Development and Validation of New Rp-Hplc Method for The Simultaneous Estimation of Lamivudine, Tenofovir and Doravirine in Pharmaceutical Dosage Form

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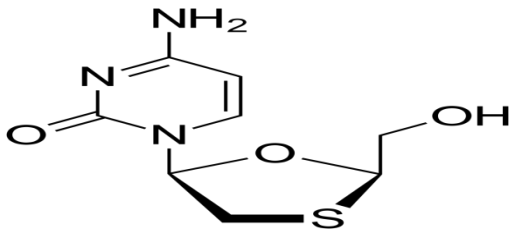
Abstracts: A simple, accurate, and precise quantitative approach for the simultaneous quantification of Lamivudine, Tenofovir, and Doravarine in pharmaceutical dose form was developed. The chromatogram was performed on an Inertsil C18, 150 * 4.6mm, 5µm column with a mobile phase containing 0.1% orthophosphoric acid and Acetonitrile in a 50:50 v/v ratio that was pumped through the column at a flow rate of 1ml/min. The temperature was kept at 30°C. The optimal wavelength was 230.0 nm. Lamivudine, Tenofovir, and Doravarine had retention times of 2.238, 2.623, and 3.088 minutes, respectively. For three medications evaluated, the % RSD of system precision and method precision was determined to be less than 2%. The mean % recovery for Lamivudine, Tenofovir, and Doravarine was 101.32 percent, 99.94 %, and 100.61 %, respectively. The method was found to be simple, accurate, sensitive,cost effective and can be useful in routine quality control analysis.

Keywords: Lamivudine, Tenofovir, Doravarine, Rp-Hplc

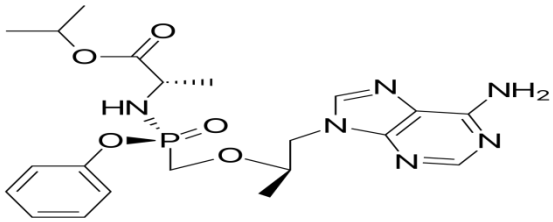
1. INTRODUCTION

Lamivudine (also known as **EPIVIR**) is a reverse transcriptase inhibitor used to treat HIV and hepatitis B infection. A reverse transcriptase inhibitor and zalcitabine analog in which a sulfur atom replaces the 3' carbon of the pentose ring. It is used to treat Human Immunodeficiency Virus Type 1 (HIV-1) and hepatitis B (HBV). Lamivudine is a synthetic nucleoside analogue and is phosphorylated intracellularly to its active 5'-triphosphate metabolite, lamivudine triphosphate (L-TP). This nucleoside analogue is incorporated into viral DNA by HIV reverse transcriptase and HBV polymerase, resulting in DNA chain termination [1-3].

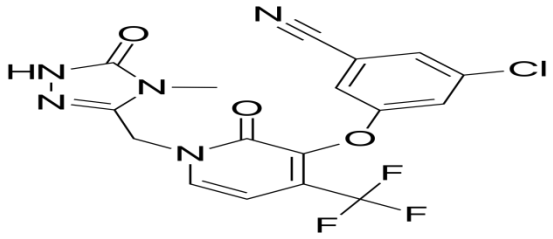
Tenofoviralafenamide is a reverse transcriptase inhibitor with a nucleoside analogue that is used to treat chronic hepatitis B virus infection in people with compensated liver damage. Both of these prodrugs were first developed to cover the polar phosphonic acid group on tenofovir by employing new oxy carbonyloxymethyl linkers in order to increase oral bioavailability and gastrointestinal diffusion. Tenofovir ([(2R)-1-(6-amino-9H-purin-9-yl) propan-2-yl]oxy methyl) phosphonic acid is an antiinfective and antiviral agent. When compared to red blood cells, tenofoviralafenamide aggregates greater in peripheral blood mononuclear cells [4-6]. Doravirine (also known as **PIFELTRO**) is a non-nucleoside reverse transcriptase inhibitor used in combination with other antiretrovirals to treat HIV-1 infections. Doravirine is an HIV-1 non-nucleoside reverse transcriptase inhibitor (NNRTI) intended to be administered in combination with other antiretroviral medicines.Doravirine is formally indicated for the treatment of HIV-1 infection in adult patients with no prior antiretroviral treatment experience, further expanding the possibility and choice of therapeutic treatments available for the management of HIV-1 infection [7-9]. **DELSTRIGO** is a three-drug combination that includes doravirine (a non-nucleoside reverse transcriptase inhibitor [NNRTI]), lamivudine, and tenofovirdisoproxilfumarate (both nucleoside analogue reverse transcriptase inhibitors). It was approved by the US Food and Medication Administration in 2018 as a complete drug regimen for HIV type 1 infection in patients who had not previously received antiretroviral therapy[12].



a) Lamivudine Structure



b) TenofovirAlafenamide



c) Doravirine structure

Figure 1. Structures of a) Lamivudine, b) Tenofovir and c) Doravirine

According to the literature, few techniques have been published for estimating Lamivudine, Tenofovir, and Doravirine alone and in combination with various dosage forms utilising UV, HPLC methods [13-15]. There is no known technique for determining assay and simultaneous estimate of Lamivudine, Tenofovir, and Doravirine in combination dose form. As a result, the current study sought to establish an accurate, precise, sensitive, selective, repeatable, and quick analytical method based on HPLC for the cost-effective measurement of Lamivudine, Tenofovir, and Doravirine in combination.

2. Materials and Methods:

Lamivudine, Tenofovir and Doravirinepure drugs were obtained from Spectrum Pharmaresearch solutions. The HPLC grade methanol and acetonitrile procured from Rankem chemical division, India. Sodium hydrogen phosphate procured from Rankem, India and Puremilli-Q water is used with the help of 0.45µ Millipore filters (Rankem, india).

2.1 Instrumentation and Chromatographic Conditions

WATERS HPLC, model:2695 SYSTEM with Photo diode array detector was used for the development and method validation, with an automated sample injector.The data was acquired using the software Empower 2.

2.2 Preparation of Solutions

Preparation of buffer

Buffer(0.01N $C_2H_7NO_2$): Accurately weighed 0.77gm of Ammonium acetate in a 1000ml of Volumetric flask add about 900ml of milli-Q water added and degas to sonicate and finally make up the volume with water then PH adjusted to 4.5 with dil. Orthophosphoric acid solution.

Preparation of Standard stock solution:

Accurately weighed 37.5mg of Lamivudine, 37.5mg of Tenofovir and 12.5mg of Doravirine were transferred to three 50ml volumetric flasks separately. 10ml of diluent was added to the flasks and sonicated for 25mins. Flasks were made up to the mark with diluent and labelled as standard stock solution 1, 2 and 3. (750µg/ml of Lamivudine, 750µg/ml of Tenofovir and 250µg/ml of Doravirine).

Preparation of Standard working solution: 1ml from each stock solution was pipetted out and taken into a 10ml volumetric flask and made up with diluent (75µg/ml of Lamivudine, 75µg/ml of Tenofovir and 25µg/ml of Doravirine).

Preparation of Sample stock solution:

10 Delstrigo tablets (label clam per one tablet: 300 mg of Lamivudine, 300 mg of Tenofovir and 100 mg of Doravirine) were weighed individually and average weight of each tablet was calculated. Weight equivalent to 1 tablet was transferred into a 100 ml volumetric flask, 25ml of diluent added and sonicated for 50 min, further the volume was made up to the mark with diluent and filtered. (3000µg/ml of Lamivudine, 3000µg/ml of Tenofovir and 1000µg/ml of Doravirine).

Preparation of Sample working solution: From the filtered solution, 0.25ml was pipetted out into a 10 ml volumetric flask and made up to 10ml with diluent. (75µg/ml of Lamivudine, 75µg/ml of Tenofovir and 25µg/ml of Doravirine).

2.3 Chromatographic conditions:

Flow rate: 1mL/min

Column : Inertsil C18 150x 4.6mm, 5µm.

wave length: 230.0 nm

Column temperature: 30°C

Injection volume: 10.0µL

Run time: 7.0minutes

Diluent: 0.1% OPA : Acetonitrile (50:50)

Lamivudine, Tenofovir and Doravirine Retention times were at 2.238 min, 2.623 min and 3.088 min respectively with good resolution (figure 2). The plate count and tailing factor were highly excellent, the technique conditions were optimized and the same conditions were used for validation.

2.4 Degradation:

To conduct the forced degradation experiment, standard stock solutions of Lamivudine, Tenofovir, and Doravirine were exposed to various stress conditions, including 1 mL of 20% H_2O_2 (for oxidative degradation), 1 mL of 2N HCL (for acidic degradation), and 1 mL of 2N NAOH (for acidic degradation) (for basic degradation). The produced solutions were refluxed for 30 minutes at 60^{o}C. To examine the deterioration, the standard solutions were also subjected to UV radiation and temperature conditions. The resulting solutions were diluted to yield 75g/ml, 75g/ml, and 25g/ml of Lamivudine, Tenofovir, and Doravirine, respectively, for degradation studies. To examine sample stability, 10µl samples were fed into the system and chromatograms were obtained.

2.5 Method Validation:

The method was validated in accordance with ICH recommendations Q2R1. System appropriateness, specificity, linearity, accuracy, precision, LOD& LOQ, and robustness are among the validation parameters.

3. RESULTS AND DISCUSSION

3.1 System suitability parameters:

The system suitability parameters were assessed by making standard solutions of Lamivudine (75g/ml), Tenofovir (75g/ml), and Doravirine (25g/ml) and injecting them six times. Peak tailing, resolution, and USP plate count were all determined. For three medications in combination, the USP Plate count exceeded 2000 and the tailing factor was less than 2. All of the system's appropriate parameters were passed and remained within the limitations. Table 1 shows the results.

3.2 Specificity:

If the methoddetermines or measures quantitatively the component of interest in the sample matrixwithout separation, it is said to be specific.In the optimised method, the interference is checked. Lamivudine, Tenofovir, and Doravirine had retention times of 2.238 minutes, 2.623 minutes, and 3.088 minutes, respectively. We found no interfering peaks in the chromatograms of blank and placebo samples during the retention periods of these medicines in our approach. As a result, this procedure was stated to be particular. Figures 3, 4, and 5 show the chromatograms for specificity.

3.3 Linearity:

Six linear concentrations of Lamivudine (18.75-112.5µg/ml), Tenofovir (18.75-112.5µg/ml) and Doravirine (6.25-37.5µg/ml) were injected in triplicate manner. Correlation coefficients obtained was 0.999 for all the three drugs. The results were shown in table 2 and figures 6, 7 and 8.

3.4 Precision:

Repeatability: Method Precision: One of the most common statistical terms employed is the standard deviation of a population of observation. Standard deviation is the square root of the sum of squares of deviations of individual results for the mean, divided by one less than the number of results in the set. The standard deviation S, is given by

$$S = \sqrt{\frac{\sum_{i=1}^{n}(x - x')^2}{(n-1)}}$$

Standard deviation has the same units as the property being measured.

The square of standard deviation is called variance (S2). Relative standard deviation is the standard deviation expressed as a fraction of the mean, i.e., S/x. It is sometimes multiplied by 100 and expressed as a percent relative standard deviation. It becomes a more reliable expression of precision.

$$\% \text{ Relative Standard Deviation} = \frac{\text{SD}}{\text{Mean}} \times 100$$

Multiple samples were taken from a sample stock solution, and six working sample solutions of the same concentrations (75g/ml of Lamivudine, 75g/ml of Tenofovir, and 25g/ml of Doravirine) were created. Each injection was given from each working sample solution, and the results are shown in table 3. The average area, standard deviation, and % RSD for the three medications were computed and found to be 0.6%, 1.7%, and 0.5%, respectively, for Lamivudine, Tenofovir, and Doravirine. The system precision was passed for this procedure since the precision limit was less than "2 %." Table 3 shows the information results.

Intermediate Precision: Multiple samples were taken from a sample stock solution, and six working sample solutions of the same concentrations (75g/ml of Lamivudine, 75g/ml of Tenofovir, and 25g/ml of Doravirine) were prepared. Each injection from each working sample solution was given on the following day of the sample preparation, and the obtained areas are listed in table 4. The average area, standard deviation, and % RSD for the three medications were computed and found to be 0.5%, 1.0%, and 0.3% for Lamivudine, Tenofovir, and Doravirine, respectively. Because the accuracy limit was less than "2%," the intermediate precision was used for this procedure. Table 4 shows the information results.

3.5 Accuracy:

The conventional addition procedure was used to create three levels of accuracy samples. Triplicate injections were administered at each degree of accuracy, and the mean percent recovery for Lamivudine, Tenofovir, and Doravirine, respectively, was 101.32 %, 99.94 %, and 100.61 %. Tables 5, 6, and 7 show the outcomes. Because satisfactory recover values were achieved, the accuracy for this approach was passed.

3.6 Robustness:

Robustness conditions such as flow minus (0.9ml/min), flow plus (1.1ml/min), mobile phase minus (55:45 v/v), mobile phase plus (45:55 v/v), temperature minus (25°C), and temperature plus (35°C) were maintained, and samples (75g/ml Lamivudine, 75g/ml Tenofovir, and 25g/ml Doravirine) were injected in duplicate. The % RSD was computed and determined to be within the acceptable range. Table 8 shows the data.

3.7 Assay:

Delstrigo tablets had a label claim of Lamivudine 300mg Tenofovir 300mg Doravirine 300mg per unit formulation. The aforementioned formulation was used for the assay. The average % assay achieved for Lamivudine, Tenofovir, and Doravirine was 99.39 %, 100.30 %, and 99.64 %, respectively.

3.8 Degradation Studies:

Degradation studies were performed with the stock standard solution and the degraded samples were analysed using proposed method. Assay percent of Lamivudine, Tenofovir and Doravirine in the injected samples was calculated and all the samples passed the limits of degradation. The results were shown in table 9.The chromatograms obtained in degradation studies are shown in figures 9, 10

11,12 and 13.

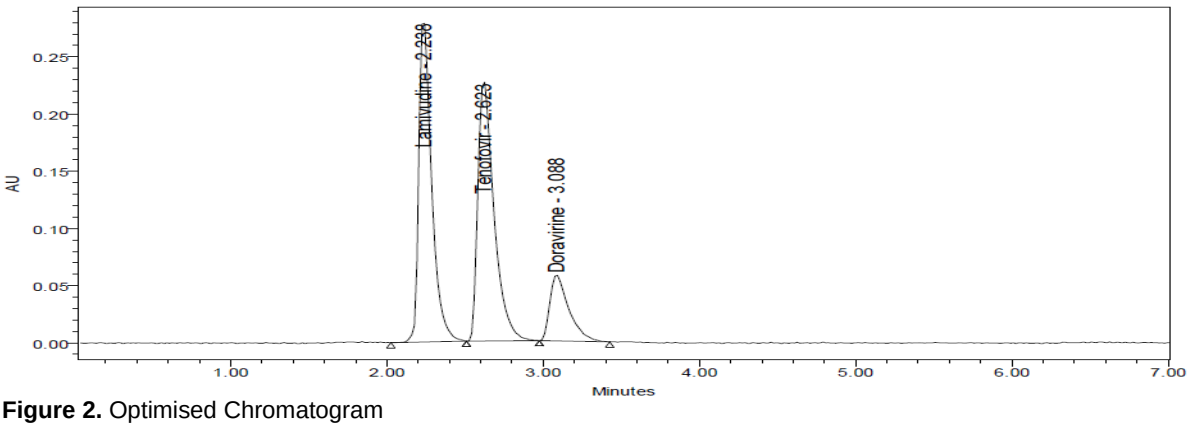


Figure 2. Optimised Chromatogram

Table 1. System suitability parameters

S No	Lamivudine			Tenofovir				Doravirine			
Inj	RT(min)	USP Plate Count	Tailing	RT(min)	USP Plate Count	Tailing	RS	RT(min)	USP Plate Count	Tailing	RS
1	2.238	5068	1.40	2.823	3690	1.6	3.6	3.351	4418	1.8	2.6
2	2.283	4989	1.42	2.826	3774	1.6	3.6	3.357	4490	1.8	2.5
3	2.285	5008	1.40	2.860	3747	1.6	3.8	3.388	4563	1.8	2.6
4	2.286	5022	1.42	2.866	3637	1.6	3.6	3.394	4374	1.8	2.6
5	2.290	4955	1.41	2.883	3764	1.6	3.7	3.420	4485	1.8	2.7
6	2.290	4932	1.42	2.902	3798	1.6	3.8	3.435	4476	1.8	2.7

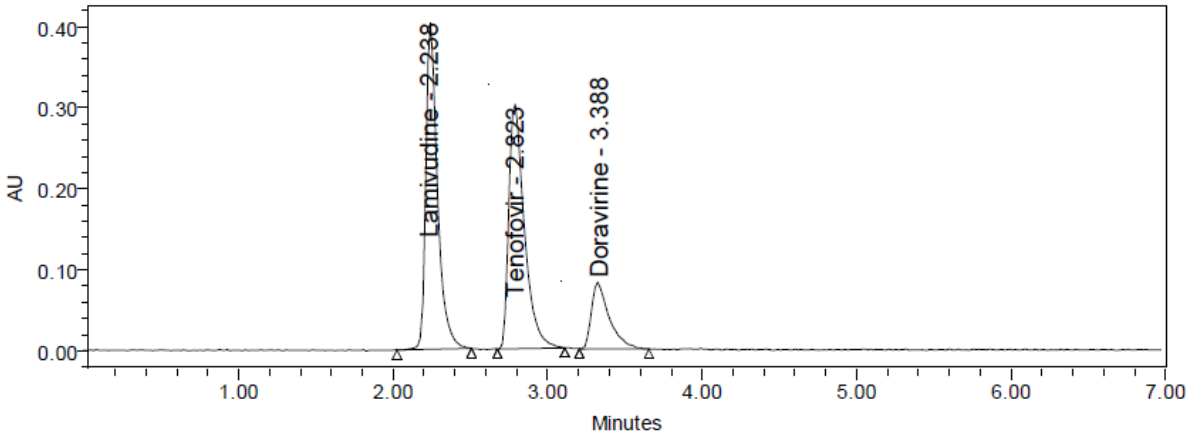


Figure 3. Standard solution chromatogram

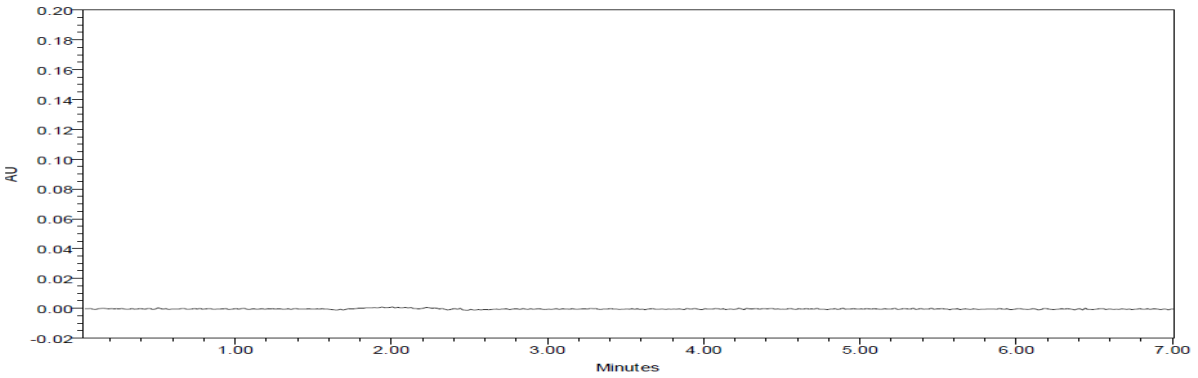


Figure 4. Blank chromatogram

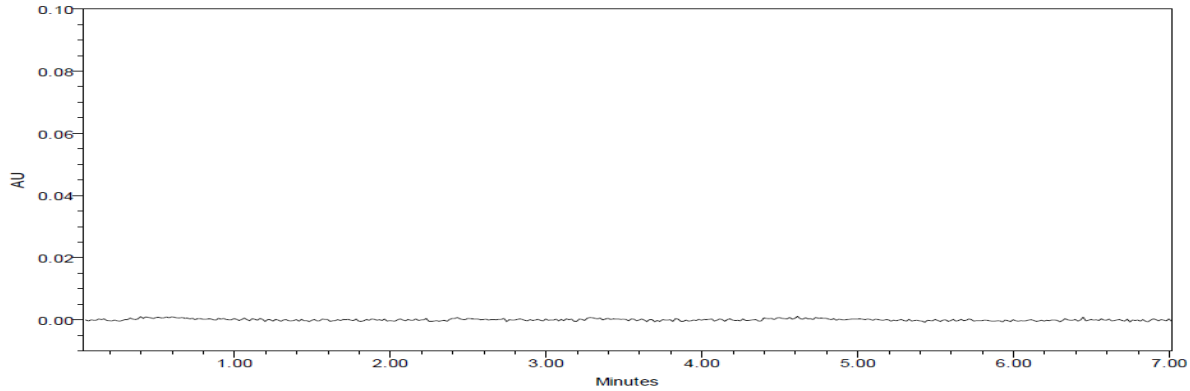


Figure 5. Placebo chromatogram

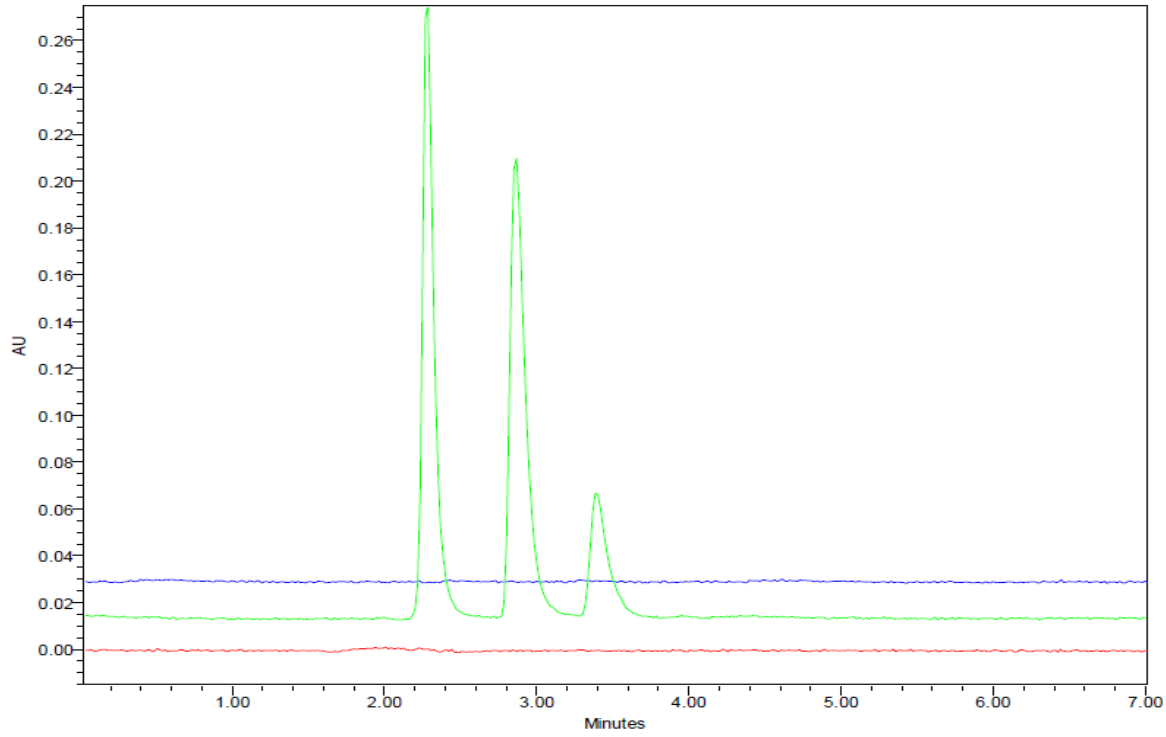


Figure 6. Overlay of Specificity chromatogram

Table 2. Linearity table for Lamivudine, Tenofovir and Doravirine

Lamivudine		Tenofovir		Doravirine	
Conc (µg/mL)	**Peak area**	**Conc (µg/mL)**	**Peak area**	**Conc (µg/mL)**	**Peak area**
18.75	576135	18.75	507414	6.25	141714
37.5	1165349	37.5	1066905	12.5	298067
56.25	1757059	56.25	1505781	18.75	438295
75	2270047	75	2015940	25	593247
93.75	2884818	93.75	2518630	31.25	728226
112.5	3454252	112.5	3036953	37.5	853001

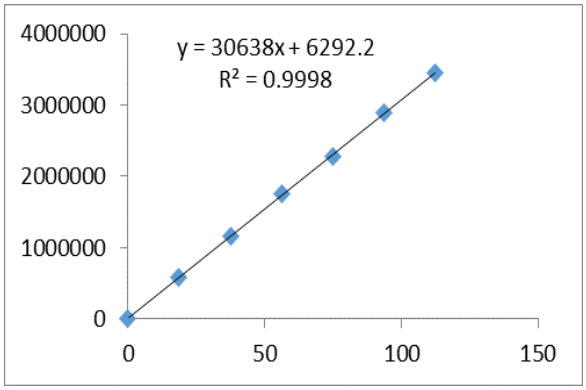


Figure 7. Calibration curve of Lamivudine

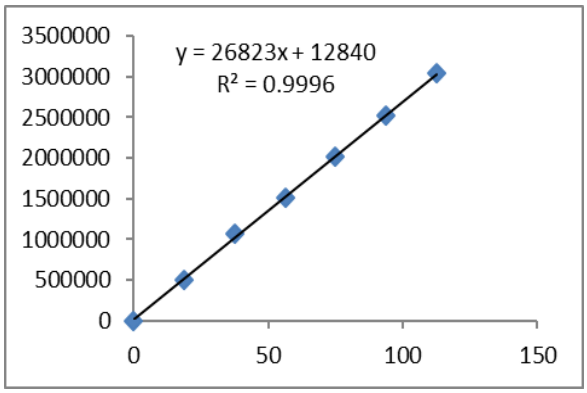


Figure 8. Calibration curve of Tenofovir

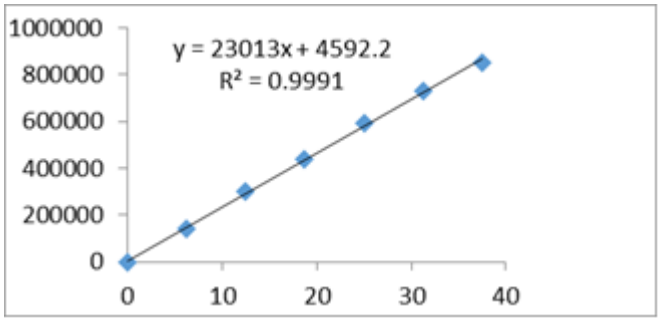


Figure 9.Calibration curve of Doravirine

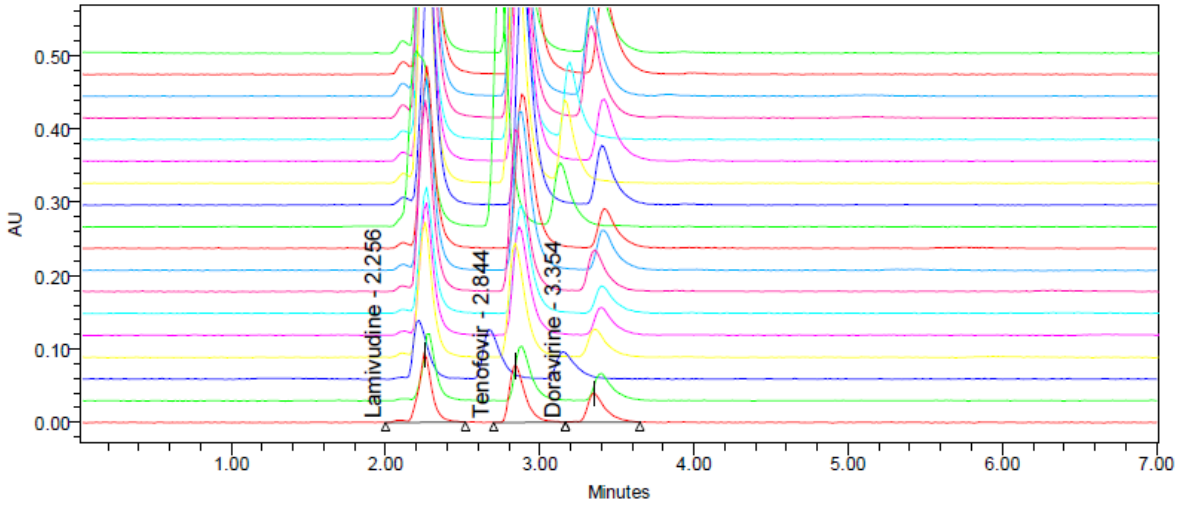


Figure 10. Overlay Linearity of Lamivudine, Tenofovir and Doravirine

Table 3. Repeatability for Lamivudine, Tenofovir and Doravirine

S.no.	Lamivudine	Tenofovir	Doravirine
1	2288233	1995962	589275
2	2299198	2015190	586275
3	2272743	2025190	579786
4	2289300	2073819	586510
5	2259300	1976724	585286
6	2275230	1994035	586699
Mean	2280667	2013487	585639
S.D	14311.3	34108.2	3158.8
%RSD	0.6	1.7	0.5

Table 4. Intermediate Precision for Lamivudine, Tenofovir and Doravirine

S.no.	Lamivudine	Tenofovir	Doravirine
1	2179863	1986858	579206
2	2195444	1989942	580381
3	2213024	1964053	581257
4	2193280	1999403	582757
5	2204419	2022827	581274
6	2196509	1975638	584626
Mean	2197090	1989787	581584
S.D	11152.9	20273.9	1893.9
%RSD	0.5	1.0	0.3

Table 5. Accuracy for Lamivudine

% Level	Amount Spiked (µg/mL)	Amount recovered (µg/mL)	% Recovery	Mean %Recovery
50%	37.5	37.42	99.79	101.32%
	37.5	38.37	102.34	
	37.5	38.04	101.46	
100%	75	75.89	101.19	
	75	75.93	101.24	
	75	76.73	102.31	
150%	112.5	114.08	101.41	
	112.5	113.56	100.95	
	112.5	113.80	101.16	

Table 6. Accuracy for Tenofovir

% Level	Amt Spiked (µg/mL)	Amount recovered (µg/mL)	% Recovery	Mean %Recovery
50%	37.5	37.31	99.52	99.94%
	37.5	37.30	99.49	
	37.5	37.70	100.55	
100%	75	75.13	100.19	
	75	75.79	101.06	
	75	76.05	101.40	
150%	112.5	111.41	99.04	
	112.5	111.69	99.28	
	112.5	111.27	98.91	

Table 7. Accuracy for Doravirine

% Level	Amt Spiked (µg/mL)	Amount recovered (µg/mL)	% Recovery	Mean %Recovery
50%	12.5	12.56	100.56	100.61%
	12.5	12.54	100.37	
	12.5	12.48	99.91	
100%	25	25.11	100.44	
	25	25.23	100.93	
	25	25.14	100.56	
150%	37.5	37.78	100.76	
	37.5	37.57	100.21	
	37.5	38.15	101.75	

Table 8. Robustness Data

S.no	Condition	%RSD of Lamivudine	%RSD of Tenofovir	%RSD of Doravirine
1	Flow rate (-) 0.9ml/min	0.9	0.9	0.4
2	Flow rate (+) 1.1ml/min	0.5	0.7	0.7
3	Mobile phase (-) 55B:45A	1.1	1.1	0.6
4	Mobile phase (+) 45B:55A	0.8	0.3	0.5
5	Temperature (-) 25°C	1.0	0.3	0.6
6	Temperature (+) 35°C	0.5	0.3	0.7

Table 9. Robustness Data

S.No.	Condition	%Degraded		
		Lamivudine	Tenofovir	Doravirine
1	**Acid**	6.61	5.68	6.18
2	**Base**	4.68	4.19	4.49
3	**Oxidation**	4.32	3.93	4.23
4	**Dry heat**	2.71	2.37	2.17
5	**UV Light**	1.80	1.23	1.43

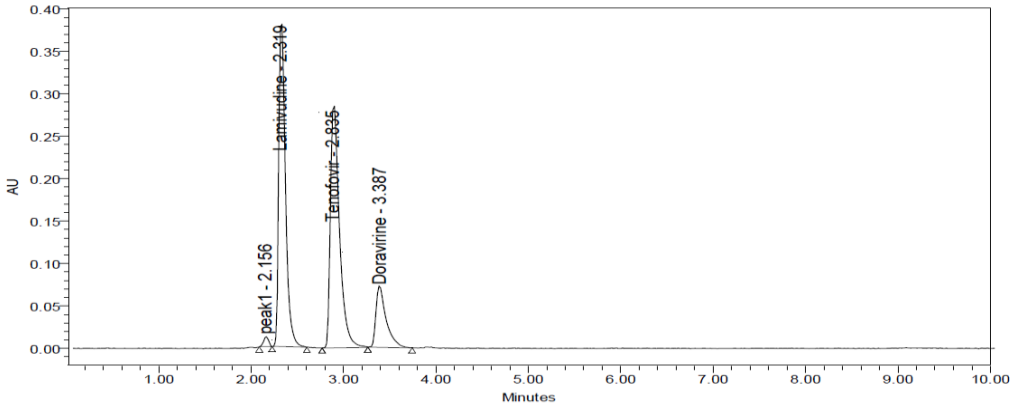


Figure 10. Acid degradation chromatogram

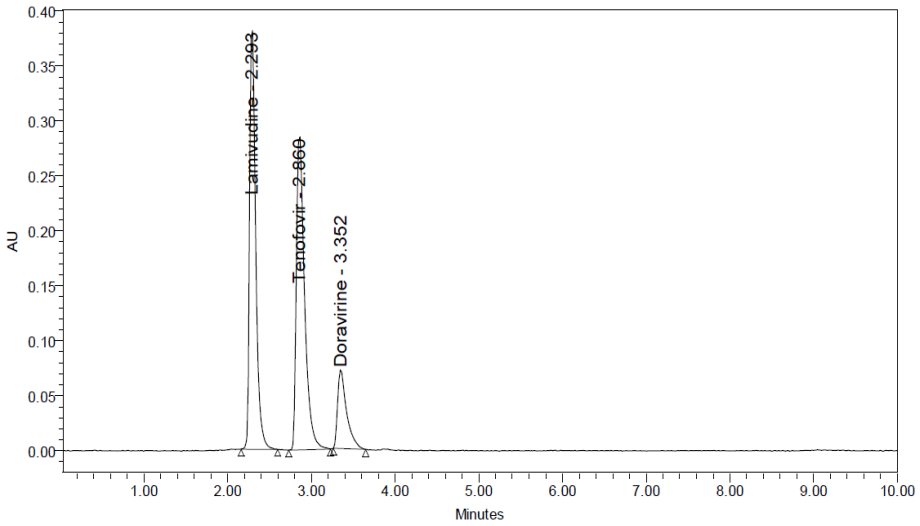
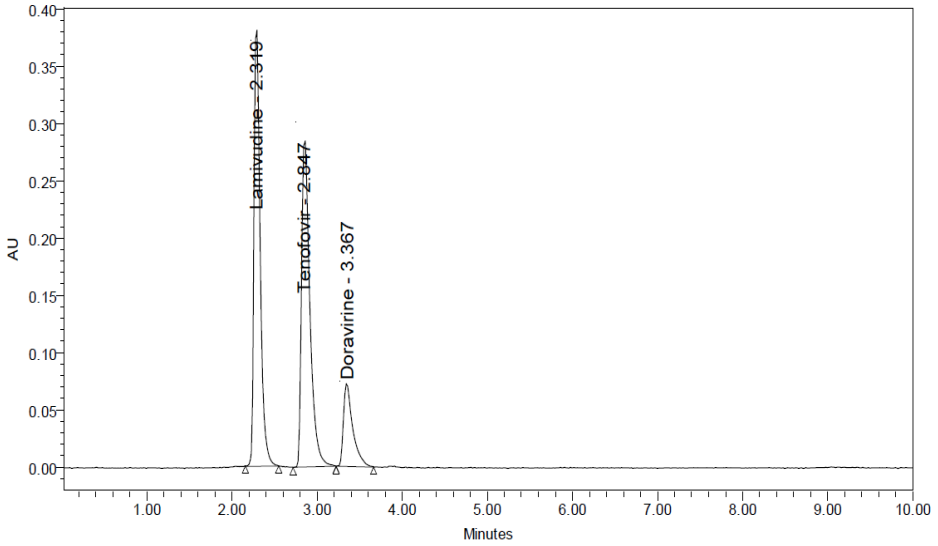


Figure 11. Base degradation chromatogram



\Figure 12. Peroxide degradation chromatogram

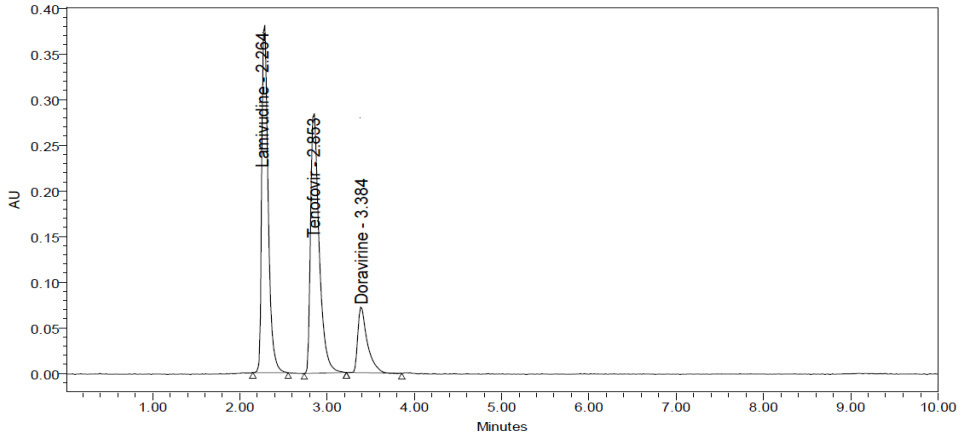


Figure 13. Thermal degradation chromatogram

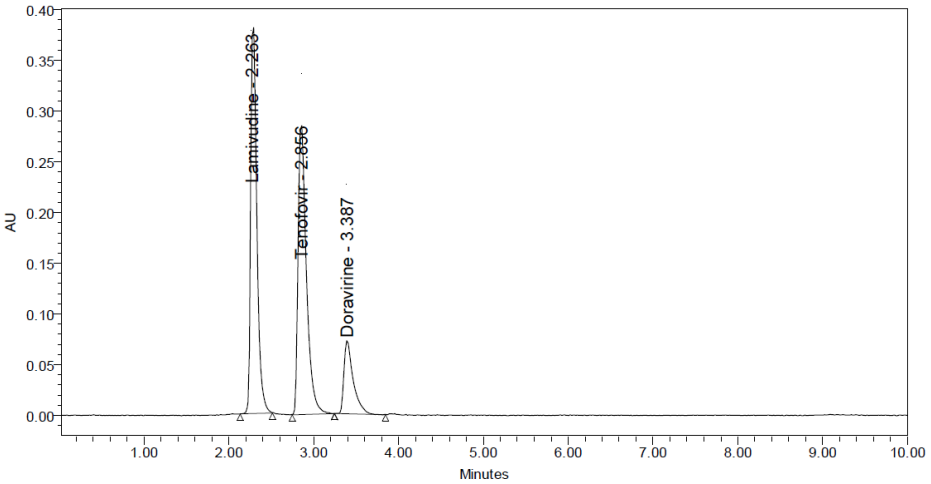


Figure 14. UV degradation chromatogram

Conclusions:

For the stability indicating RP-HPLC technique for the simultaneous determination of Lamivudine, Tenofovir, and Doravirine in pharmaceutical dosage form, a simple, accurate, and exact approach was derived. The created approach met all of the validation criteria, and the results were found to be within the acceptable range. When compared to previous studies, the current technique reduces retention times. As a result, the method created was simple and cost-effective, and it may be used in routine quality control tests in industries.

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