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New Green Stability Indicating Rp-Hplc Method Development And Validation Of Fixed Drug Combination Of Emtricitabine, Tenofovir Alafenamide And Dolutegravir Sodium In Marketed Formulation By Gradient Elution Programme

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Abstract
In this work, a new, sensitive and accurate RP-HPLC method for determining the Emtricitabine, Tenofovir Alafenamide, and Dolutegravir sodium used for the treatment of viral disease was developed and validated. Shimadzu, C18 (4.6 mm X 100mm, 3.0 µm) column was used for the separation. Gradient elution programme of the mobile phase is composed of a solution containing 20 mM potassium dihydrogen phosphate and methanol was used for separation. UV detector was used and detection was performed at 254nm. The method showed linearity across the concentration range. The method was validated and effectively useful for the commercial dosage forms of the combination drug. The method has been proven to be highly accurate, precise, and robust according to the statistical analysis. The greenness profiles of the developed HPLC method were evaluated by AGREE evaluation method. The findings indicated that the described method aligns with the principles of green analytical chemistry.

Key words*:Dolutegravir, Emtricitabine, Gradient elution, Greenness, RP-HPLC, Stability indicating method, Tenofovir alafenamide*

Introduction

In the unending fight against HIV-1 infection, effective treatment options are essential for maintaining patient health and quality of life. The combination of DTG, EMT, and TAF has proven to be a powerful therapy, providing substantial benefits for both adults and children.

DTG, EMT, and TAF constitute a three-drug regimen comprising DTG (an integrase strand transfer inhibitor [INSTI]), EMT, and TAF. The combination is prescribed for both HIV nucleoside analog reverse transcriptase inhibitors (NRTIs) and is recommended for use alone as a complete regimen for treating HIV-1 infection in adults and pediatric patients weighing at least 40 kg.[1]

Antiretroviral therapy (ART), involving the administration of HIV medicines, including combinations from different drug classes, is recommended for all individuals with HIV to suppress viral replication and prevent disease progression .[2]

Antiretroviral therapy (ART) refers to the treatment regimen involving HIV medicines. It is recommended for all individuals diagnosed with HIV, emphasizing the importance of initiating ART promptly upon diagnosis. Those undergoing ART adhere to a daily regimen comprising a combination of HIV medicines, known as an HIV treatment regimen. Typically, the initial ART regimen consists of three HIV medicines selected from at least two distinct drug classes designed to effectively manage HIV infection .[2]

Emtricitabine:

Emtricitabine, chemically named 4-amino-5-fluoro-1-[(2R,5S)-2-(hydroxymethyl)-1,3-oxathiolan-5-yl]pyrimidin-2-one, has a molecular formula C8H10FN3O3S and a molecular weight of 247.25 g/mol.[3] It acts as a cytidine analog that competes with deoxycytidine 5'-triphosphate for HIV-1 reverse transcriptase. Upon phosphorylation to EMT 5'-triphosphate, it incorporates into viral DNA strands, inhibiting further nucleotide incorporation and leading to viral DNA chain termination. This mechanism inhibits viral RNA transcription into DNA, thereby reducing viral load [4.]

Tenofovir alafenamide:

Tenofovir alafenamide, with the IUPAC name propan-2-yl (2S)-2-[[[(2R)-1-(6-aminopurin-9-yl) propan-2-yl] oxymethyl-phenoxy phosphoryl]amino] propanoate, has a molecular formula C21H29N6O5P and a molecular weight of 476.5 g/mol.[5] Compared to TAF disoproxil, it exhibits 91% lower plasma concentration but achieves about 20-fold higher intracellular accumulation due to prolonged systemic exposure. Once intracellularly, it is converted to TAF diphosphate, which accumulates primarily in peripheral blood mononuclear cells, inhibiting viral polymerase and viral replication. [6]

Dolutegravir sodium:

DTG's chemical name is sodium;(3S,7R)-13-[(2,4-difluorophenyl)methylcarbamoyl]-7-methyl-9,12-dioxo-4-oxa-1,8-diazatricyclo[8.4.0.03,8]tetradeca-10,13-dien-11-olate, with a molecular formula of C20H18F2N3NaO5 and a molecular weight of 441.4 g/mol.[7] It functions as an HIV-1 antiviral agent by inhibiting HIV integrase, thereby prevent the integration of viral DNA into the host cell genome. This inhibition disrupts the viral replication cycle, leading to reduced viral activity.[8]

Analytical methods such as assay method by HPLC [9, 10, 11] Stability-indicating assay methods by HPLC[12], chemometric method [13], and LCMS/MS in human plasma [14, 15] have been reported for the quantification of these drugs. However, there remains a need to develop a new green stability-indicating method capable of separating all relevant degradant impurities. The greenness of the method was assessed by AGREE software .[16]

2. Materials and Methods

2.1 Reagents and chemicals

EMT, TAF, and DTG, which are active drug substance, were provided as a complimentary sample from Emcure Pharmaceuticals Ltd, Ahmedabad. The pharmaceutical dosage form under the brand name Spegra– containing 25 mg TAF, 50 mg DTG and 200 mg EMT), which is fixed drug combination was purchased from a local medical store.

All the solvents and chemicals used for the analysis were HPLC and analytical grade and used from local distributor of Merck India.

2.2 Instrument

The HPLC analysis was done using the Shimadzu LC30, containing a Quaternary solvent delivery pump with a UV detector, sample injector. Shimadzu, C18 (4.6 mm X 100mm, 3.0 µm) column was selected. Lab solution software was used for the data acquisition.

2.3 Solution preparation

2.3.1 Diluent: Water: Acetonitrile (50:50%v/v) was chosen as diluent to prepare all the samples for analysis.

2.3.2 Buffer preparation

20mM potassium dihydrogen phosphate (pH-4.5)

Weigh accurately 2.72 gm of potassium dihydrogen phosphate, transfer into 1000 mL volumetric flask and add diluent to the flask up to the mark. Filter the buffer with 0.45 µ filter paper and sonicate it.

2.3.3 Mobile phase

Mobile phase A: 20mM Potassium dihydrogen phosphate buffer

Mobile phase B: Methanol

Time programme for gradient elution using mobile phase is mentioned in table-1

2.3.4 Preparation of standard solution

(125 µg/mL TAF, 250µg/mL DTG and 1000 µg/mL EMT)

In clean dry 50 mL volumetric flask an accurately measured quantity of EMT (50 mg), TAF (11.4 mg), and DTG (12.5 mg) drug substance was added. The compounds were then dissolved in 35 ml of diluent, sonicated the solution to make all three drug substance to completely dissolve. Diluent was added to the flask up to the mark to achieve the desired concentration.

2.3.5 Preparation of test solutions

(125 µg/mL TAF, 250µg/mL DTG and 1000 µg/mL EMT)

Transferred 1 tablet to 200 mL of volumetric flask. About 70% diluent was added. Sonicate the flask to completely dissolve the content with intermittent shaking for 30 minutes. After sonication dilute the volume of flask with diluent.

2.4 Force degradation[19]

Force degradation was performed on different condition and preparation of the sample mention.

Degradation sample preparation

Accurately weighed the fine powder equivalent to 50 mg of EMT, 6.25 mg of TAF, and 12.5 mg of DTG and transferred into a 50 mL volumetric flask. Approximately 35 mL of diluent was added, and the mixture was sonicated with intermittent shaking for 30 minutes to ensure dissolution. Following sonication, different degradation conditions were applied to the drugs (Refer table-3). After applied different conditions, dilute the flask up to the mark with diluent. From prepared solution, 10 µl sample was injected into the HPLC and chromatograms were recorded to evaluate the stability of different stress sample.

Note: Similarly blank sample also prepared for all stress conditions.

2.5 Method Validation [20]

It is essential to validate the developed analytical method in order to ensure that the method is suitable for quality control of the formulation. Therefore the developed HPLC method was validated as per ICH Q2 (R1) guidelines. various validation parameters such as specificity, system adaptability, linearity, accuracy, precision, limit of detection and quantification, as well as conducting a robustness study.

2.5.1 Specificity

Specificity refers to the capability to accurately identify the analyte even in presence of other expected components. Typically, these could involve placebo,excipients, impurities, degradants, matrix etc. There should not be any interference observed at the retention time of the all three analytes. The specificity test evaluated by injecting a standard solution and blank. Chromatogram were recorded and compared the response of both chromatograms.

2.5.2 System suitability parameters

Standard solutions of EMT, TAF, and DTG was prepared to determine system suitability parameters. These solution underwent six injections, and various parameters including retention time, resolution, peak tailing, and theoretical plates were evaluated.

2.5.3 Linearity

Linearity was performed on different level from 20% to 150% on for all the component. Linearity range for EMT, TAF, and DTG were in the series of 200–1620 µg/mL, 25–200 µg/mL, and 50–400 µg/Ml

Standard stock solutions preparation for linearity

Weighed accurately and transferred 200.0 mg, 41.5 mg & 50.0 mg of EMT, TAF and DTG drug substance into a 50 mL volumetric flasks, 15 ml of diluent was added, sonicated until all drug substance get dissolved. Volume of the flask were made up to the mark with water and acetonitrile (50:50) and labelled as Standard stock solution.

Preparation of different concentration of linearity solutions are mentioned in table-2

All the prepared concentration in developed method and recorded response and monitor r2 of all the three component.

2.5.4 Precision

Method Precision: (Repeatability)

The concept of repeatability refers to the precision within the same chromatographic conditions over a short period of time. It is also known as intra-assay precision. To assess repeatability, a minimum of 6 determinations at 100% of the test concentration were conducted. The obtained results were used to monitor the % Assay and %RSD.

Intermediate Precision

The variations that need to be studied encompass days, analysts, equipment, and so on. There's no need to analyze these effects separately. Intermediate precision was evaluated at 100% in triplicate, both on the same and on different days. Based on the obtained results monitor the % Assay and %RSD was monitored.

2.5.5 Accuracy:

The accuracy parameter was performed at 50%, 100%, and 150% concentration levels to validate the method. Three times and recorded the chromatogram to calculate accuracy. The mean percentage recovery of the sample was calculated in this parameter.

2.5.6 Sensitivity

The detection limit and quantification limit for EMT, TAF, and DTG were established using the calibration curve method. Prepared solutions of these compounds within their respective linearity ranges and injected them in triplicate. After analyzing the data, Plotted the graph of average peak area from the three analyses against the concentration.

2.5.7 Robustness

The robustness was studied by knowingly adjusted the optimized conditions slightly. Parameters like the column oven temperature (± 5ºC), the flow rate (± 0.2 mL/min), and the wavelength (±2 nm) were deliberately adjusted within small margins. Based on results % Assay and % RSD were reported.

Evaluation of the greenness of chromatographic methods

The greenness of chromatographic methods was assessed using the AGREE-Analytical greenness metric software. AGREE provides a metric system for evaluating the environmental impact of analytical procedures based on fundamental principles. The software is user-friendly and incorporates 12 basic principles in greenness assessment, allowing for flexible weight assignment. It generates easily interpretable color pictogram outputs illustrating strengths and weaknesses. The Analytical Greenness score, which represents the weighted average of the benchmark scores, is displayed in the center of the graph and rounded to two decimal places, with a value range from 0.0 (lowest score) to 1.0 (perfect score). The graph visually depicts the score, benchmark scores, and benchmark weights.[17,18]

3. Result and discussion

The three analytes were found to be polar molecules, leading to the development of a reverse phase HPLC method utilizing an Inertsil ODS-3V C18 (4.6 mm X 100mm, 3.0 µm) column. In order to expedite the HPLC process, a 100 mm column was selected. Wavelength selection involved the analysis of UV spectra for EMT, TAF, and DTG, ultimately resulting in the selection of 254 nm for further chromatographic optimization.

3.1 The Optimization of Chromatographic Parameters

In order to simultaneously estimate EMT, TAF, and DTG, a reliable HPLC method was developed by optimizing the chromatographic conditions using a gradient time programme. The analytical method was designed for use at a temperature of 30°C to accommodate room temperature conditions. After conducting numerous trials, we successfully optimized the method by using an Inertsil ODS 3V C18 column and a mobile phase composed of a 20 mm potassium hydrogen phthalate buffer and methanol with a gradient time program of 14 minutes (table-1). The flow rate was set at 1.0 mL/min, and an injection volume of 10 µL was chosen. The detailed chromatographic conditions can be found in table -1, and the resulting chromatogram is depicted in Figure 2.

3.2 Force degradation

When conducting a stability-indicating method, it's crucial to ensure that the acceptable percentage of degradation does not surpass 20%. To assess degradation, we compared the peak area of the 100% level working standard concentration under both normal and stress conditions.

3.2.1: Acid Hydrolysis

Upon acid degradation studies 10.8% of EMT, 19.2% TAF and 14.2 % DTG was degraded. 3 degradant peaks in acidic media are observed at RT 3.833; 13.150; 14.233 minutes. Chromatogram was mentioned in Figure 3 and % degradation was illustrated in table-3.

3.2.2: Alkali Hydrolysis

EMT observed stable upon alkali degradation while 15.9% TAF and 10.8% DTG was degraded. Peaks of 5 degradant impurities observed at retention time 3.850; 4.708; 7.450; 8.058; 13.125 minutes. Chromatogram was mentioned in Figure-4 and % degradation was illustrated in table-3.

3.2.3: Peroxide degradation

Upon oxidation reaction 9.4 % of EMT, 11.1% TAF and 5.9% DTG was degraded. Peaks of 2 degradant impurities at retention time 3.733; 7.425 were observed. Chromatogram was mentioned in Figure-5 and % degradation was illustrated in table-3.

3.2.4: Thermal degradation

Three molecules were observed stable upon thermal degradation. So no degradation was obtained in this condition. Chromatogram was mentioned in Figure-6 and % degradation was illustrated in table-3.

3.2.5: Photolytic degradation

Three molecules were observed stable upon photolytic degradation. So no degradation was obtained in this condition. Chromatogram was mentioned in Figure-7 and % degradation was illustrated in table-3.

3.2.6: Heat/humidity degradation

Three molecules were observed stable upon degradation. So no degradation was obtained in this condition. Chromatogram was mentioned in Figure-8 and % degradation was illustrated in table-3.

The degradation percentages of the three analytes in stressed solutions are detailed in Table 3. Chromatograms illustrating acid degradation, alkali degradation, peroxide degradation, photo degradation, thermal degradation, and Heat/Humidity can be found in Figures 3, 4, 5, 6,7 and 8 respectively. These findings validate the method's capability to indicate stability.

3.3 Method Validation

3.3.1 Specificity

The method displayed exceptional specificity, as there was no evident interference from blank or placebo samples at the retention times of the target analytes. Therefore, the developed method is considered to be highly specific. The chromatogram in figure-2 illustrates the specificity of the developed method.

3.3.2 System Suitability Parameters

System suitability was thoroughly assessed before method validation, and the outcomes met the required criteria. The retention times for EMT, TAF, and DTG were 5.692, 10.01, and 10.758 minutes, respectively. The plate counts went over 2000, the peak tailing was under 2, and the percentage relative standard deviation (%RSD) of peak areas from six injections was ≤ 2% (refer to Table 4). These findings confirm that the method is appropriate for regular analysis.

3.3.3 Linearity and Range

The method demonstrated a linear relationship for EMT, TAF, and DTG across the concentration ranges of 200–1620 µg/mL, 25–200 µg/mL, and 50–400 µg/mL, Linearity was calculated by the R^2 values from linear regression plots of concentration versus peak area (refer to table 5 and figure-10). The regression lines, established using the least squares method, exhibited correlation coefficients (R^2) greater than 0.996, indicating linearity (see Table 5). Refer figure-9 for overlay chromatogram of linearity solution.

3.3.4 Precision

Precision was assessed by examining six replicate samples at the identical concentration within the same day and over two separate days. The %RSD for EMT, TAF, and DTG remained within the acceptable limit of ≤ 2%, demonstrating reproducibility across different days, analysts, and columns (Table 6).

3.3.5 Accuracy

The accuracy was confirmed through recovery studies; yielding percentage recoveries of 101.16% for EMT, 99.8% for TAF, and 99.9% for DTG at different concentration level. (Table 7). These results validate the accuracy of the proposed method.

3.3.6 Limit of Detection (LOD) and Limit of Quantification (LOQ)

Based on the calibration curves, the LOD and LOQ were determined using the slope and Y-intercept. The values were obtained for EMT, the LOD was 3.0µg/mL and the LOQ was 10.0µg/mL for TAF, the LOD was 0.9µg/mL and the LOQ was 1.25µg/mL for DTG, the LOD was 0.75µg/mL and the LOQ was 2.50µg/mL. For more details, refer to table-8.

3.3.7 Robustness

The robustness of the method was assessed by analyzing sample and standard solutions of EMT, TAF, and DTG under varied conditions. Upon comparison to the results obtained under optimized conditions, it was observed that there were no major changes in the %RSD, number of theoretical plates, retention time, or tailing factor (refer to table- 9).

3.3.8 Assay

The method developed was used to analyze the content of EMT, TAF, and DTG in pharmaceutical dosage forms. The percentage purities were 99.80% for EMT, 99.89% for TAF, and 100.35% for DTG.

3.3.9 Agree Analytical Greenness Calculator

The developed method achieved a total score of 0.8 (Figure 11). Additionally, the method's environmental friendliness was assessed using the analytical eco-scale and the green analytical process index, demonstrating strong alignment with green metrics principles.

List of Figures:

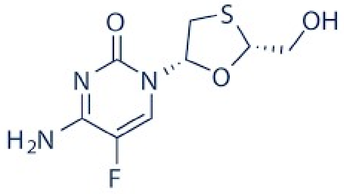	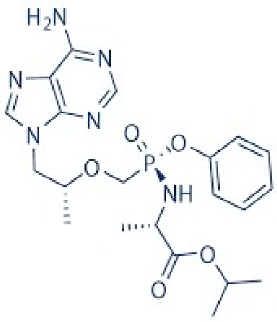	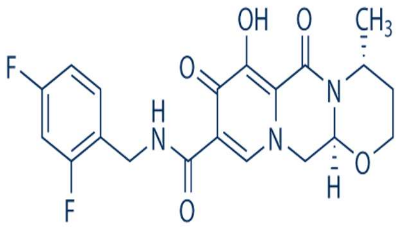
EMT	TAF	DTG

Figure 1: Chemical Structure of EMT, TAF, DTG

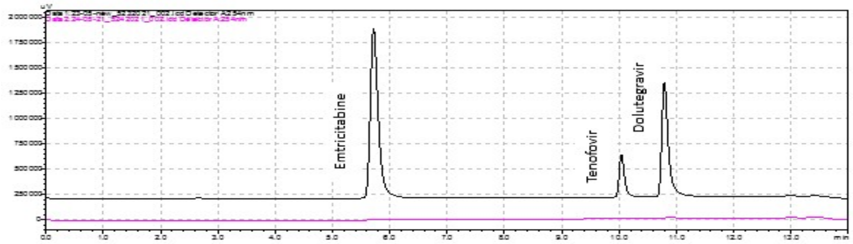

Figure 2: Optimized chromatogram of Standard with overlay with Blank

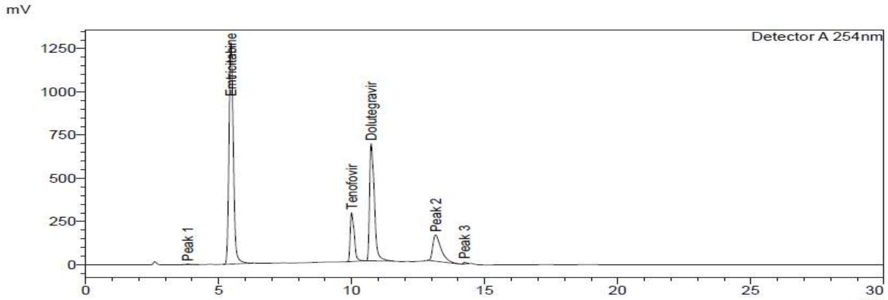

Figure 3: Optimized chromatogram of Acid hydrolysis.

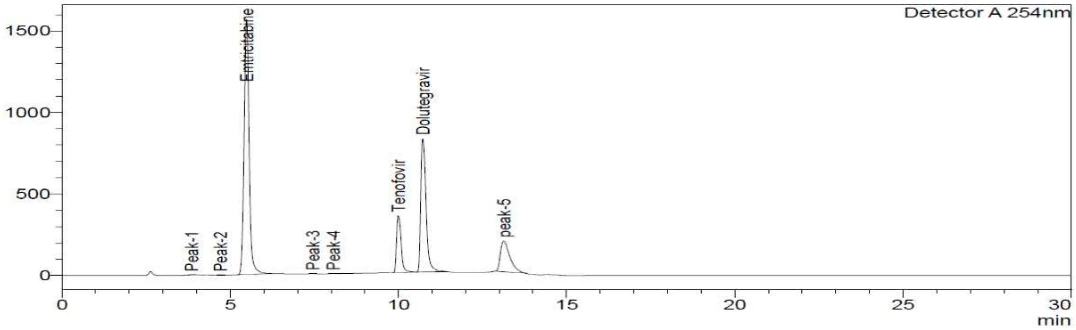

Figure 4: Optimized chromatogram of Alkali hydrolysis.

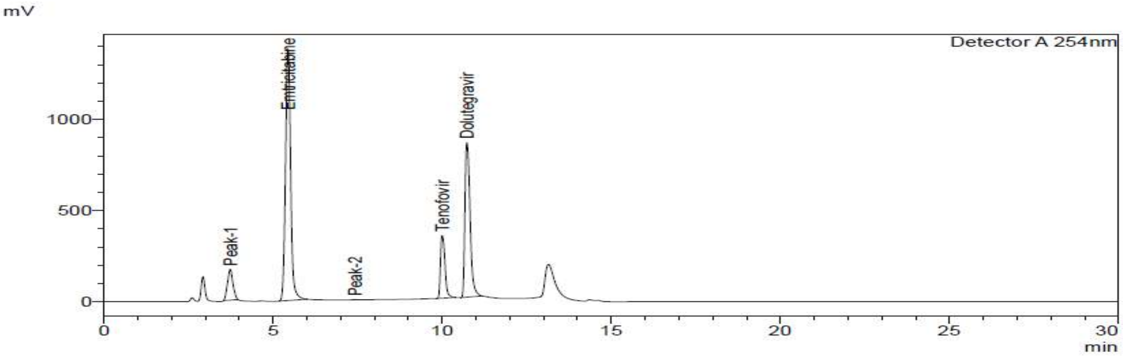

Figure 5: Optimized chromatogram of peroxide degradation

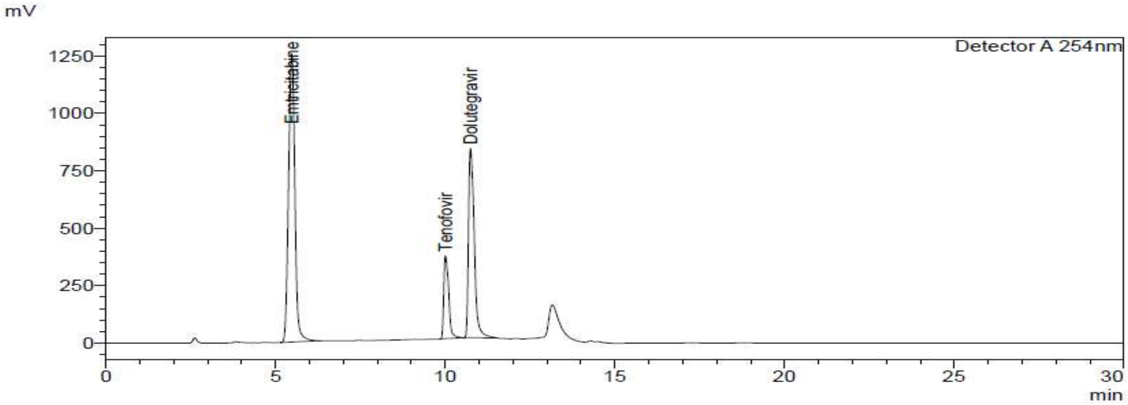

Figure 6: Optimized chromatogram of Thermal degradation

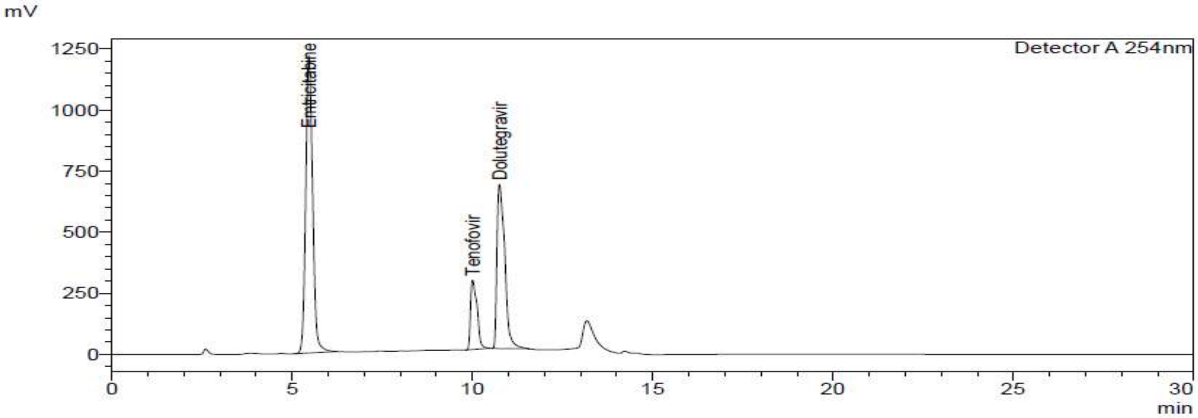

Figure 7: Optimized chromatogram of photolytic degradation

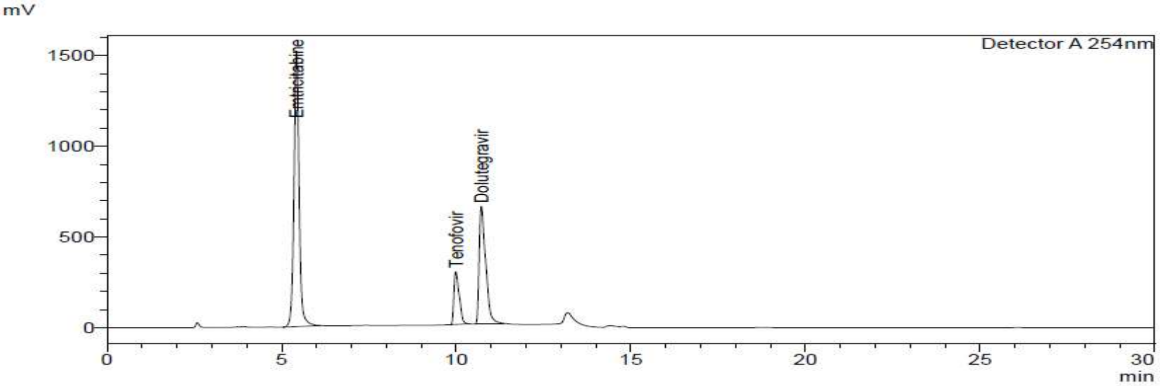

Figure 8: Optimized chromatogram of heat/ humidity degradation

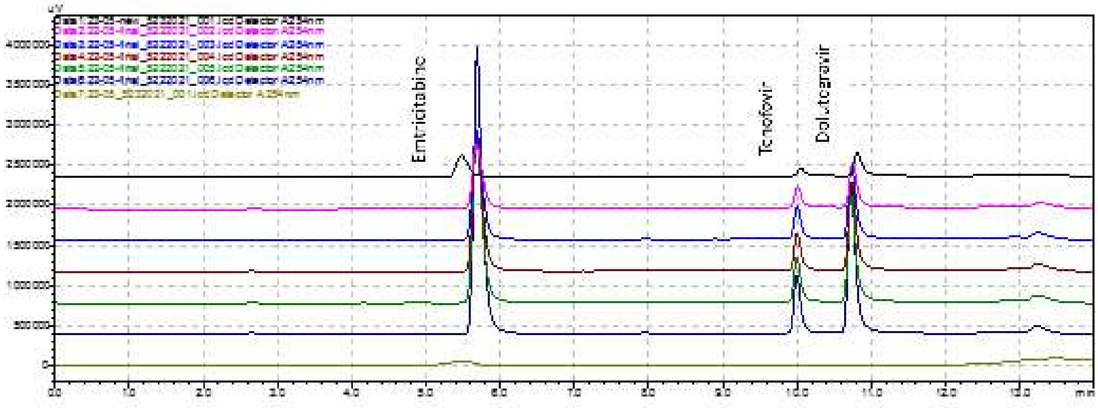

Figure 9: Overlay chromatogram of Linearity

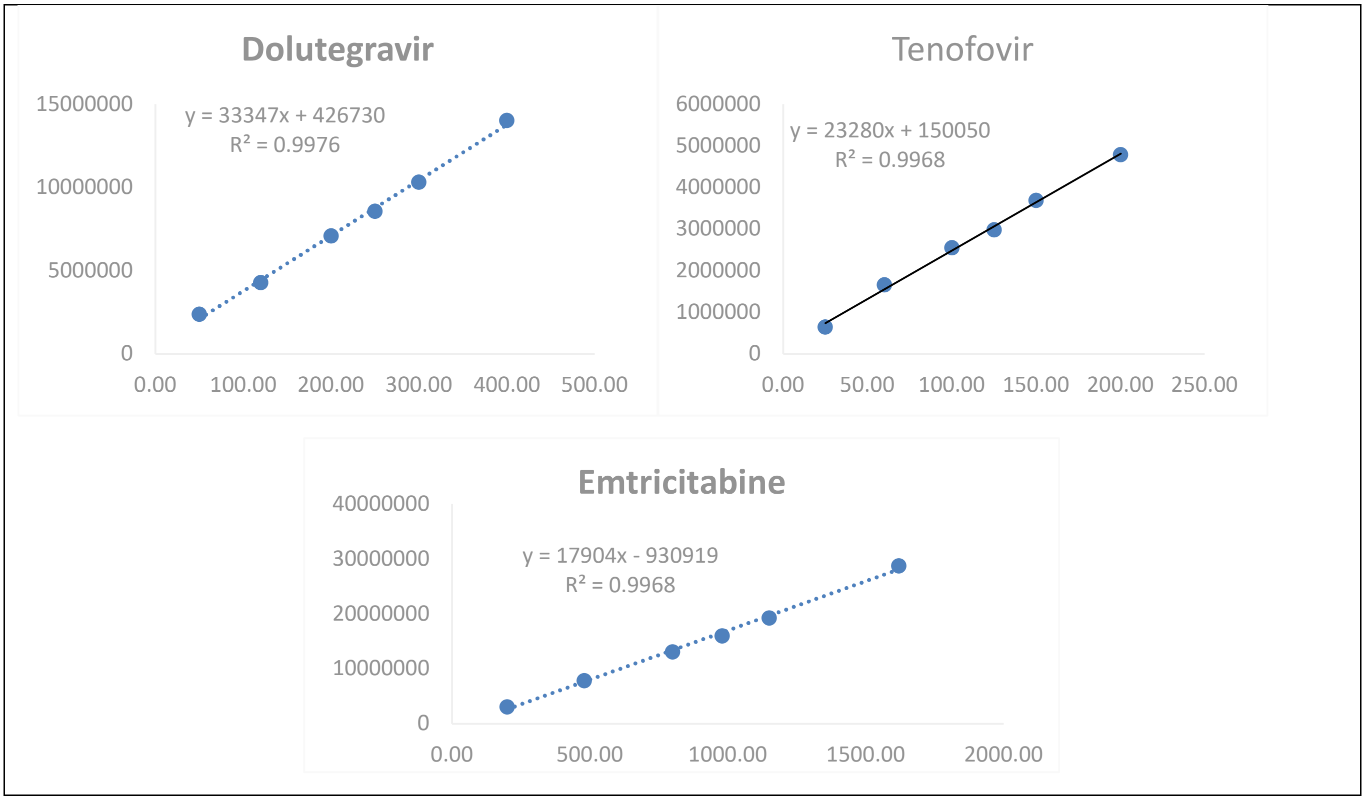

Figure 10: Calibration curve for Linearity results

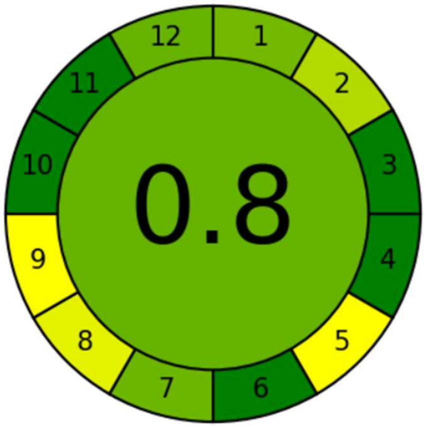

Figure 11: Green assessment results of the proposed HPLC method.

List of Tables:

Table 1: Optimized chromatographic condition.

Chromatographic conditions:			
Column	Shimadzu,C18 (4.6 mm X 100mm, 3.0 µm)		
Flow rate	1.0 mL/min		
Column Temp (°C)	30°C		
Injection volume	10 µL		
Detection wavelength	254 nm		
Diluent	Water : Acetonitrile (50:50% v/v)		
Run time	14 minutes		
Mobile phase A	20 mM potassium dihydrogen phosphate		
Mobile phase B	Methanol		
Gradient time programme	**Time (Minutes)**	**% Mobile phase A**	**% Mobile phase B**
	0	75	25
	6	20	80

	8	20	80
	9	10	90
	10	90	10
	12	75	25
	14	75	25

Table 2: Linearity solution preparation

<table>
<tr><th colspan="6">Linearity stock solution preparation</th></tr>
<tr><th>Name</th><th>Standard weight (mg)</th><th>Diluted upt0(mL)</th><th>potency of standard</th><th>Final concentration (mg/mL)</th><th>Final concentration (µg/mL)</th></tr>
<tr><td>TAF</td><td>41.5</td><td rowspan="3">50</td><td rowspan="3">100</td><td>0.50</td><td>500</td></tr>
<tr><td>DTG</td><td>50</td><td>1.00</td><td>1000</td></tr>
<tr><td>EMT</td><td>200</td><td>4.00</td><td>4000.0</td></tr>
<tr><th colspan="6">Linearity solutions preparation</th></tr>
<tr><td>Level</td><td>Linearity stock taken (mL)</td><td>Diluted upto (mL)</td><td>Name</td><td colspan="2">Final concentration (µg/mL)</td></tr>
<tr><td rowspan="3">Level-1 (20%)</td><td rowspan="3">0.5</td><td rowspan="3">10</td><td>TAF</td><td colspan="2">25.02</td></tr>
<tr><td>DTG</td><td colspan="2">50.00</td></tr>
<tr><td>EMT</td><td colspan="2">200.00</td></tr>
<tr><td rowspan="3">Level-2 (50%)</td><td rowspan="3">3</td><td rowspan="3">25</td><td>TAF</td><td colspan="2">60.04</td></tr>
<tr><td>DTG</td><td colspan="2">120.00</td></tr>
<tr><td>EMT</td><td colspan="2">480.00</td></tr>
<tr><td rowspan="3">Level-3 (80%)</td><td rowspan="3">2</td><td rowspan="3">10</td><td>TAF</td><td colspan="2">100.06</td></tr>
<tr><td>DTG</td><td colspan="2">200.00</td></tr>
<tr><td>EMT</td><td colspan="2">800.00</td></tr>
</table>

Level-4 (100%)	5	20	TAF	125.08
			DTG	250.00
			EMT	1000.00
Level-5 (120%)	3	10	TAF	150.10
			DTG	300.00
			EMT	1200.00
Level-6 (150%)	4	10	TAF	200.13
			DTG	400.00
			EMT	1600.00

Table 3: Force degradation results

Degradation sample	Stress condition	EMT		DTG		TAF	
		% Assay	% Degradation	% Assay	% Degradation	% Assay	% Degradation
As Such	NA	100.4	NA	100.9	NA	101.4	NA
Acid	5mL-0.02 N HCL RT- 30 minutes Neutralize with 0.02 N NaOH	89.6	10.8	81.7	19.2	87.2	14.2
Alkali	2mL-0.02 N NaOH RT- 10 minutes Neutralize with 0.02 N HCl	100.4	NA	85.0	15.9	90.6	10.8
Peroxide	2mL-3%H2O2 RT- 30 minutes	91.0	9.4	89.8	11.1	95.5	5.9

Photolytic	U.V Rays for 7 Days	100.9	NA	100.5	NA	99.7	NA
Thermal	80°C for 7 days	101.4	NA	101.7	NA	103.4	NA
Heat / Humidity	40°C/75% RH For7 Days	101.2	NA	NA	99.4	101.0	NA

Table 4 Results of system suitability parameters of 100% level standard solution

Standard Injections	**EMT**				**TAF**				**DTG**			
Injection	RT	Area	T.F	NTP	RT	Area	T.F	NTP	RT	Area	T.F	NTP
1	5.683	16020746	1.4	52854	10.000	3044899	1.5	335714	10.733	8565099	1.6	295862
2	5.692	16096576	1.5	54413	10.025	3085692	1.5	328975	10.775	8542022	1.5	295837
3	5.692	15927000	1.5	54342	10.017	3087191	1.4	332963	10.758	8545451	1.6	295500
4	5.692	15934770	1.4	54255	10.025	3042582	1.3	336199	10.767	8535594	1.5	295668
5	5.700	15900575	1.5	54314	10.017	3064415	1.5	334516	10.767	8542880	1.5	294132
Mean		15975933				3064956				8546209		
%RSD		0.5				0.7				0.1		

Table 5 Peak area of linearity standard solution of EMT, TAF, DTG

Level	EMT		TAF		DTG	
	Concentration (µg/mL)	Area	Concentration (µg/mL)	Area	Concentration (µg/mL)	Area
Level-1	200.00	3072413	25.02	638083	50.00	2357443
Level-2	480.00	7860070	60.04	1652888	120.00	4265298
Level-3	800.00	13072962	100.06	2545213	200.00	7078618

Precision	**Sample**	**EMT % Assay**	**TAF % Assay**	**DTG % Assay**
Method Precision	Set-1	98.8	101.4	102.1
	Set-2	101.3	99.3	102.3
	Set-3	101.7	99.2	100.9
	Set-4	100.6	100.4	99.9
	Set-5	100.0	100.8	99.7
	Set-6	98.5	100.6	100.0
	% Mean	100.2	100.3	100.8
	% RSD	1.3	0.9	1.1
Interday Precision	Set-1	98.8	101.4	102.1
	Set-2	101.3	99.3	102.3
	Set-3	101.7	99.2	100.9
	% Mean	100.6	100.0	101.8
	% RSD	1.6	1.2	0.8
Intraday Precision	Set-1	100.9	98.4	99.5
	Set-2	101.8	99.6	99.0
	Set-3	100.8	101.4	101.2
	% Mean	101.2	99.8	99.9
	% RSD	0.5	1.5	1.2

Level-4	980.00	16020746	125.08	2973972	250.00	8565099
Level-5	1150.00	19272568	150.10	3681120	300.00	10298624
Level-6	1620.00	28755650	200.13	4783964	400.00	14013645

Table 6: Precision studies of EMT, TAF, and DTG

Table 7: Recovery studies of EMT, TAF, and DTG

	EMT			TAF			DTG		
Level	**Amount added (µg/mL)**	**Amount Recovered (µg/mL)**	**% Recovery**	**Amount added (µg/mL)**	**Amount Recovered (µg/mL)**	**% Recovery**	**Amount added (µg/mL)**	**Amount Recovered (µg/mL)**	**% Recovery**
50%	500.0	504.6	100.9	62.5	61.3	98.1	125.0	124.7	99.8
	500.0	504.6	100.9	62.5	61.6	98.6	125.0	122.8	98.3
	500.0	504.8	101.0	62.5	61.6	98.6	125.0	125.3	100.3
	Mean		100.9	**Mean**		98.4	**Mean**		99.5
	%RSD		0.0	**% RSD**		0.3	**% RSD**		1.1
100 %	1000.0	1017.9	101.8	125.0	124.6	99.8	250.0	247.6	99.1
	1000.0	1009.6	101.0	125.0	124.0	99.2	250.0	247.6	99.0
	1000.0	1027.1	102.7	125.0	124.7	99.8	250.0	247.5	99.0
	Mean		101.8	**Mean**		99.6	**Mean**		99.0
	%RSD		0.9	**%RSD**		0.3	**%RSD**		0.0
150 %	1500.0	1526.8	101.8	187.5	190.0	101.4	375.0	376.7	100.5
	1500.0	1499.9	100.0	187.5	191.2	102.0	375.0	381.9	101.9
	1500.0	1509.8	100.7	187.5	188.9	100.8	375.0	380.1	101.4
	Mean		100.8	**Mean**		101.4	**Mean**		101.2
	%RSD		0.9	**%RSD**		0.6	**%RSD**		0.7

Table 8: LOD and LOQ results of EMT, TAF, and DTG

	EMT	**TAF**	**DTG**
LOD (µg/mL)	3.0	0.9	0.75
LOQ (µg/mL)	10	1.25	2.50

Table 9: Robustness studies of EMT, TAF, and DTG

Method parameter	**Conditions**	**% Assay**			**% RSD**		
		EMT	**TAF**	**DOL**	**EMT**	**TAF**	**DOL**
Flow rate ± 0.2 mL/min	0.8 mL/min	99.1	100.8	100.1	0.5	1.0	0.5
	1mL/min	98.6	100.4	102.0	0.5	0.7	0.1

	1.2 mL/min	98.8	99.2	100.4	0.9	1.1	0.5
Wave length ± 2 nm	252	100.4	99.8	101.3	0.3	0.4	0.3
	254	99.1	101.4	102.1	0.5	0.7	0.1
	256	100.6	102.2	100.6	0.5	0.7	0.3
Column oven temperature ±5˚	30˚C	99.2	100.3	100.4	0.8	0.7	0.9
	25˚C	100.8	101.4	102.1	0.5	0.7	0.1
	35˚C	100.4	101.7	100.2	0.5	1.2	0.7

List of abbreviations:

EMT: Emtricitabine

TFA: Tenofovir Alafenamide

DTG: Dolutegravir Sodium

RT: Retention time

LOD: Limit of detection

LOQ: Limit of quantification

mM : Mili molar

%v/v: Percentage volume by volume

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