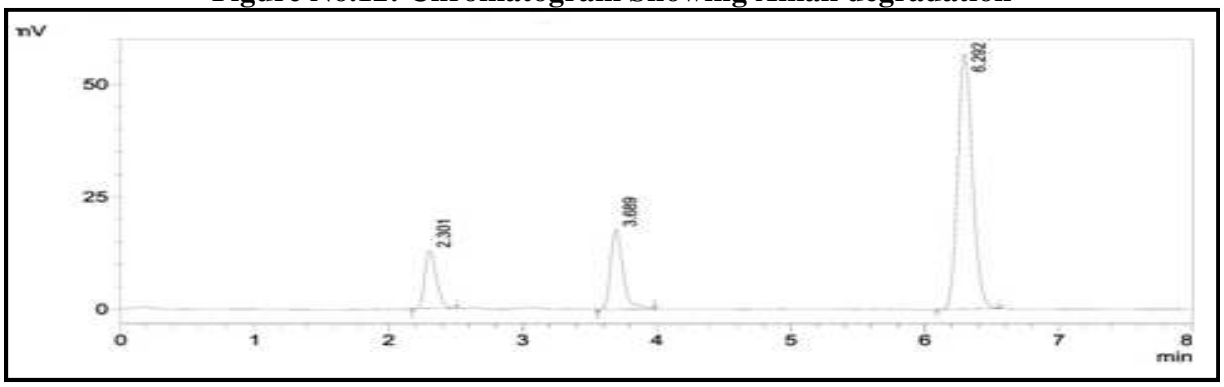


Figure 13: Chromatogram Showing Peroxide Condition

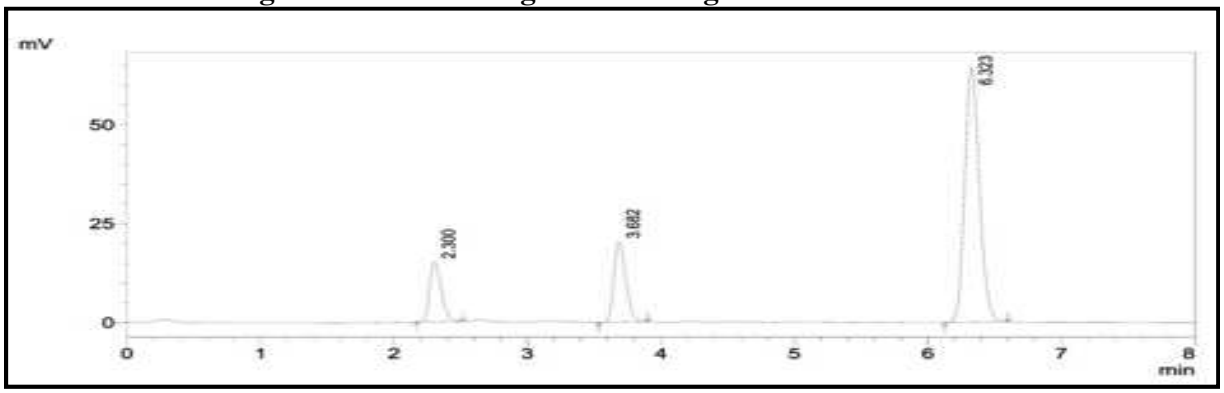


Figure No.14: Chromatogram Showing Thermal Condition

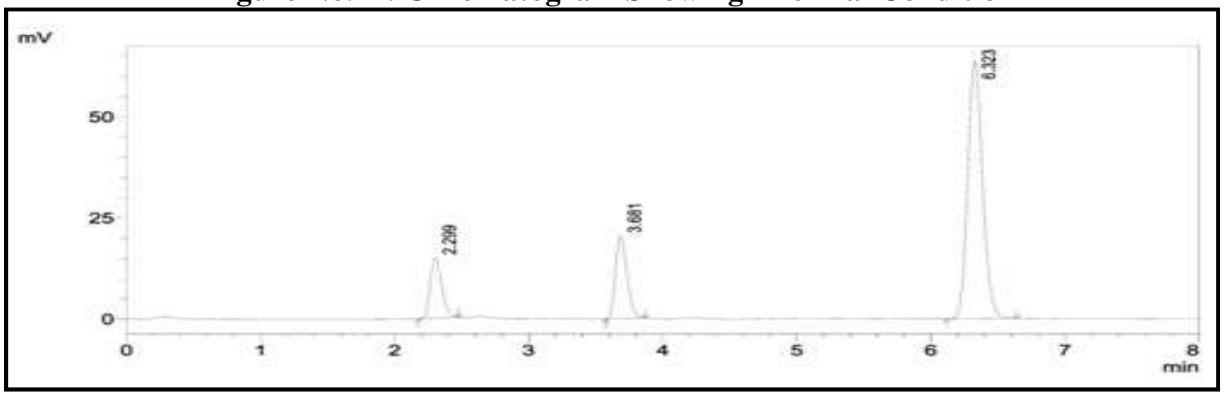


Figure No.15: Chromatogram Showing Photolytic Condition

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 Rilpivirine, Emitricitabine, and Tenofovir alafenamidein presence of its degraded product. It can successfully applied for estimation of Rilpivirine, Emitricitabine, and Tenofovir alafenamidein its pharmaceutical dosage form andbio-pharmaceutical and bio-equivalence studies and in clinical pharmacokinetic studies in near future.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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