IJCRT.ORG ISSN : 2320-2882

INTERNATIONAL JOURNAL OF CREATIVE RESEARCH THOUGHTS (IJCRT)

An International Open Access, Peer-reviewed, Refereed Journal

ANALYTICAL METHOD DEVELOPMENT AND VALIDATION OF ANTIVIRAL DRUG BY RP-HPLC WITH QBD APPROACH

S.S.Salunkhe*, A.S.Kulkarni , R.D.Chakole , M.S.Charde

Department of pharmaceutical chemistry

c, Vidyanagar, Karad pin- 415124

Abstract –

Work aims to develop a simple rapid and reliable method for reverse-phase high-performance liquid chromatography (RP-HPLC) for simultaneous estimation of antiviral drugs Tenofovir and Emtricitabine. Separation is done by using mobile phase methanol and 0.05% orthophosphoric acid in a ratio of 61:39. The separation was carried out on Agilent C18 column (4.6×250mm,5µm)

The flow rate was set at 0.9ml/min. The injection volume was 20µl and the UV detector was Operated at 269nm. the retention time for tenofovir and emtricitabine was 3.009min and 5.361min respectively. The standard calibration plot was found linear over a range of 5 to 25µg/ml and the coefficient of correlation was found to be (r2 =0.999). The % RSD value of intraday and interday precision. The LOD and LOQ were found to be 0.529 and 1.605 for tenofovir 0.0595 and 1.803 for emtricitabine. The developed method was eventually applied for quantification of the marketed formulation satisfactory result were obtained. The developed method was validated according to an international conference of harmonization (ICH) guidelines.

Keyword-Tenofovir, Emtricitabine, RP -HPLC, Chromatogram, validation

Introduction (1)(2)(3)-

Emtricitabine is an orally administrated nucleoside reverse transcriptase inhibitor. Emtricitabine is effective against the Human Immune deficiency virus and hepatitis B virus. Emtricitabine is a 5-fluorinated derivative of lamivudine approved for the treatment of HIV infection (1)(2)

Tenofovir is an acyclic nucleotide analog of adenosine used in combination with other agents in the therapy of human immune deficiency virus and as a single agent in hepatitis B virus (HBV) infection. It is a member of a class of phosphonic acid that is methyl phosphonic acid in which one of the methyl hydrogens is replaced by [(2R)-1-(6-amino 9H purine 9yl)] oxy group (3).

Analytical method development is selecting an accurate assay procedure for each ingredient present in pharmaceutical dosage form, either individually or complex dosage formulation containing several therapeutically and chemically compatible drugs with very similar chemical nature is a monumental undertaking.

Analytical method validation is the process of demonstrating that analytical procedures are suitable for their intended use and provide accurate test results that evaluate a product against its defined specification and quality attribute.

Table No. 1 Drug Profile Tenofovir

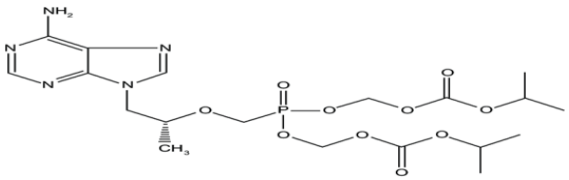
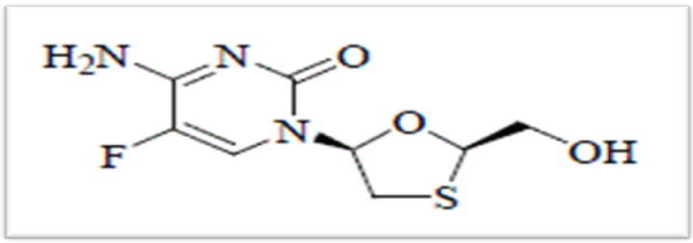
Sr.No.	Name of Drug:	Tenofovir
1	Structure	
2	Molecular formula	C9H14N5O4P
3	Molecular weight	287.21
4	Chemical name	[(2R)-1-(6-aminopurin-9-yl) propan-2-yl] oxymethylphosphonic acid
5	Description	white to off-white crystalline powder
6	Melting point	277-279 °c
7	Solubility	a solubility of 13.4 mg/mL in distilled water at 25 °C
8	Mode of action	Tenofovir diphosphate prevents HIV-1 activity. Via competition with the natural substrate deoxyadenosine 5'-triphosphate, followed by DNA chain termination following integration. Tenofovir the mammalian DNA polymerases and mitochondrial DNA polymerases can be slightly inhibited by diphosphate.
9	Adverse effect	Diarrhea, headache, depression, rash, itching, fever, difficulty falling asleep or staying asleep
10	Category	antiviral agent

Table 2 Drug Profile Emtricitabine

Sr,.No.	Name of Drug:	EMTRICITABINE
1	Structure	
2	Molecular formula	C8H10FN3O3S
3	Molecular weight	247.24.
4	Chemical name	5-fluoro-1-(2R,5S)-[2-(hydroxymethyl)-1,3oxathiolan-5-yl] cytosine
5	Description	white to off-white powder

6	Melting point	137-138 °c
7	Solubility	approximately 112 mg/mL in water at 25 °C
8	Mode of action	a cytidine analog competes with deoxycytidine 5'-triphosphate for HIV-1 reverse transcriptase when it is phosphorylated to form Emtricitabine 5'- triphosphate. Emtricitabine prevents the incorporation of additional nucleotides into the DNA strands being formed by HIV-1 reverse transcriptase,
9	Adverse effect	Headache, upset stomach; diarrhea; trouble sleeping; darkening skin color on palms of hands and soles of feet
10	Category	antiviral agent

2) material and method

2.1 Chemicals

Table no 3: List of Chemicals

Sr.No.	Ingredients	Grade	Suppliers
1	Emtricitabine	API	R.S.I.T.C Jalgaon
2	Tenofovir	API	R.S.I.T.C Jalgaon
3	Orthophosphoric acid (OPA)	HPLC	Avantor Performance material India Ltd. Thane, Maharashtra
4	MEOH	HPLC	Merck Specialties Pvt. Ltd. Shiv Sager Estate 'A' Worli, Mumbai
5	Water	HPLC	Merck Specialties Pvt. Ltd. Shiv Sager Estate 'A' Worli, Mumbai

2.2 Equipment

Table 4: List of instruments

Sr. no	Name of Instrument	Company Name
1	HPLC Instrument	Agilent 1100 with autosampler Chemstation software
2	UV-Spectrophotometer	Analytical Technologies Limited
3	Column(C18)	Agilent C18 (100mmX 4.6mm,5µm)
4	pH meter	VSI pH meter (VSI 1-B)
5	Balance	WENSAR™ High-Resolution Balance
6	Sonication	Ultrasonic electronic instrument

3)Method development and optimization of chromatographic condition –

3.1] selection of chromatographic condition-(4)

Selection depends upon whether the drug is an ionic ionizable or neutral molecule.

It also depends upon the solubility and molecular weight of the drug selected for study i.e., Tenofovir and Emtricitabine both are polar in nature so reverse phase HPLC was selected for the separation due to its suitability selection of wavelength.

Tenofovir and Emtricitabine combination prepared in a mixture of water and acetonitrile in n ratio 1:1. The spectrum was recorded by scanning indidrugsal drug and the combination in the range of 200nm to 400nm for the analysis of data obtained from the spectrum wavelength of 269nm was selected.

3.2] selection of wavelength-(5)

Tenofovir and emtricitabine combination prepared in a mixture of water and acetonitrile in a ratio of 1:1. The spectrum was recorded by scanning individual drugs and the combination in a range of 200nm The. The analysis of data obtained from a spectrum wavelength of 269nm was selected.

3.3] Selection of stationary phase-(6)

C18 (Agilent)2 stationary phases are used for separation because of its hydrophobic interaction. as solutes the in mobile phase travel through silica pores they can be attracted and held by hydrocarbons through the stationary phase was C18(Agilent) SN: B13151408-2. The mobile phase was methanol and 0-0.5% OPA in a ratio 61:39wavelenth selected for detection was 269nm; the flow rate for separation was 0.9ml/min. the temperature of the column was 15 DC and the particle size was 20ul

3.4] Selection of mobile phase-(7)

Methanol and 0.05% orthophosphoric acid in a combination of a ratio of 61:39 was selected as the mobile phase.

Menthol allows Л-Л interaction which allows better separation and 0.05% orthophosphoric acid is used as a buffer because pH could be adjusted between 3-6.5 for the C18 column otherwise column gets damaged.

3.5] Selection of flow rate (8)

0.9 ml/min flow rate was selected for a high flow rate to reduce retention time so component molecules have little time to interact with the stationary phase as they pass quickly through the column.

The developed method is represented below-

The standard solution contains 500ug/ml emtricitabine and 750ug/ml tenofovir.

The stalk solution contains 500ug/ml emtricitabine and 750ug/ml tenofovir.

Method of validation-

Validation is the procedure by which it is set up by the research facility that the execution qualities of strategy meet the prerequisite for the proposed application parameter for the method validation are as follows-

1] Linearity (10)-

Linearity indicates the ability to produce results that are directly proportional to the analytical sample. From Emtricitabine standard stock solution, a different working standard solution was prepared in the mobile phase likewise from the Tenofovir standard stock solution different working standard solution was prepared in the mobile phase 20µl of sample solution was injected into the chromatographic system using a fixed volume loop injector. Chromatograms were recorded. The area for each concentration was recorded.

2] Precision (11)-

The precision of an analytical method is the degree of agreement among individual test results when the method is repeated to multiple sampling of homogenous samples precision is usually expressed as the standard deviation of a series of measurements. It is indicated by RSD i.e., relative standard deviation.

a] Repeatability-

Use of analytical procedure within laboratory over a short period using same analyst, and equipment- carried out on a minimum of nine samples.

Intra-day precision:

Sample solutions containing 5 mg of Emtricitabine and 7.5 mg of Tenofovir in three different concentrations (10 µg/ml, 15 µg/ml, 20 µg/ml) concentration of Emtricitabine and (7.5µg/ml, 22µg/ml,37.5 µg/ml) of

Tenofovir. Emtricitabine and Tenofovir were analyzed three times on the same day and %R.S. D was calculated.

Inter-day precision:

Sample solutions containing 5 mg of Emtricitabine and 7.5 mg of Tenofovir in three different concentrations (10 µg/ml, 15 µg/ml, 20 µg/ml) concentration of Emtricitabine and (7.5 µg/ml, 22 µg/ml, 37µg/ml) of Tenofovir. Emtricitabine and Tenofovir were analyzed for three days and %R.S. D was calculated.

▪ **Acceptance criteria:**

The Relative Standard Deviation should not be more than 2%.

4] Accuracy (12)-

The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted as a reference value. accuracy should be assayed using a minimum of nine determinations over a minimum of three concentration levels.

Accuracy should be reported as percentage recovery by the assay of the known added amount of analyte in the sample.

Accept the criteria for RSD <2%

5] Robustness (13)-

ICH and USP guidelines define the robustness of analytical procedure as a measure of its capacity to remain unaffected by small but deliberate variation parameters.

During a robustness study method, parameters are varied intentionally to see if the method results are affected. For example;

1) Change in mobile phase composition

2) Change in buffer composition.

3) Change in the mobile phase.

4) Change in temperature.

5) Change in flow water.

6) Change in wavelength likewise.

6) Ruggedness (14)-

It is the capacity to yield exact result presence of small changes of experimental conditions that might occur during the utilization of these procedures. For example;

1) Different laboratories.

2) Different analysts.

3) Different instruments.

4) Different Days.

7) Limit of detection (LOD) (15)-

The limit of detection is the lowest possible concentration at which the method can be detected (but not quantity) usually limit of detection is determined only for qualitative determination.

The limit of detection can be evaluated in different ways-

1) Visual examination- eg- calculating zone of inhibition.

2) Determination of single-to-noise ratio- It is measured by comparing the signal of a sample containing a low concentration of analyte against the signal of blank and determining g minimum concentration at which the analyte signal could be reliably detected acceptable signal to noise ratio-3:1

Standard deviation calculated by the formula –

LOD = 3.3 × σ/s

Where, σ= SD of the obtained result

S= slope of the calibration curve.

8) Limit Quantification (LOQ) (15)-

LOQ is the lowest possible concentration of analyte that can be quantified LOD is determined by

1) Visual examination-

Ex- Titration-A known concentration of analyte is added until of color of the mixture changes.

2) Determination of signal-to-noise ratio signal of know concentration of analyte is compared to blank.

The S/N ratio of 10:1 is acceptable.

3) Standard deviation-

LOQ is calculated by the formula

LOQ= 10× σ/s

σ= SD of obtained result s= Slop of calibration curve.

Result and discussion-

a) Development and optimization of stability indicating the HPLC method

1] Method development-

The following chromatographic conditions were established by trial and error and were kept constant throughout the experimentation.

Table no 5 Chromatographic condition

Sr no.	Parameter	Description
1	HPLC	AGILET (1100) auto sampler
2	FORMT ware	CHEMSTATION.
3	Column	-id 4.6*250mm length
4	Particle size packing	- 5um
5	Stationary phase	C18(Agilent)2
6	Mobile phase	0.05% OPA = 61:39
7	Detection wavelength	269nm
8	Flow rate	0.9ml/min
9	Temperature	25°c
10	Sample size	20µl
11	Buffer	0.05%OPA

preliminary studies on Emtricitabine and Tenofovir Melting point-

The melting point of Tenofovir and Emtricitabine is as follows

The melting point of tenofovir: 277-279 ^{o}c

The melting point of emtricitabine-137-138 ^{o}c

Table 6: Solubility of the drug in different solvents

Sr no	solvent	Tenofovir	Emtricitabine
1	water	+	+
2	acetonitrile	+	+
3	0.1N NAOH	+	+
4	0.1HCL	+	+
5	Methanol	+	+

UV spectroscopy

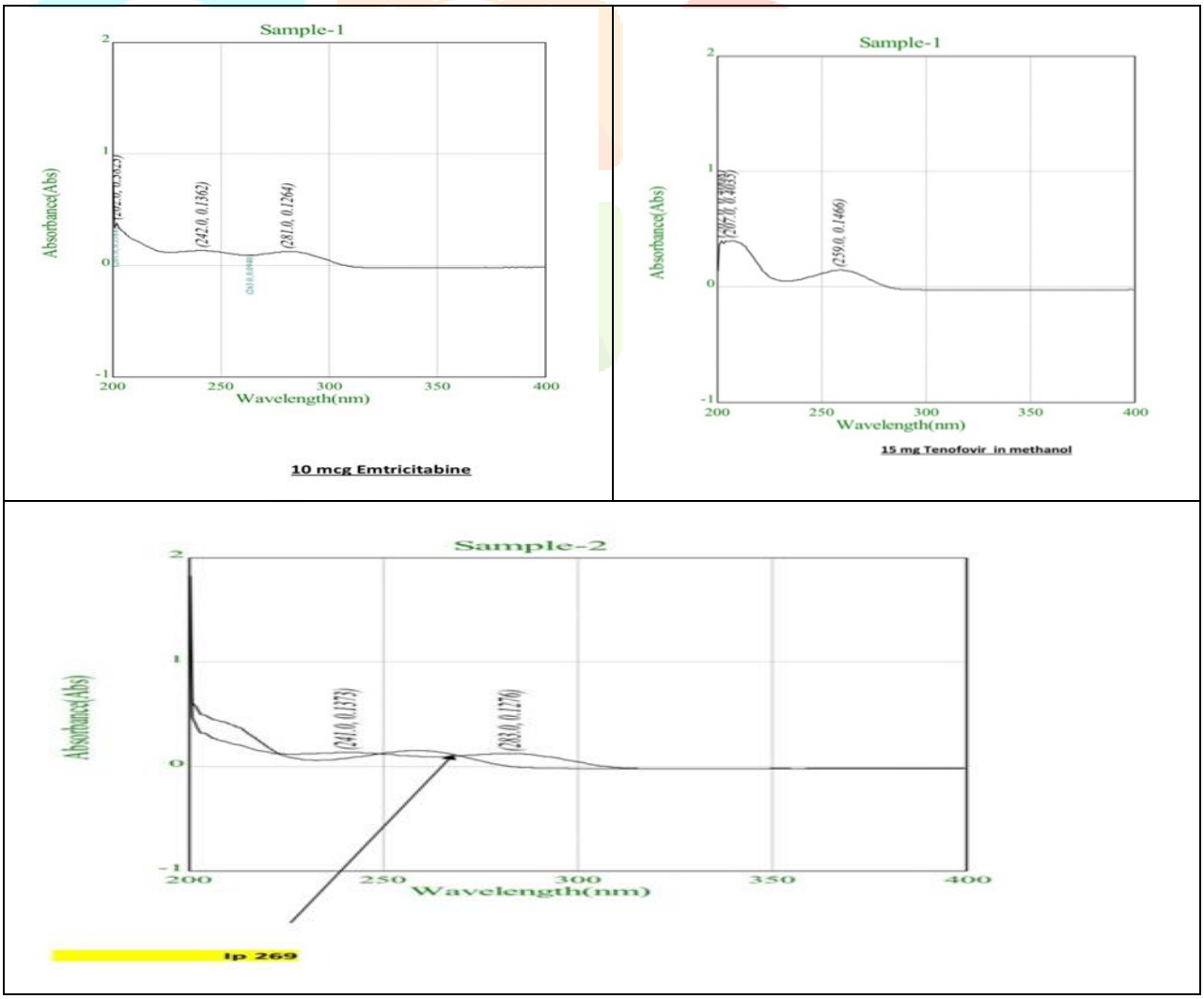


Figure 1: λmax and isosbestic point of drugs

Selection of wavelength and calibration curve UV absorption of 20mcg solution of Emtricitabine and Tenofovir in MeOH was generated and absorbance was taken in the range of 200-400 nm. λ max of Emtricitabine and Tenofovir in MeOH was found to be 241and 259 nm respectively

METHOD DEVELOPMENT BY HPLC:

Screening and optimization in advance Analysis of Data The important elements were determined by preliminary tests using the Taguchi screening Method, and their levels (maximum and minimum) were selected for the experimental design. Design Expert software and the numerical optimization technique, the forecast of the Optimum analytical condition was also carried out. Validating the Box-Behnken Design The grid search data was used to pick fifteen runs, which were then prepared according to the composition(s) specified and tested for the three key quality characteristics (CQA) of theoretical plates (TP), assay, and tailing factor (TF). Comparing the anticipated and actual responses, linear correlation graphs were created. The residual plots were also created, and the percent bias (error) about the observed responses was determined. Assay, TP, and TF

Table No. 7: Selection of Mobile Phase

Sr.no	Mobile phase
1	[85% MeOH +15% Water (pH 3.0 adjusted with OPA) Flow 1 ml/min abs at 269 nm (column 250 mm X 4.6, 2.5µm)
2	[70% MeOH +30% Water (pH 3.0 adjusted with OPA) Flow 0.8 ml/min abs at 269nm (column 250 mm X 4.6, 2.5µm)
3	[60% MeOH +40% Water (pH 3.0 adjusted with OPA) Flow 0.8 ml/min abs at 269nm (column 250 mm X 4.6, 2.5µm)

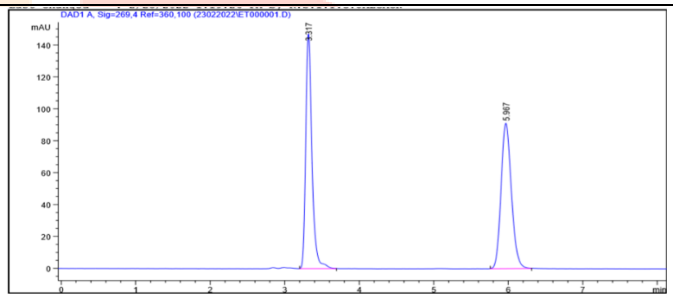
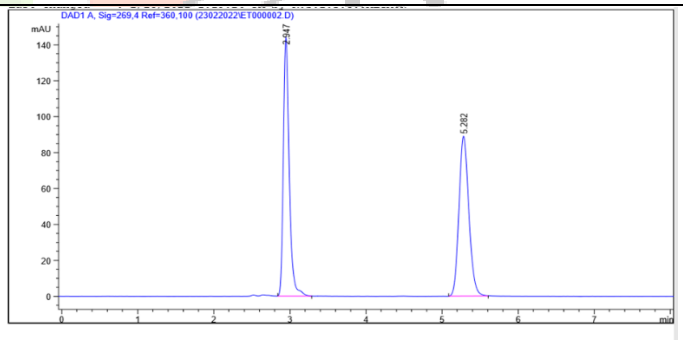
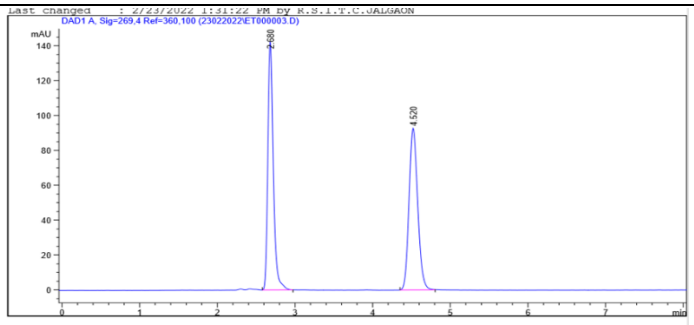
Studies on the chromatographic behavior of Emtricitabine and Tenofovir

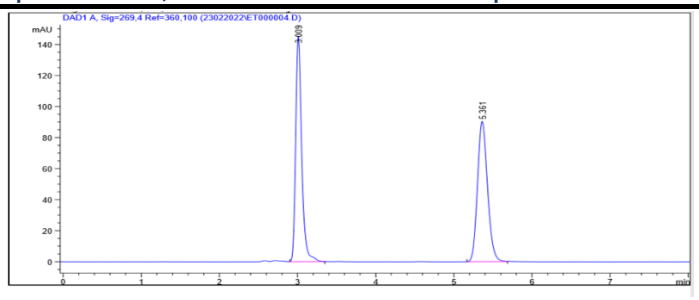
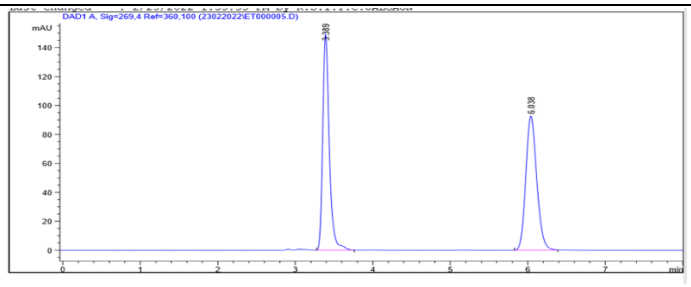
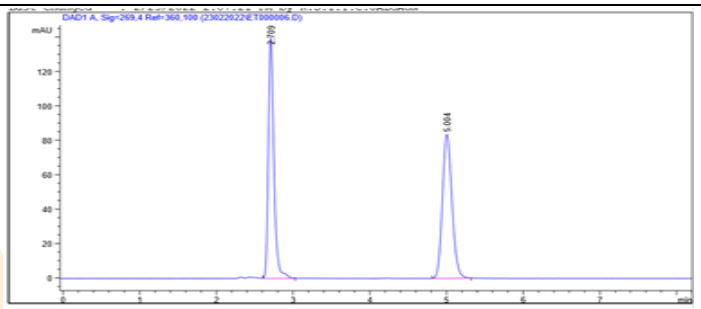
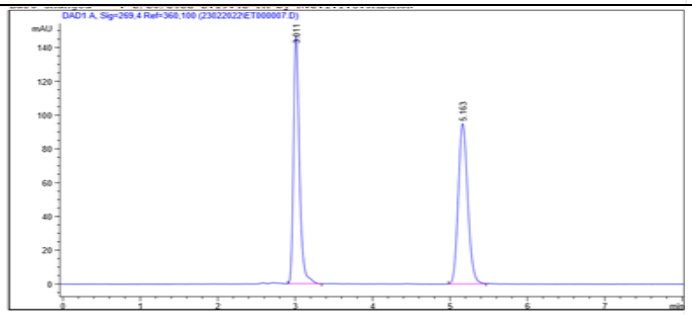
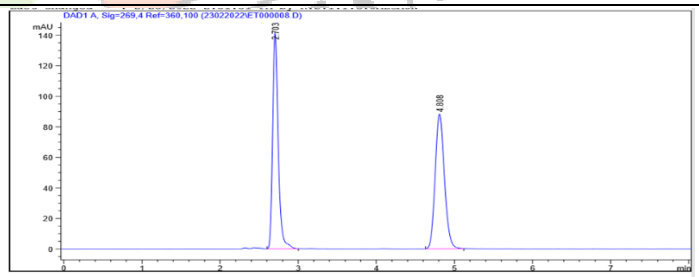
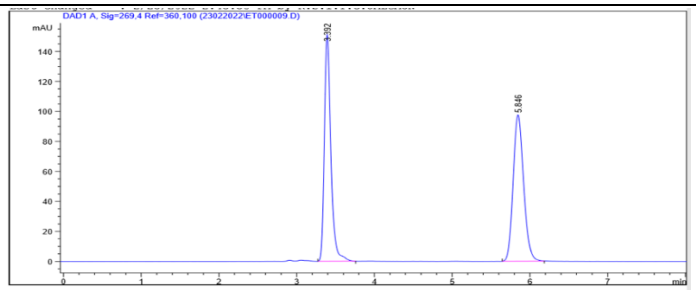
Table No.8: Chromatographic behavior of Emtricitabine and Tenofovir mobile phase composition

Sr.no.	Mobile phase	Retention time (min)		Remark
		Emtri	Teno	
1	[60% MeOH +40% Water (pH 3.0 adjusted with OPA) Flow 0.8 ml/min abs at 269nm (column 250 mm X 4.6, 2.5µm)	5.96	3.317	Rejected
2	[60% MeOH +40% Water (pH 3.0 adjusted with OPA) Flow 0.9ml/min abs at 269nm (column 250 mm X 4.6, 2.5µm)	5.282	2.947	Rejected
3	[62% MeOH +38% Water (pH 3.0 adjusted with OPA) Flow 1ml/min abs at 269nm (column 250 mm X 4.6, 2.5µm)	4.520	2.680	Rejected

4	**[61% MeOH +39% Water (pH 3.0 adjusted with OPA) Flow 0.9ml/min abs at 269 nm (column 250 mm X 4.6, 2.5µm)**	**5.361**	**3.009**	**Accepted**
5	[61% MeOH +39% Water (pH 3.0 adjusted with OPA) Flow 0.8 ml/min abs at 269 nm (column 250 mm X 4.6, 2.5µm)	5.038	2.389	Rejected
6	[60% MeOH +40% Water (pH 3.0 adjusted with OPA) Flow 0.8 ml/min abs at 269 nm (column 250 mm X 4.6, 2.5µm)	5.004	2.709	Rejected
7	[62% MeOH +38% Water (pH 3.0 adjusted with OPA) Flow 0.9 ml/min abs at 269nm (column 250 mm X 4.6, 2.5µm)	5.163	3.011	Rejected
8	[61% MeOH +39% Water (pH 3.0 adjusted with OPA) Flow 1ml/min abs at 269 nm (column 250 mm X 4.6, 2.5µm)	4.808	2.703	Rejected
9	62% MeOH +38% Water (pH 3.0 adjusted with OPA) Flow 0.8 ml/min abs at 269 nm (column 250 mm X 4.6, 2.5µm)	5.846	3.392	Rejected

Table No. 9: QBD Trials

SR, NO	TRIAL	CHROMATOGRAM
1	60%methanol+0.05%orthophosphoric acid Flow rate-0.8ml/min Wavelength- 269nm	
2	60%methanol+0.05%orthophosphoric acid Flow rate-0.9ml/min Wavelength- 269nm	
3	62%methanol+0.05%orthophosphoric acid Flow rate-1ml/min Wavelength- 269nm	

4	61%methanol+0.05%orthophosphoric acid Flow rate-0.9ml/min Wavelength- 269nm	
5	61%methanol+0.05%orthophosphoric acid Flow rate-0.8ml/min Wavelength- 269nm	
6	60%methanol+0.05%orthophosphoric acid Flow rate-1ml/min Wavelength- 269nm	
7	62%methanol+0.05%orthophosphoric acid Flow rate-0.9ml/min Wavelength- 269nm	
8	61%methanol+0.05%orthophosphoric acid Flow rate-1ml/min Wavelength- 269nm	
9	62%methanol+0.05%orthophosphoric acid Flow rate-0.8ml/min Wavelength- 269nm	

Optimized method

Table no: 8 optimized chromatographic condition

parameter	Description
Mode of operation	Isocratic
Diluents	Methanol:0.05%ortho phosphoric acid
column	Id4.6×250mm length (3)
Mobile phase	Methanol:0.05&OPA (61:39)
Stationary phase	C18(Agilent)2
Particle size	20µg/ml
Flow rate	0.9ml/min
wavelength	259nm
temperature	25^0C
Injection volume	20µl
Run time	20min
detector	G-13148
Pump unit	G1310AISOpump
Maximum pressure	400bar
Discharge rate	0.001to5ml
Pressure limit range	400 bar
Pressure display accuracy	5%
Pump unit HP	1100 reciprocating pump

Optimized batch

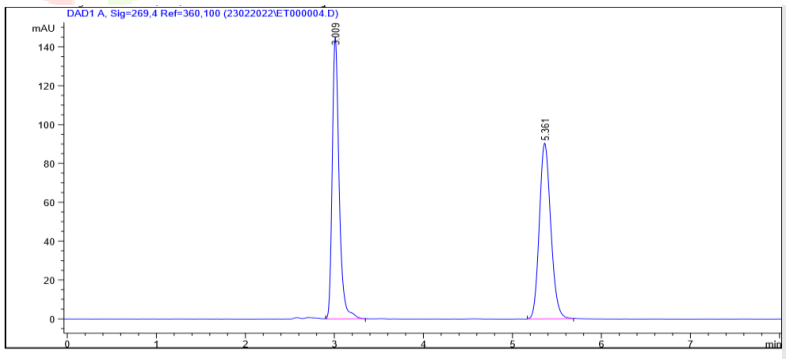


Figure no-2 Chromatogram of standard Combination of Emtricitabine and Tenofovir

61%methanol+0.05%orthophosphoric acid

Flow rate-0.9ml/min

Wavelength- 269nm

Table no: 10 optimized chromatographic condition result

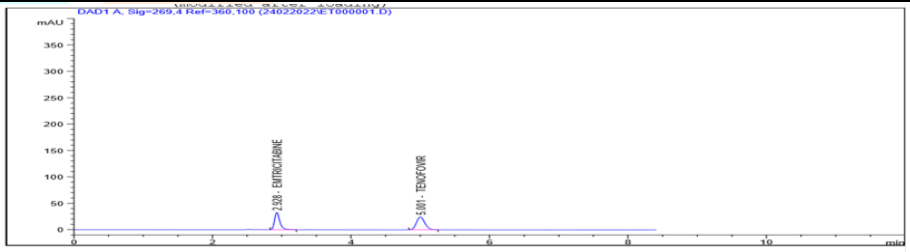
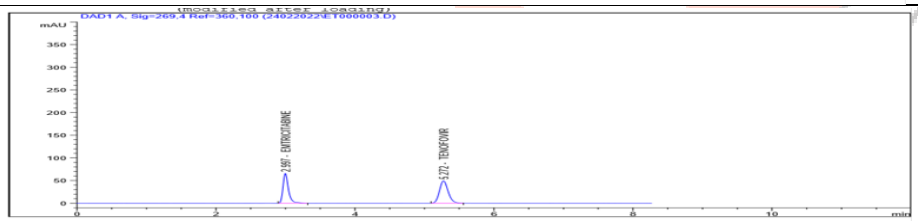
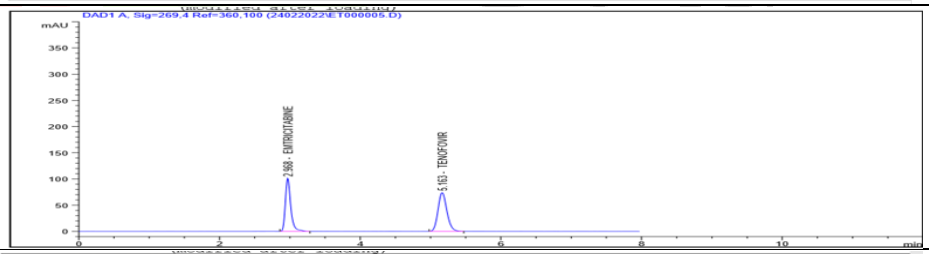
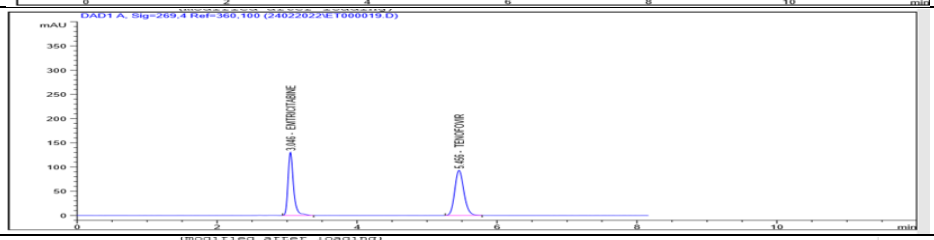
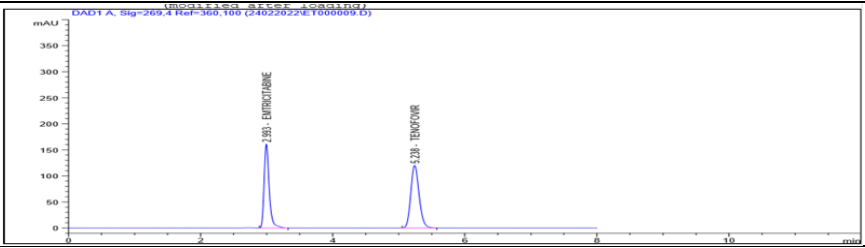
Retention time(min)	Area	Height	symmetry	width	plates	Resolution	selectivity
3.009	779.71	145.27	0.71	0.077	8290	-	-
5.361	807.21	90.39	0.82	0.135	8737	12.99	1.78

2] Method validation-

The developed method was evaluated using ICH guidelines for specificity, linearity, range, accuracy, precision, LOD, LOQ, and robustness.

Linearity: As the concentration of the drug increases area under the curve also increases.

Table no: 11 linearity chromatographs

concentration	chromatogram
5+7.5mcg microgram/ml-1	
10+15mcg microgram/ml-1	
15+22.5mcg microgram/ml-1	
20+30 mcg microgram/ml-1	
25+37.5mcg microgram/ml-	

2)Precision-

Intraday, Interday, and repeatability were carried out for both drug and observation as follows-

Table no: 12 Intraday and intraday precision data

METHOD	Drug	Conc (µg/ml)	Interday Precision		Intraday Precision	
			Mean± SD	%Amt Found	Mean± SD	%Amt Found
Rp-HPLC METHOD	TENO	7.5	210.8±2.44	98.75	211.15± 1.4	98.88
		22.5	643.9±4.18	101.83	641.8±0.7	101.49
		37.5	1057.±1.16	100.54	1054±5.16	100.27
	EMTRI	5	179.9±2.33	99.70	178.8±2.97	99.03
		15	536.4±1.08	102.96	533.82±3.68	102.60
		25	883.3±0.94	102.59	881.2±0.69	102.36

INTERDAY PRECISION

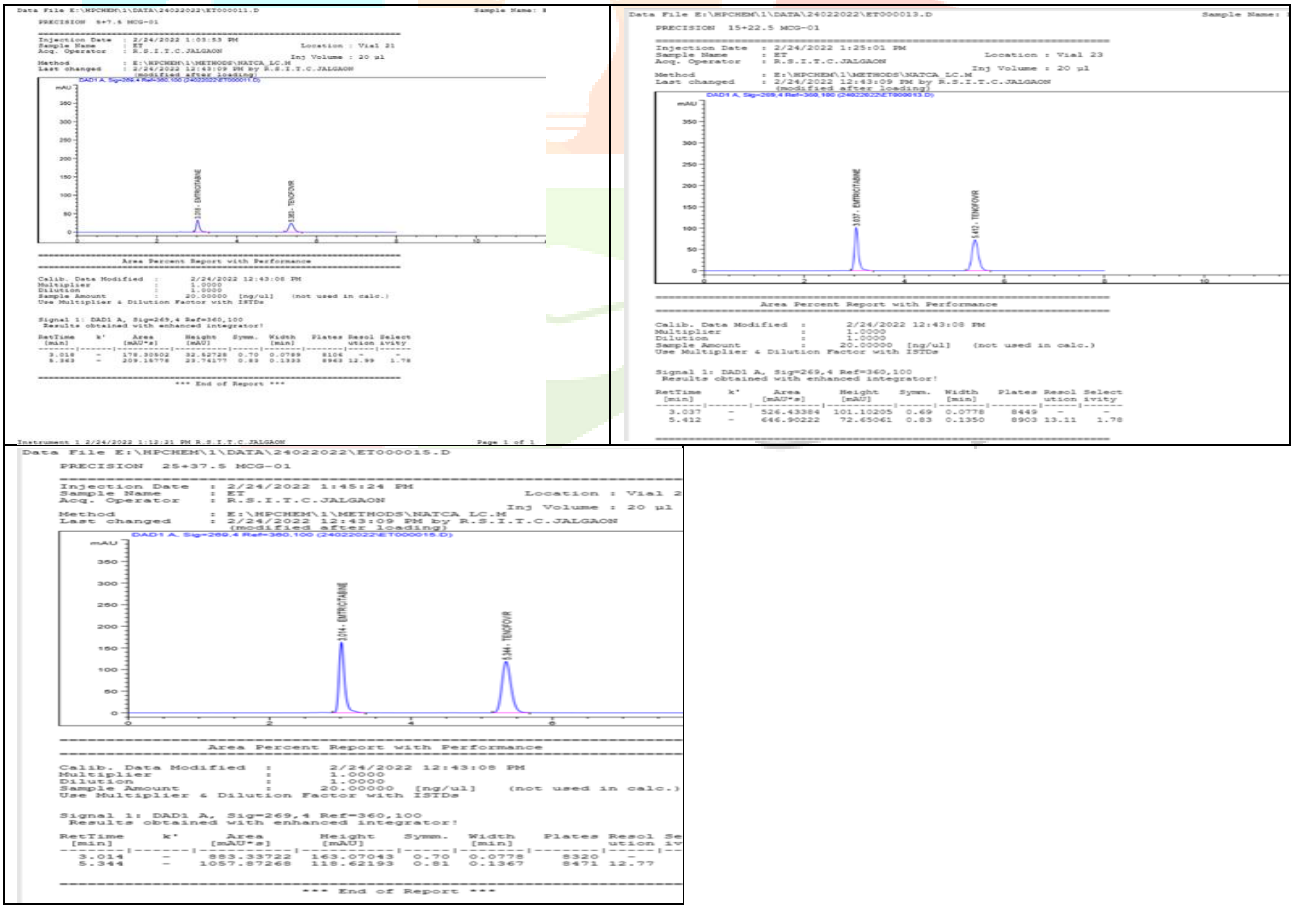
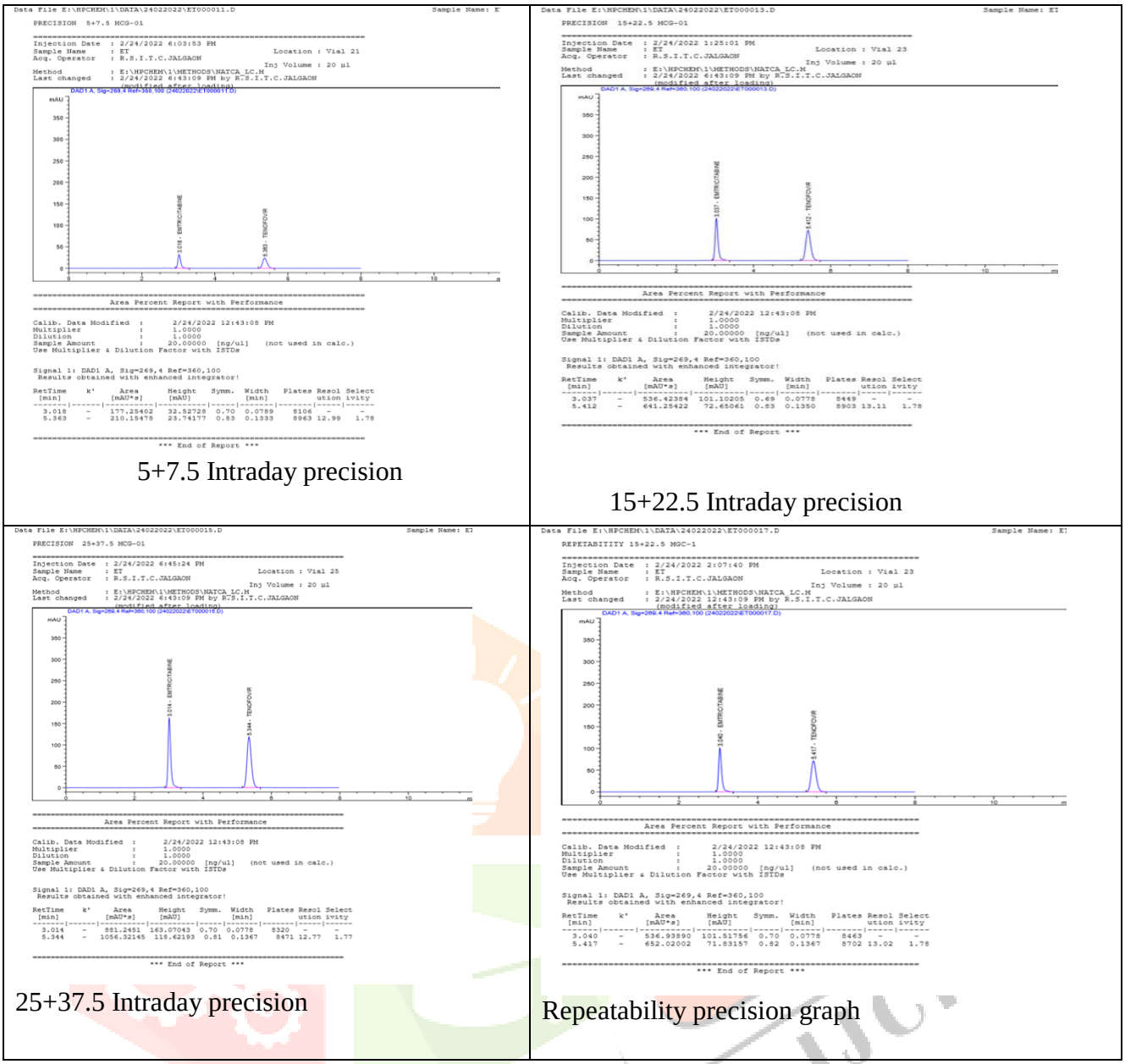


FIGURE NO -3 INTERDAY PRECISION GRAPHS

Intraday Precision graph-



5+7.5 Intraday precision

15+22.5 Intraday precision

25+37.5 Intraday precision

Repeatability precision graph

FIGURE NO - 4 INTERDAY PRECISION GRAPH

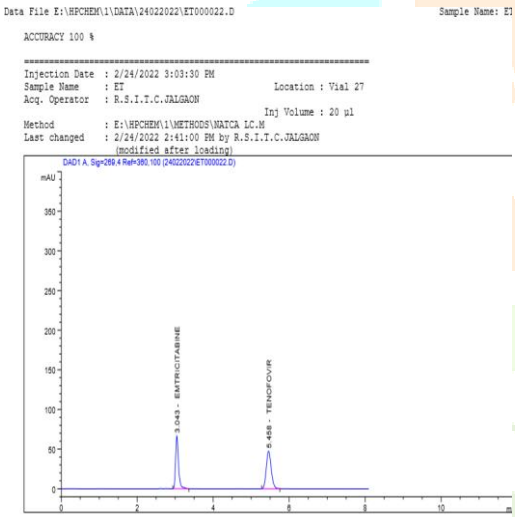
3)Accuracy -

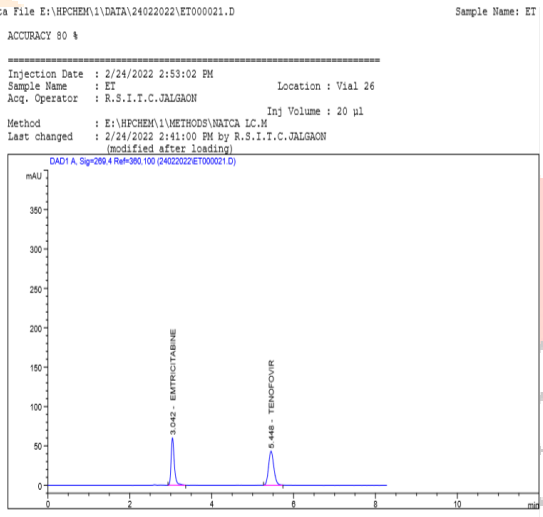
As per Q2(R1), The accuracy for assay of a drug substance can be studied from 80 to 120% of the test solution.

Table no: 13 accuracy data

METHOD	Drug	Level (%)	Amt. taken (ug/ml	Amt. Added (ug/ml	Absorbance Mean* ± S.D.	Amt. recovered Mean *±S.D.	%Recovery Mean *± S.D.
RP-HPLC Method	TENO	80%	7.5	6	13.40±0.010	5.90±0.010	98.50±0.17
		100%	7.5	7.5	15.10±0.031	7.60±0.031	101.02±0.41
		120%	7.5	9	16.69±0.039	9.19±0.039	102.39±0.44
	Emtri	80%	5	4	8.99±0.004	3.99±0.004	99.80±0.10
		100%	5	5	9.99±0.005	4.99±0.005	99.72±0.09
		120%	5	6	11.05±0.044	6.05±0.044	100.83±0.74

Graphs of accuracy –





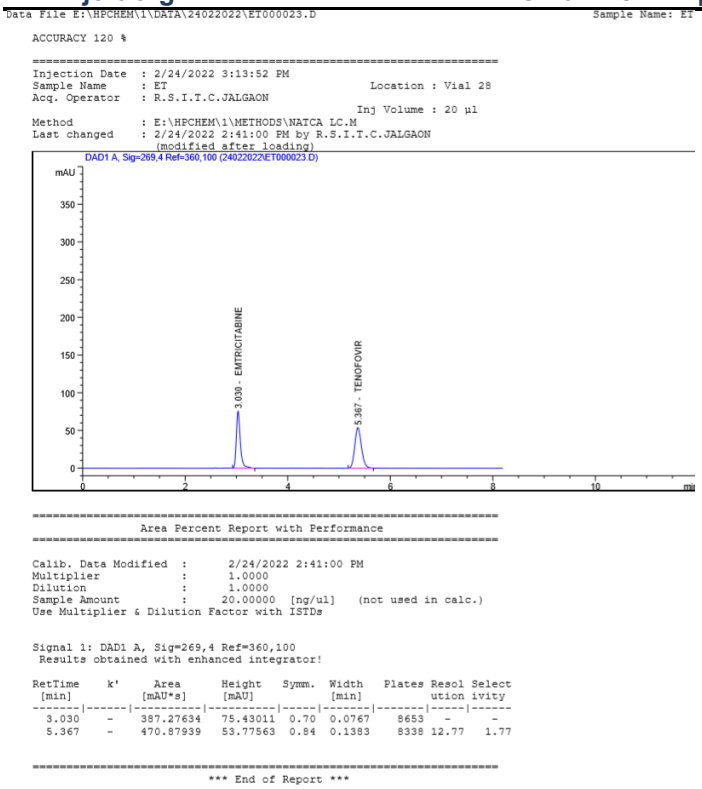


figure no – 5 accuracy graphs

Robustness-

The robustness of both drugs was carried out by the change in the composition of mobile phase, wavelength, and flow rate.

Table no: 14 Robustness data

Parameters	Conc.(µg/ml)	Amount of detected (mean ±SD)	%RSD	Amount of detected (mean ±SD)	%RSD
		For Tenofovir		For Emtricitabine	
Chromatogram of flow change 0.8 ml	15+10	378.51±2.33	0.61	333.1±16.90	5.07
Chromatogram of flow change 1 ml	15+10	481.90±0.81	0.17	402.73±0.84	0.21
Chromatogram of comp change wavelength change 268 nm	15+10	446.56±1.66	0.37	350.9±1.12	0.32
Chromatogram of comp change wavelength change 270 nm	15+10	409.17±2.84	0.71	364.12±0.64	0.18
Chromatogram of mobile phase change 60+40 ml	15+10	425.76±0.62	0.15	358.96±3.18	0.89
Chromatogram of mobile phase change 62+38 ml	15+10	409.12±2.89	0.71	3.55±2.40	0.68

LOD and LOQ

Table no: 15 LOD & LOQ data

Emtricitabine	**Tenofovir**
LOD = 3.3 X Avg.SD/ Slope =0.5950818 **LOQ** = 10 X Avg SD/Slope =1.8032782	**LOD**= 3.3 X Avg.SD/ Slope =0.5298081 **LOQ** = 10X Avg SD/Slope = 1.6054792
LOD of Emtricitabine: 0.59508 ug/mL LOQ of Emtricitabine: 1.80327 ug/mL	LOD of Tenofovir: 0.52980 ug/mL LOQ of Tenofovir: 1.60547 ug/mL
slope- 34.01 Inercept-10.41 Regression-0.999	slope- 27.93 Intercept-4.017 Regression -0.999

Conclusion-

This developed RP- HPLC method was validated according to ICH guidelines in terms of linearity, precision, accuracy, and repeatability. All validation parameters were found to be within the allowed limit according to ICH guidelines. The method was successfully applied for the simultaneous estimation of Tenofovir and Emtricitabine. So we can conclude that the developed RP-HPLC method is precise, accurate, sensitive, and reproducible for quantitative estimation of Tenofovir and Emtricitabine bulk and its formulation.

References

1] James E Frampton et al; Emtricitabine a review of its use; 2005,65(10):1427-48
2] James T. Muller et al; Emtricitabine national library of medicine. Jan 2022.
3] John E. Bannet MD et al; An tirero viral therapy for human immunodeficiency virus infection; principle and practice of infectious disease; 128,1739-1760.
4] Maryam Bavand Savadkouhi et al; RP-HPLC method development and validation. Iranian Journal of pharmaceutical research 2017spring 16(2); 490-497
5] Dhara S. Bhavsar et al; RP-HPLC method for simultaneous estimation; pharm methods; 2012 Jul-Dec 3(2) 73-78
6] Ozlem Coskum- separation techniques; chromatography. Northern clics of Istambul 216; 3(2):156-160
7] Barry E Boyes et al; modern trend and best practices in mobile phase selection in reverse phase chromatography. LCGC North America vol 36 issue 10:752-768
8] Noopur K Gandhi- Stability indicating analytical method development using QBD Research Journal of pharmacy and technology pp 5937-5944
9] Centre of drug evaluation and research U.S. food and drug administration, validation of chromatographic method FDA Rockville, MD Nov 1994.
10] Europian medicinal agency; ICH topic Q2 (R1) validation of analytical procedure June 1995
11] P.C. Gupta –method validation of analytical procedure; pharmatutor; 2015; 3(1); 32-39
12] Dr. Raja Abhilash- analytical method validation. Slide share 2012
13] Michael E Wartz. Method validation and robustness LCGC North America 2006 vol. 24 issue 5, 480-490
14] Y Vander Heyden et al; Review of the use of robustness and ruggedness in analytical chemistry. ChemOAC, Pharmaceutical institute B-109 Brussels, Belgium
15] Shrivastava and Gupta; method for LOD and LOQ determination; chronicles of your scientists vol. 2; issue 1; 2011