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Development and Validation of a Reverse Phase High-Performance Liquid Chromatography (RP-HPLC) Dissolution Method for the Simultaneous Quantification of Emtricitabine and Tenofovir Alafenamide in Tablet Formulations

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Reversed Phase-High Performance Liquid Chromatography (RP-HPLC) is an isocratic method that is simple, specific, accurate, fast, and inexpensive. It was created and approved for the quantitative measurement of tenofovir alafenamide and emtricitabine in pharmaceutical tablet dosage forms. An in vitro dissolving study of tablets containing the aforementioned medications was conducted using the HPLC method. Phenomenex C18 (250 mm X 4.6 mm, 5 μm) was used in the development of the RP-HPLC technique. Methanol and water made up the mobile phase (65:35% v/v). The UV-Vis detector was used to monitor the responses at 216 nm while a flow rate of 1.0 mL/min was used. With a USP Apparatus II at 37°C and 500 mL of 50 mM Sodium Citrate buffer (pH 5.50) as the dissolution medium, the dissolution test was carried out at 75 rpm. It was discovered that the retention periods for EMT and TNF were 3.13 and 4.82 minutes, respectively. EMT (correlation coefficient 0.99998) in the 10–150 μg/mL range and TNF (correlation coefficient 0.99994) in the 1.25–18.75 μg/ml range were found to be linear. Limit of detection, limit of quantitation, precision, accuracy, robustness, specificity, and linearity were all validated. The proposed method is suitable for routine quality control.

Keywords: Dissolution; Emtricitabine (EMT); Isocratic; Method Validation; RP-HPLC; Tenofovir Alafenamide (TNF).

Emtricitabine (EMT) is a nucleoside reverse transcriptase inhibitor utilized in the course of treatment and prevention of HIV-1 and it works by inhibiting viral DNA synthesis, leading to chain termination. Commonly combined with other antiretrovirals, it is known for its effectiveness and favourable safety profile[1]. In terms of chemistry, it's known as 4-amino5-fluoro-1-[(2R,5S)-2-(hydroxymethyl)-1,3-oxathiolan-5-yl]Pyrimidine-2-one[2].

Tenofovir alafenamide (TNF) is a nucleotide reverse transcriptase inhibitor used in the HIV-1 treatment and chronic hepatitis B. It is a prodrug of tenofovir, offering improved Safety profiles for bones and kidneys in comparison to tenofovir disoproxil fumarate[3]. TAF is often combined with other antiretrovirals for enhanced therapeutic efficacy [4]. Chemically, it is described as propan-2-yl (2S)-2-[[[[2R)-1-(6-aminopurin-9-

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yl)propan-2-yl]oxymethyl-phenoxyphosphoryl] amino] propanoate[5].

Additionally, understanding the safety profiles of these agents is crucial for clinical decision-making[6,7]. Research continues to evolve regarding the long-term impacts of NRTIs on patients' health [8]. As more data becomes available, clinicians can better tailor therapies to individual patient needs [9, 10]. Recent advancements in HPLC methodologies also support the need for consistent and reliable drug analysis[11,12].

A few analytical techniques for measuring TNF and EMT alone and together have been published[13]. However, there is currently no published method for estimating TNF and EMT in combination dosage forms for in vitro dissolution[14]. Thus, the development of a robust dissolution method is critical to ensure bioavailability and therapeutic effectiveness. A dissolution method utilizing a high performance liquid chromatography (HPLC) technique has been proposed, which allows for the simultaneous quantification of both TNF and EMT[15]. This method can be optimized for various parameters such as pH, temperature, and agitation speed to mimic physiological conditions[16].

The present study reports on the invention and validation of a novel RP-HPLC analytical method that is easy to use, quick to perform, and repeatable for the measurement of TNF and EMT in bulk and pharmaceutical tablet formulations. In compliance with ICH guidelines, the established method underwent validation for robustness, accuracy, precision, linearity, specificity, LOD, and LOQ. [17]. Further studies will also focus on the pharmacokinetics and interactions of these drugs in combination therapies[18, 19, 20].

MATERIALS AND METHODS

Reagents and Chemicals

Cipla Ltd. (Mumbai, India) graciously provided the reference samples of the emtricitabine and tenofovir alafenamide standards as gift samples. Analytical grade chemicals were all used. The marketed pharmaceutical tablets of TAFERO-EM, purchased from the pharmacy, have a batch number of HH2303088 and an expiry date of February 2025. The tablet contains 200 mg of EMT and 25 mg of TNF, which are both produced by Hetero Healthcare Ltd. Thermo Fisher Scientific's Qualigens supplied methanol, Hydrochloric acid (HCl), and acetonitrile of HPLC grade, while Moreshwar Enterprises supplied HPLC-grade water.

Instrumentation

Chromatographic separation was carried out using the HPLC-Agilent (Model-1260 Infinity II) system, which has a UV detector, an integrated auto sampler, and a column oven. The Open lab Ezchrom workstation software was used. The column utilized was Phenomenex ODS-3, 250 mm x 4.6 mm, 5 ìm. A Bio Technics India ultra sonicator was utilized to enhance the drug's solubility. The solution's pH was adjusted using a Labman pH meter. The samples were weighed with an Aczet balance.

Method development

EMT standard stock solution preparation

20 milligrams of emtricitabine should be precisely weighed before being transferred to a 25 mL volumetric flask. Add 20 mL of water, sonicate until entirely dissolved, and then add water to bring the volume up to the desired level. (Emtricitabine 800 PPM)

TNF standard stock solution preparation

Transfer 28 mg of tenofovir alafenamide fumarate, which is equal to 12.5 mg of tenofovir alafenamide, to a 50 mL volumetric flask after precisely weighing it. Add 30 milliliters of water, sonicate until thoroughly dissolved, and then top off the volume with water (250 parts per million of tenofovir alafenamide).

Preparation Standard

Transfer 2.5 mL of the Emtricitabine stock solution and 1 mL of the Tenofovir alafenamide stock solution via pipette into a 20 mL volumetric flask, adding mobile phase to bring the volume up to the desired level. (Emtricitabine 100 PPM and Tenofovir alafenamide 12.5 PPM)

Preparation of dissolution media

Take 5 litre beaker and add 1000 mL of distilled water. Weighed and transferred about 73.5 gm of sodium citrate (50 mM Sodium Citrate buffer, pH 5.5 as dissolution media.) in beaker and dissolved in same 1000 mL of distilled water by means of sonication. Mix with distilled water to dilute to 5000 mL. Adjust the pH to 5.50±0.05 by using HCl solution. This produced a 50 mM Sodium Citrate buffer, pH 5.5, for use as dissolution media. (FDA, Guidance for Industry: Dissolution

Table 1. Optimized Chromatographic Conditions

Parameter	Description
Mode	Isocratic
Column Name	Phenomenex C18, 250 mm X 4.6mm ID, 5 ìm
Detector	UV Detector
Injection Volume	20 µl
Wavelength	216 nm
Column Oven temp	38ºC
Mobile Phase	Methanol : Water (65:35%V/V)
Flow Rate	1.00 ml/min
Run time	09 Minutes

Table 2. System suitability test

Criteria	EMT	TNF
Retention time	3.13	4.82
Number of Theoretical Plate	8822	14606
Tailing factor	1.20	1.17
Resolution	NA	11.84

Testing of Emtricitabine/Tenofovir Alafenamide Fumarate Tablets, July 28, 2016.).

Tablet Sample preparation for Dissolution

Add about 500 mL of dissolution media in each dissolution bowl and set bath temperature at 37°C. Attach the paddle apparatus and set the RPM at 75. When dissolution media temperatures

Table 3. Filter study results

Sample	Area		% Absolute difference	
	EMT	TNF	EMT	TNF
Unfiltered	81325964	9364527	NA	NA
0.45 µ PVDF filter	80908628	9342631	0.51	0.23
0.45 µ Nylon filter	80596873	9302567	0.90	0.66

Table 4. Solution Stability results of Emtricitabine

Time point	Solution of EMT Sample		Solution of EMT Standard	
	Area	Absolute difference (%)	Area	Absolute difference (%)
Initial	82543109	NA	88520163	NA
12-Hours	82359637	0.22	88225639	0.33
24-Hours	81593976	1.15	87259341	1.42

Table 5. Solution Stability results of Tenofovir alafenamide

Time point	Solution of TNF Sample		Solution of TNF Standard	
	Area	Absolute difference (%)	Area	Absolute difference (%)
Initial	9450251	NA	10024751	NA
12-Hours	9412418	0.40	9976529	0.48
24-Hours	9366524	0.89	9919856	1.05

reached to 37°C, Place one tablet in each dissolving bowl, and use the dissolution device in accordance with the guidelines. Withdraw 10 mL of aliquot at each time point (5, 10, 15, 20, 30 and 45 Minutes) and add 10 mL of dissolution media. 3 mL of the filtrate should be discarded after passing the aliquot through an appropriate 0.45 µ syringe filter. Using dissolution media, further dilute 2.5 ml of filtrate to 10 ml. (100 PPM of Emtricitabine and 12.5 PPM of Tenofovir alafenamide)

Placebo Preparation

Take 800 mg of Lactose (filler), 50 mg of Starch (binder), 50 mg of Magnesium Stearate (lubricant), 50 mg of Talc (glidant), and 50 mg of Crospovidone (disintegrant) to prepare 10 grams of placebo. Accurately weigh each ingredient and mix them thoroughly to ensure a uniform blend. The resulting placebo can then be used for comparison and analysis of the unknown excipients in the test sample.

RESULT AND DISCUSSION

Validation

System Suitability

To verify the system's reproducibility, re-injections of drug solution at 100 µg/ml EMT and 12.5 µg/ml TNF concentrations were used

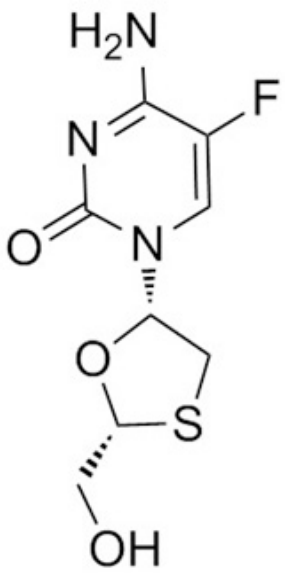

Fig. 1. Structure of Emtricitabine

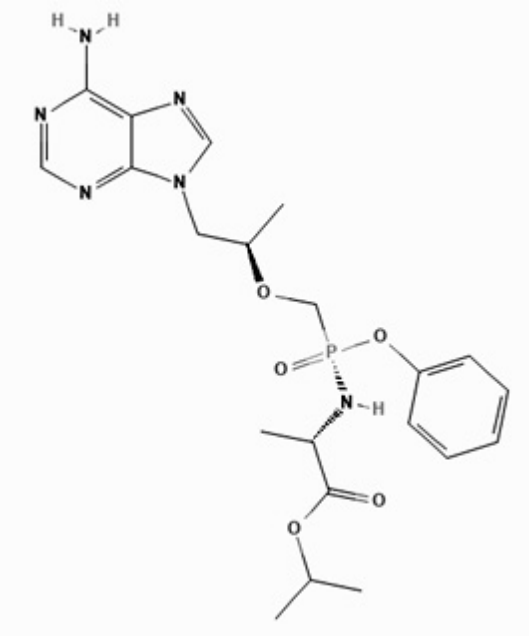

Fig. 2. Structure of Tenofovir alafenamide

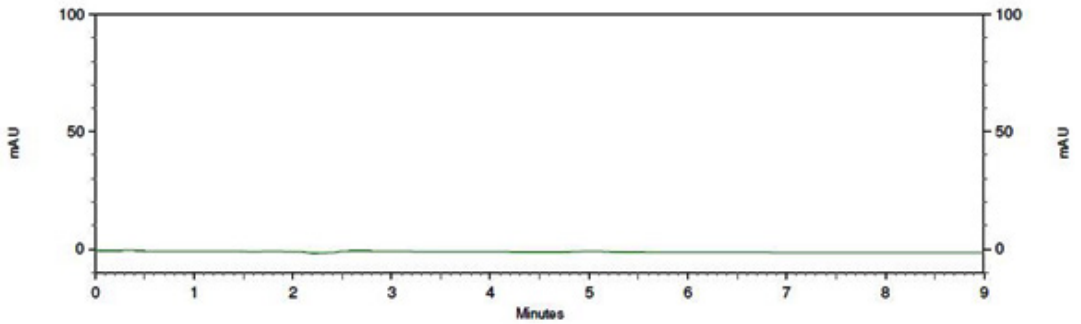

Fig. 3. Blank Chromatogram

to check the system suitability test parameters. Parameters of system suitability such as tailing factor, retention time, and theoretical plate count (N), the resolution of repetitive injections were studied. The results shown in Table 1.

Filter Study

Dissolution study performed on one bowl only for 45 minutes. 500 mL of dissolution media added and set bath temperature at 37°C. The paddle apparatus attached with RPM at 75. When dissolution media temperature reached to 37°C, one tablet transferred in dissolution bowl and subject the dissolution for 45 minutes. After 45 minutes, 3 aliquots were processed as follows:

1) A 10 mL aliquot was taken out and centrifuged for five minutes at 5000 RPM. 2.5 mL of supernatant was further diluted with dissolving media to reach 10 mL.

2) After discarding the first 3 mL of filtrate, 10 mL of the aliquot was removed and filtered using a 0.45 µ PVDF syringe filter. 2.5 mL of the filtrate was further diluted with dissolving media to 10 mL.

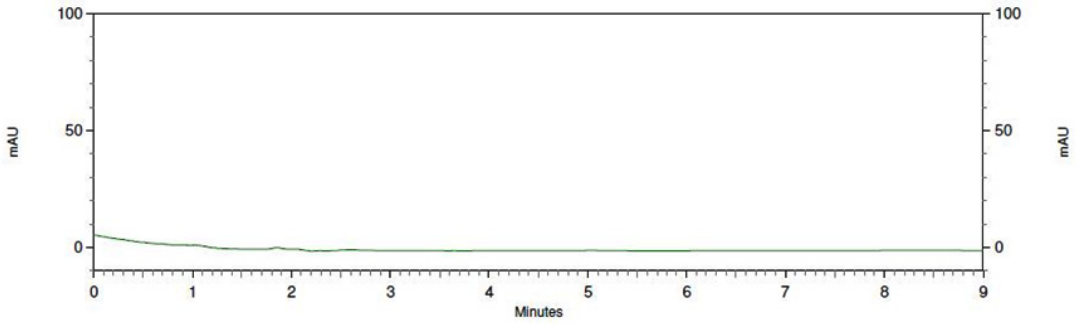

Fig. 4. Placebo Chromatogram

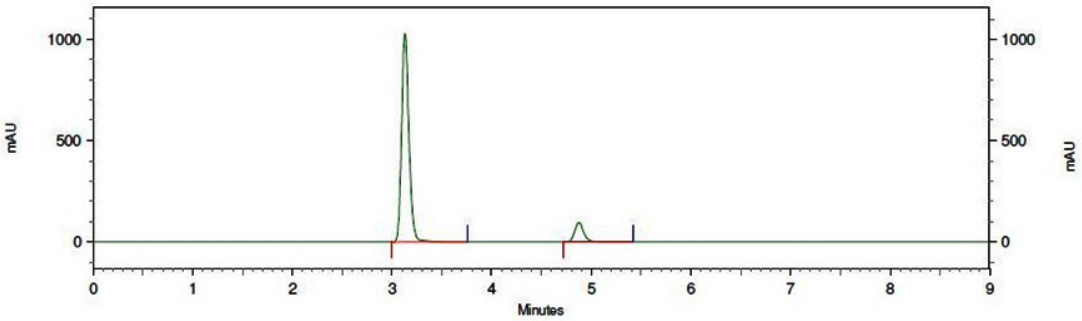

Fig. 5. Standard Chromatogram

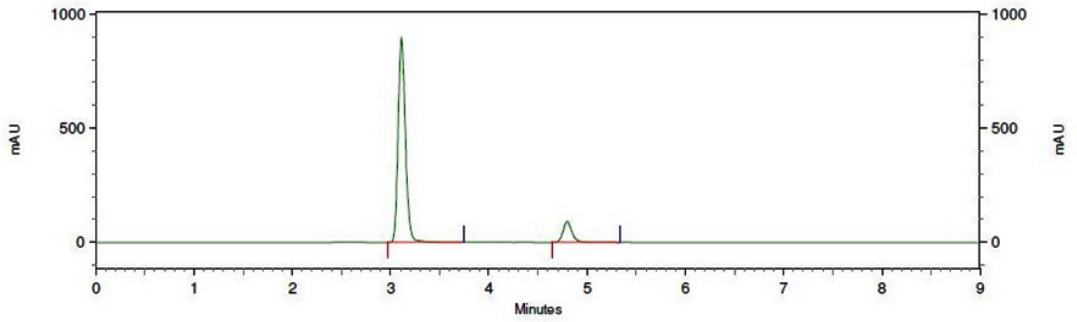

Fig. 6. Sample Chromatogram

3) After discarding the first 3 mL of filtrate, 10 mL of the aliquot was taken out and filtered using a 0.45 µ nylon syringe filter. 2.5 mL of the filtrate was further diluted with dissolving media to 10 mL.

Both PVDF and nylon filters meet the requirements for the filter research, thus they can be employed. We used PVDF filter as it shows less difference as compare to Nylon filter.

Stability of the Solution

At the beginning, twelve, and twenty-four hours, standard and sample solutions were injected. Calculated as a percentage absolute difference from the initial area. Dissolution study performed on one bowl only for 45 minutes. Sample prepared by using 500 mL of dissolution media at 37°C. The paddle apparatus attached with RPM at 75. When dissolution media temperature reached to 37°C, one tablet transferred in dissolution bowl and subject the dissolution for 45 minutes. After forty-five minutes, a 10-milliliter aliquot was taken out and filtered through an appropriate 0.45-inch syringe filter, discarding the first three milliliters of filtrate. Dissolving media were used to further dilute 2.5 mL of filtrate to 10 mL.

Specificity

To verify for peak purity, the following solutions were injected: blank, placebo, standard solution, and tablet sample solution at 45-minute intervals. At R.T. of Tenofovir, blank and placebo do not interfere. Peak purity was within acceptable limits for both the standard and the sample. The sample solution's R.T. is identical to the standard solution's. Consequently, the developed

Table 6. Linearity of EMT and TNF

% level	Emtricitabine		Tenofovir Alafenamide	
	Concentrations (µg/ml)	Area of Peak	Concentrations (µg/ml)	Area of Peak
10%	10.00	8813587	1.25	1033969
50%	50.00	44443206	6.25	5066403
100%	100.00	88761076	12.50	10064681
125%	125.00	110880382	15.63	12490352
150%	150.00	132194485	18.75	15162975

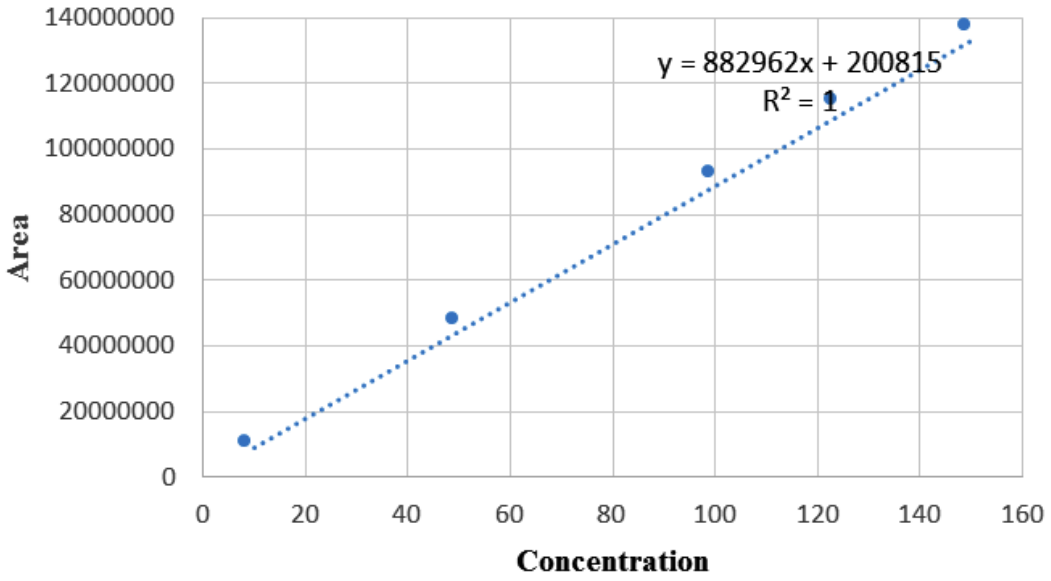

Fig. 7. Linearity plot of Emtricitabine

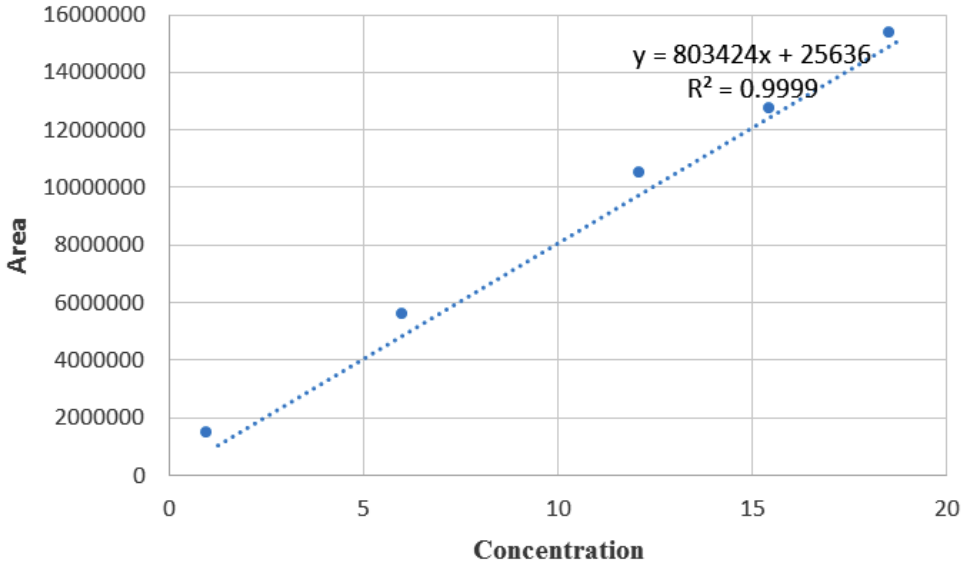

Fig. 8. Linearity plot of Tenofovir Alafenamide

Table 7. Recovery Sample Preparation

Level (%)	EMTAPI (mg)	TNF API (mg)	Wt of Placebo (mg)	Diluted to (mL)	Vol. taken	Diluted to (mL)	EMT Added Conc. (µg/mL)	TNF Added Conc. (µg/mL)
50	100.8	28.2	99.3	200	1.6	20	40.32	5.03
	100.2	28.4	99.5	200	1.6	20	40.08	5.07
	99.8	28.3	99.6	200	1.6	20	39.92	5.05
100	201.2	56.2	99.7	200	1.6	20	80.48	10.03
	200.5	56.3	99.2	200	1.6	20	80.20	10.04
	200.7	56.2	99.6	200	1.6	20	80.28	10.03
150	300.2	84.2	99.8	200	1.6	20	120.08	15.02
	300.4	84.1	98.9	200	1.6	20	120.16	15.00
	300.8	84.5	99.6	200	1.6	20	120.32	15.07

Table 8. Results of emtricitabine accuracy study

Level (%)	Area	EMT Recovered Conc. (µg/mL)	EMT Added Conc. (µg/mL)	% Recovery	Mean % Recovery	% RSD	Overall Recovery	% RSD for over all recovery
50	34852145	40.73	40.32	101.01	100.14	1.166	100.02	0.819
	34501297	40.32	40.08	100.59				
	33754185	39.44	39.92	98.81				
100	68452741	79.99	80.48	99.39	100.32	0.862		
	68953524	80.58	80.20	100.47				
	69452873	81.16	80.28	101.10				
150	102352610	119.60	120.08	99.60	99.61	0.407		
	102853065	120.19	120.16	100.02				
	102150445	119.37	120.32	99.21				

chromatographic and dissolving method satisfied the specificity requirements.

Linearity

Five levels, ranging from 10% to 150% of working concentration, were prepared. Three injections of each level. Conc. vs. Mean Area displayed on a linearity graph. Table 5 presented the findings.

Accuracy

Three levels of recovery were carried out. Prepared at 50%, 100%, and 150% levels. Three copies of each level were prepared. At all three levels, the percentage recovery was found to be well within the acceptable range.

Recovery sample preparation

500 mL of dissolution media added in each dissolution bowl and set bath temperature at 37°C. The paddle apparatus attached and the RPM was set to 75. When dissolution media temperature reached to 37°C, about 99.3 mg of placebo weighed and transferred in each dissolution bowl. Subject the dissolution for 45 minutes. After 45 minutes (last time point), 10 mL of aliquot has

Table 9. Results of tenofovir alafenamide accuracy study

Level (%)	Area	TNF Recovered Conc. (µg/mL)	TNF Added Conc. (µg/mL)	% Recovery	Mean % Recovery	% RSD	Overall Recovery	% RSD for over all recovery
50	3925241	5.00	5.03	99.37	100.56	1.029	100.14	0.977
	4026524	5.13	5.07	101.21				
	4008361	5.10	5.05	101.11				
100	7812149	9.95	10.03	99.23	100.17	0.965		
	7895214	10.05	10.04	100.11				
	7963528	10.14	10.03	101.16				
150	11895241	15.15	15.02	100.85	99.70	1.129		
	11615362	14.79	15.00	98.60				
	11795611	15.02	15.07	99.65				

Table 10. Results of Repeatability of Emtricitabine

Time Point (Minutes)	5	10	15	20	30	45
Tablet 1	41.40	67.53	83.95	91.28	92.83	92.06
Tablet 2	38.20	64.46	89.59	91.59	91.43	90.93
Tablet 3	41.10	67.62	83.05	92.36	92.15	91.74
Tablet 4	41.60	69.53	85.29	93.91	93.68	93.17
Tablet 5	42.40	69.45	84.89	94.00	93.58	93.27
Tablet 6	38.80	67.58	86.45	92.93	92.26	91.65
% Average Release	40.58	67.70	85.54	92.68	92.66	92.14

Table 11. Results of Repeatability of Tenofovir Alafenamide

Time Point (Minutes)	5	10	15	20	30	45
Tablet 1	40.70	60.41	75.91	97.42	97.45	97.25
Tablet 2	43.90	54.68	76.09	94.22	95.68	95.41
Tablet 3	39.00	57.78	74.66	97.49	97.35	97.05
Tablet 4	38.30	60.97	82.22	96.24	96.02	95.82
Tablet 5	41.50	62.33	83.25	95.97	95.92	95.72
Tablet 6	43.10	65.76	78.22	95.56	95.41	95.31
% Average Release	41.08	60.32	78.39	96.15	96.31	96.09

been withdrawn from each bowl and 3 mL of the filtrate was discarded after the solution was filtered using an appropriate 0.45 µ syringe filter. Using dissolution media, further dilute 2.5 ml of filtrate to 10 ml.

Precision

Repeatability

Repeatability Precision performed by preparing 6 test sample (Tablet solution samples)

Sample preparation

About 500 mL of dissolution media added in each dissolution bowl and set bath temperature at 37°C. Attach the paddle apparatus and the RPM was set to 75. When dissolution media temperature reached to 37°C, one tablet transferred in each dissolution bowl and subject the dissolution apparatus as per parameters. 10 mL of aliquot has been withdrawn at each time point and 10 mL

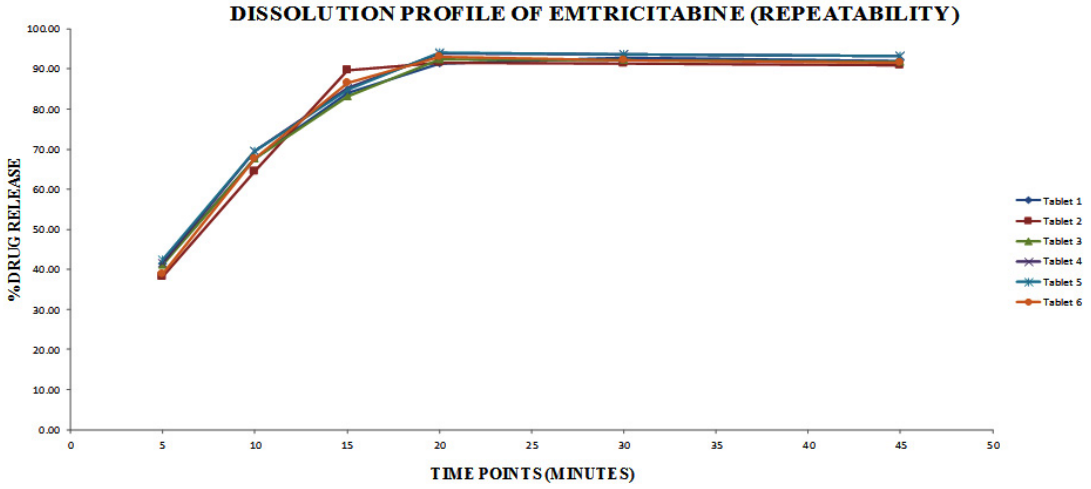

Fig. 9. Dissolution Profile of Emtricitabine (Repeatability)

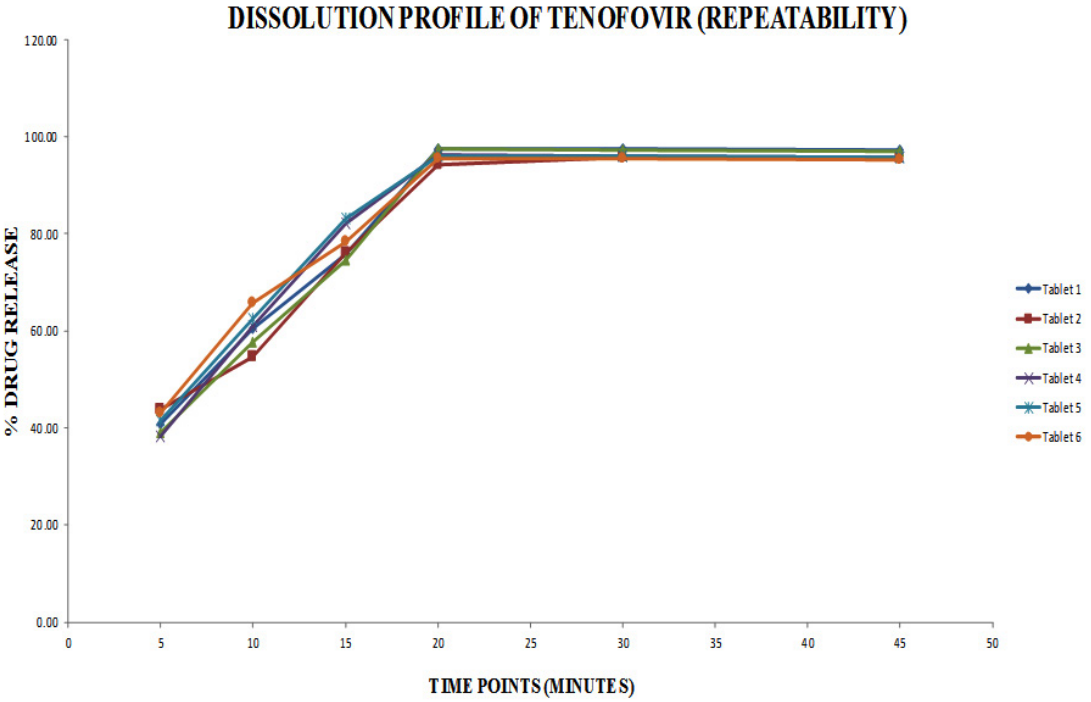

Fig. 10. Dissolution Profile of Tenofovir Alafenamide (Repeatability)

of dissolution media added. Three milliliters of filtrate were discarded after the aliquot was passed through an appropriate 0.45 µ syringe filter. Used dissolve media to further dilute 2.5 ml of filtrate to 10 ml. (100 PPM of Emtricitabine and 12.5 PPM of Tenofovir alafenamide).

Intermediate Precision

Six test samples were prepared on different days by different analysts to achieve intermediate precision. It is also called as Ruggedness. Intermediate Precision passes the criteria, no variation found.

Table 12. Results of Intermediate Precision of Emtricitabine

Time Point (Minutes)	5	10	15	20	30	45
Tablet 1	37.70	65.95	86.42	93.83	93.88	93.78
Tablet 2	38.80	66.48	82.33	90.85	91.12	89.72
Tablet 3	39.70	66.99	84.34	90.09	92.20	92.04
Tablet 4	40.80	70.02	84.10	93.88	93.18	92.36
Tablet 5	41.80	72.94	85.36	92.01	91.64	91.43
Tablet 6	43.30	70.77	84.42	91.09	91.52	91.33
% Average Release	40.35	68.86	84.50	91.96	92.26	91.78

Table 13. Results of Intermediate Precision of Tenofovir Alafenamide

Time Point (Minutes)	5	10	15	20	30	45
Tablet 1	38.00	58.96	72.28	93.25	94.77	94.40
Tablet 2	40.20	59.40	72.89	94.26	96.99	96.84
Tablet 3	42.30	62.75	74.66	95.39	96.81	96.34
Tablet 4	37.00	63.54	73.17	93.96	96.58	96.23
Tablet 5	36.30	57.93	72.26	94.35	93.69	93.67
Tablet 6	41.00	60.72	76.21	94.62	96.59	96.53
% Average Release	39.13	60.55	73.58	94.31	95.91	95.67

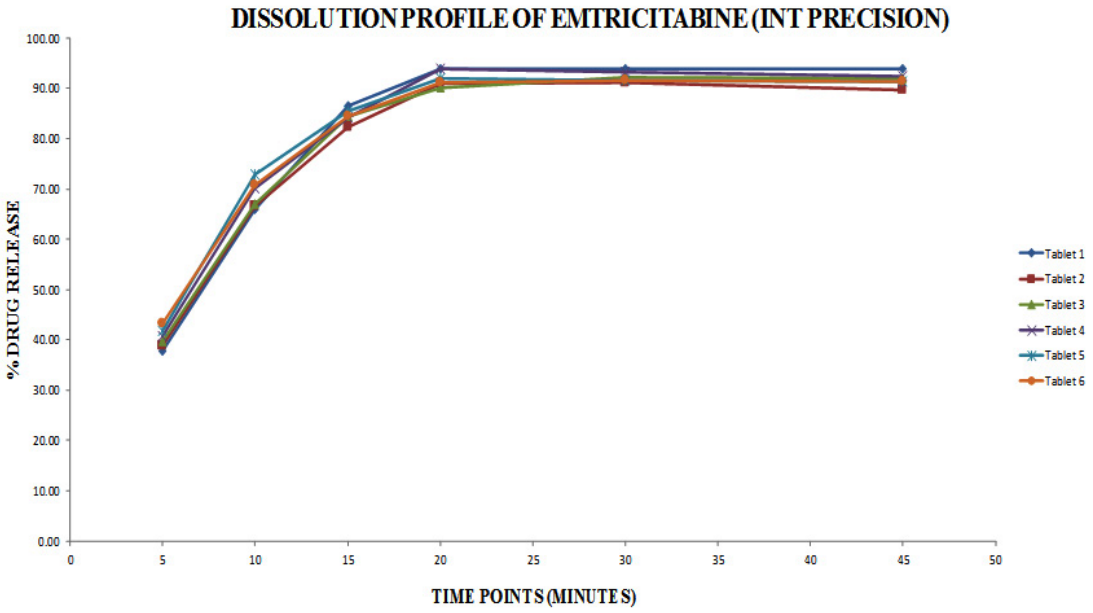

Fig. 11. Dissolution Profile of Emtricitabine (Intermediate precision)

Sample preparation

500 mL of dissolution media was added in each dissolution bowl and bath temperature was set at 37°C. The paddle apparatus attached and the RPM was set at 75. When dissolution media temperature reached to 37°C, one tablet transferred in each dissolution bowl and subject the dissolution apparatus as per parameters. 10 mL of aliquot has been withdrawn at each time point and 10 mL of dissolution media was added. Three milliliters of filtrate were discarded after the aliquot was passed through an appropriate 0.45 µ syringe filter. Used dissolve media to further dilute 2.5 ml of filtrate to 10 ml. (100 PPM of Emtricitabine and 12.5 PPM of Tenofovir alafenamide).

Table 14. Absolute difference between repeatability and intermediate precision results at each time point

	Time Point (Minutes)	5	10	15	20	30	45
EMT	Repeatability % release (Avg. value)	40.58	67.7	85.54	92.68	92.66	92.14
	Intermediate % release (Avg. value)	40.35	68.86	84.50	91.96	92.26	91.78
	Abs Difference	0.23	1.16	1.04	0.72	0.4	0.36
TNF	Repeatability % release (Avg. value)	41.08	60.32	78.39	96.15	96.31	96.09
	Intermediate % release (Avg. value)	39.13	60.55	73.58	94.31	95.91	95.67
	Abs Difference	1.95	0.23	4.81	1.84	0.4	0.42

Table 15. Robustness results of Emtricitabine

Change in Parameter	% Dissolution at 45 minutes	Absolute difference
Repeatability	92.14	NA
Minus 2 RPM (73 RPM)	92.03	0.10
Plus 2 RPM (77 RPM)	90.40	1.74
Minus 0.20 pH (5.30 pH)	89.87	2.27
Plus 0.20 pH (5.70 pH)	92.63	0.50
Minus 5% Volume (475 mL)	91.37	0.76
Plus 5% Volume (525 mL)	90.01	2.13

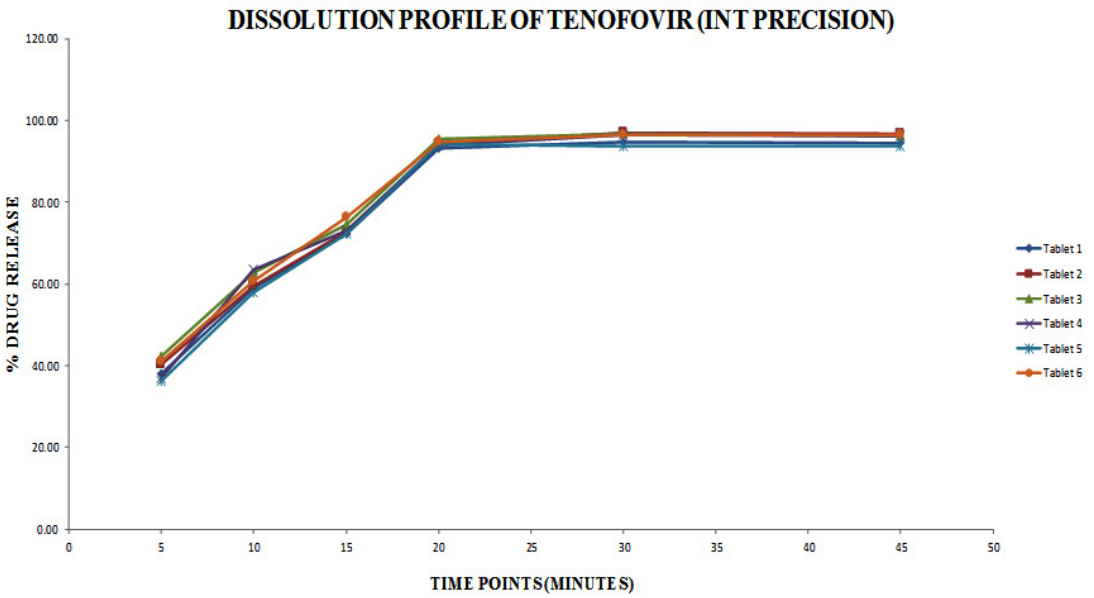

Fig. 12. Dissolution Profile of Tenofovir Alafenamide (Intermediate precision)

Table 16. Robustness results of Tenofovir Alafenamide

Change in Parameter	% Dissolution at 45 minutes	Absolute difference
Repeatability	96.09	NA
Minus 2 RPM (73 RPM)	92.90	3.19
Plus 2 RPM (77 RPM)	94.67	1.43
Minus 0.20 pH (5.30 pH)	92.93	3.16
Plus 0.20 pH (5.70 pH)	93.73	2.36
Minus 5% Volume (475 mL)	94.15	1.95
Plus 5% Volume (525 mL)	94.44	1.66

Robustness

Three Changes made under Robustness for dissolution method:

Change in RPM ±2 RPM

Dissolution performed for 3 tablet unit at the RPM of 73 and 77. Aliquot withdrawn directly at 45 minutes. % Drug release at 45 minutes compared % drug release at 45 minutes under repeatability study and absolute difference calculated w.r.t. % dissolution results of repeatability.

Change in ±0.2 pH

Dissolution performed for 3 tablet unit by change in dissolution media pH by ±0.20 pH. Aliquot withdrawn directly at 45 minutes. % Drug release at 45 minutes compared % drug release at 45 minutes under repeatability study and absolute difference calculated w.r.t. % dissolution results of repeatability**.**

Change in Dissolution media volume (± 5%)

Dissolution performed for 3 tablet unit by change in dissolution media volume by ± 5%. Aliquot withdrawn directly at 45 minutes. % Drug release at 45 minutes compared % drug release at 45 minutes under repeatability study and absolute difference calculated w.r.t. % dissolution results of repeatability.

LOD & LOQ

The LODs for TNF and EMT were found to be 0.25ìg/ml and 1.22ìg/ml, respectively. The LOQs for TNF and EMT were found to be 0.75ìg/ml and 3.69ìg/ml, respectively.

CONCLUSION

A novel validated RP-HPLC method has been developed for the quantitative assessment of Emtricitabine and Tenofovir alafenamide in pharmaceutical tablet dosage forms and bulk. The findings of the statistical analysis indicate that the suggested approach has good accuracy and precision. The approach was thoroughly validated, and the findings for each method validation parameter are satisfactory. It was found that the created approach was not only quick due to its short run time, but also reliable, simple, linear, accurate, sensitive, affordable, and repeatable. Emtricitabine and Tenofovir alafenamide tablets can also be easily tested for dissolving using the established approach.

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Conflict of interest

The authors do not have any conflict of interest.

Data Availability Statement

This statement does not apply to this article.

Ethics Statement

This research did not involve human participants, animal subjects, or any material that requires ethical approval.

Informed Consent Statement

This study did not involve human participants, and therefore, informed consent was not required.

Clinical Trial Registration

This research does not involve any clinical trials.

Author Contributions

Shankar Sahebrao Yelmame : had done the method development and validation; Sunil Vishvanath Amrutkar : helped in drafting the manuscript.

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