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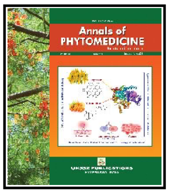

Original Article : Open Access

Validated RP-HPLC method for the simultaneous estimation of emtricitabine and tenofovir disoproxil fumarate in pure drug form with PDA detection

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Abstract

This research outlines the development and validation of a reverse phase high performance liquid chromatography method using a photo diode array (PDA) detector for the simultaneous determination of emtricitabine and tenofovir disoproxil fumarate in pharmaceutical products. The technique involves a mobile phase composed of methanol and potassium dihydrogen phosphate buffer in a volume of 70:30 v/v ratio. Orthophosphoric acid is utilized to adjust the buffer's pH to 3.4. The separation using chromatography was conducted at a flow rate of 1.0 ml per minute utilizing an inertsil octadecylsilane C-18 column (250 × 4.6 mm, 5 µm). The detection was recorded at a wavelength of 247 nm. The retention durations for emtricitabine and tenofovir disoproxil fumarate were 3.049 and 4.317 min, respectively.The technique showed high accuracy, yielding average recoveries of 99.83% for emtricitabine and 100.02% for tenofovir disoproxil fumarate. For both substances, linearity has been demonstrated over the range of concentrations of 20-80 µg/ml. An excellent linear response is indicated by a correlation value (r^2) of 0.999. The limits of detection (LOD), used to illustrate the method's sensitivity, were found to be 0.22 µg/ml for emtricitabine and 0.25 µg/ml for tenofovir disoproxil fumarate. For emtricitabine and tenofovir, the limits of quantification (LOQ) were found to be 0.65 µg/ml and 0.75 µg/ml, respectively. Testing regarding robustness, which involved intentionally varying the flow rate (± 0.2 ml/min), yielded relative standard deviation (% RSD) values less than 2%, demonstrating the method's reliability and consistency. This method is applicable for the analysis of tablet formulations as well as for conducting stability studies.

1. Introduction

Emtricitabine is often referred to as 5-fluoro 1-(2R,5S) 2-(hydroxymethyl) [3,2-d]pyrimidin-4-yl-1-(1H-pyrrolo) -1H-pyrimidine 2,4-dione, is classified as a nucleoside reverse transcriptase inhibitor (NRTI) and is commonly employed in the management of HIV infection. It is a synthetic derivative of cytidine and, once inside the host cell, is converted into its pharmacologically active form emtricitabine 5'-triphosphate". This active metabolite competes with the naturally occurring nucleoside cytidine for integration intoviral DNA during reverse transcription. When incorporated, it causes premature cessation of DNA chain, consequently viral multiplication. Tenofovir disoproxil fumarate, chemically named 9-[(R)-2-(phosphonomethoxy)propyl]adenine, is a nucleotide reverse transcriptase inhibitor and serves as a prodrug of tenofovir. After administration, it undergoes conversion to its active metabolite, tenofovir diphosphate. This active form competes with natural deoxyribonucleotides for HIV reverse transcriptase to incorporate into viral DNA. It causes an early termination of the DNA chain following incorporation, effectively halting viral replication. Emtricitabine and tenofovir disoproxil fumarate are frequently used together as part of antiretroviral therapy for HIV treatment (Karunakranth *et al.,* 2018; Madhuri *et al.,* 2019; Shymala *et al.,* 2018; Rao *et al.,* 2016)

This work represents a novel RP-HPLC technique for the analysis of drugs combination, offering precision, cost-effectiveness, and sensitivity. Compared to previously reported methods (Gandhi *et al.,* 2015; Lavanya *et al.,* 2022; Rao *et al.,* 2017; Sahoo *et al.,* 2024; Shah *et al.,* 2016), the developed method demonstrated higher sensitivity, as indicated by lower limits of detection (LOD) and quantification (LOQ). The method was validated in accordance with the ICH Q2 (R1) (2005) guidelines.

2. Materials and Methods

2.1 Chemicals

Methanol of HPLC standards was bought from Merck Pvt. Ltd., Mumbai. Similarly, HPLC - grade potassium dihydrogen phosphate buffer was sourced from the same supplier. The working standards tenofovir disoproxil fumarate and emtricitabine are provided as gift samples by Active Pharma Pvt. Ltd., Hyderabad.

2.2 Instruments

A water HPLC system was employed for the chromatographic analysis (Model ID: 2690/5 Compact System) equipped along with an inertsil ODS column C-18. An electronic equilibrium device (Sartorius) was utilized for accurate measurements and sample preparation was facilitated by a sonicator (Clean Fast).

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2.3 Buffer preparation

Potassium dihydrogen phosphate, precisely weighed at 2.7218 g, was dissolved in HPLC grade water and make up to 1000 ml. And the prepared solution was then passed through a 0.22 µm membrane filter, and its pH was carefully adjusted to 3.4 using orthophosphoric acid.

2.4 Preparation of mobile phase

Methanol and the prepared buffer were combined in a v/v 70:30 ratio and sonicated for 30 min to ensure proper mixing and degassing. Before being used, the resultant mobile phase was filtered through a 0.45 µm membrane filter.

2.5 Preparation of standard stock solution

Standard stock solutions were prepared by carefully weighing 25 mg of tenofovir disoproxil fumarate and 20 mg of emtricitabine, then transferring them into individual 100 ml volumetric flasks. For twenty min, the liquids were given a sonic treatment to dissolve each component.The mobile phase was then added to bring the volume up to the required level after 10 ml of each stock solution was put into a single 50 ml volumetric flask. The final mixture was sonicated for an additional 10 min to ensure uniform distribution.

2.6 Standard working solution preparation

Standard working solutions of emtricitabine and tenofovir disoproxil fumarate were prepared by diluting the stock solutions to concentrations range between 20 and 80 µg/ml. Prior to analysis, the solutions underwent sonication and filtration through a 0.45 µm membrane filter for ensuring uniformity and clarity.

3. Results

3.1 Optimized conditions

To achieve optimal separation with proper resolution, tailing factor, and other system suitability parameters, a lot of studies were conducted using different mobile phase mixtures, including water : acetonitrile, acetonitrile : methanol, and water : methanol, in various proportions. Different columns, such as the kromasil C-18 and inertsil C-18 column, were tested. The optimal chromatographic conditions were achieved using an inertsil C-18 column (250 × 4.6 mm, 5 µm) with a mobile phase of methanol and potassium dihydrogen phosphate buffer (70:30 v/v), with a 1.0 ml/min flow rate. The study carried out at a detection wavelength of 247 nm, with a total 10 min run time. Under these conditions, tenofovir disoproxil fumarate and emtricitabine have been reported to have retention durations of 4.317 min and 3.049 min, respectively. Figure 1 is optimized chromatogram.

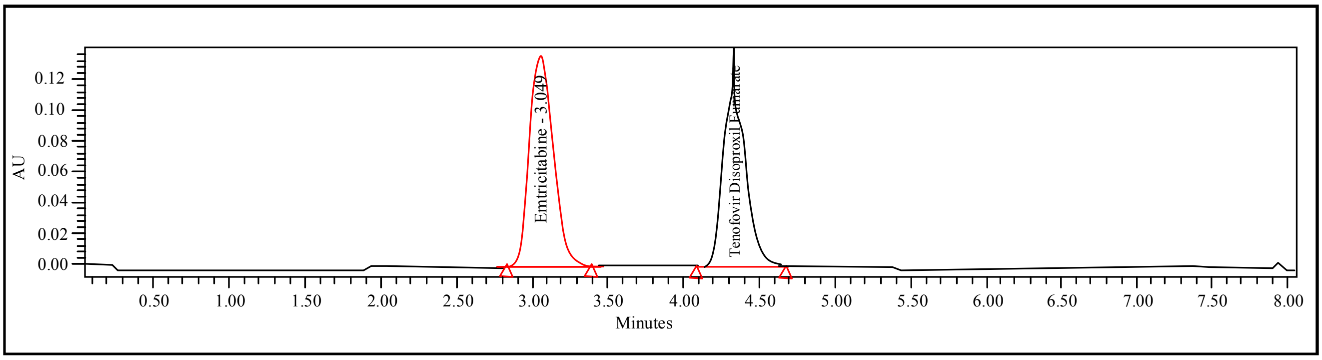

Figure 1: Optimized chromatogram.

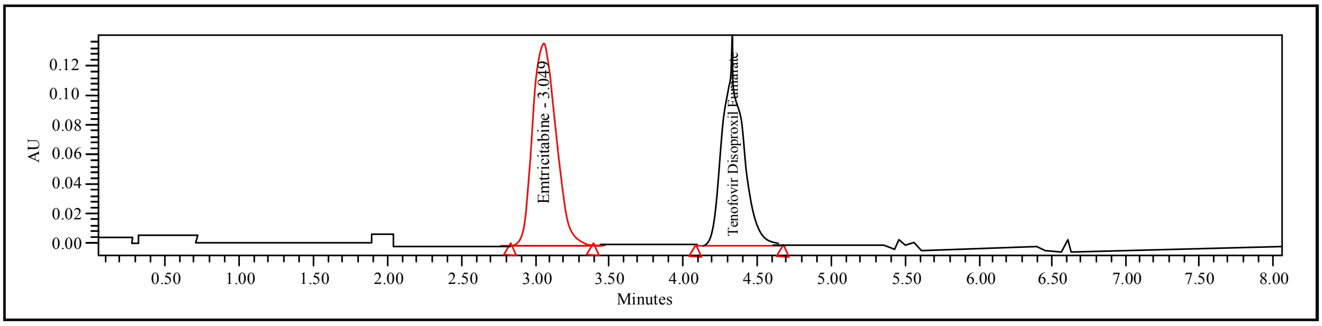

Figure 2: Typical chromatogram of specificity.

3.2 Specificity

An analytical method's specificity is its capacity to precisely identify the target analyte free from interference by excipients, degradation products, or contaminants. Both emtricitabine and tenofovir, no interference was detected at their respective retention times, demonstrating that the method developed is specific. This demonstrates that the method is reliable and suitable for accurate measurement in pharmaceutical applications. Figure 2 is typical chromatogram of specificity.

3.3 Linearity

The concentration range for emtricitabine and tenofovir was 20-80 µg/ml, with both drugs showing an r^2 value of 0.999, indicating outstanding linearity. Table 1 indicating linearity results for both the drugs. Figures 3 and 4 are representing calibration curve of emtricitabine and tenofovir, respectively.

Table 1: Results of linearity

Emtricitabine		Tenofovir		Statistical analysis			
Concentration (µg/ml)	Area of the peak	Concentration (µg/ml)	Area of the peak	Emtricitabine		Tenofovir	
20	588735	20	343650	Slope	30432x	Slope	16575
30	885434	30	498630	Intercept	15666	Intercept	4265
40	1214943	40	674665	r^2	0.999	r^2	0.999
50	1489197	50	829406				
60	1794937	60	992122				
70	2101821	70	1160122				
80	2450946	80	1336708				

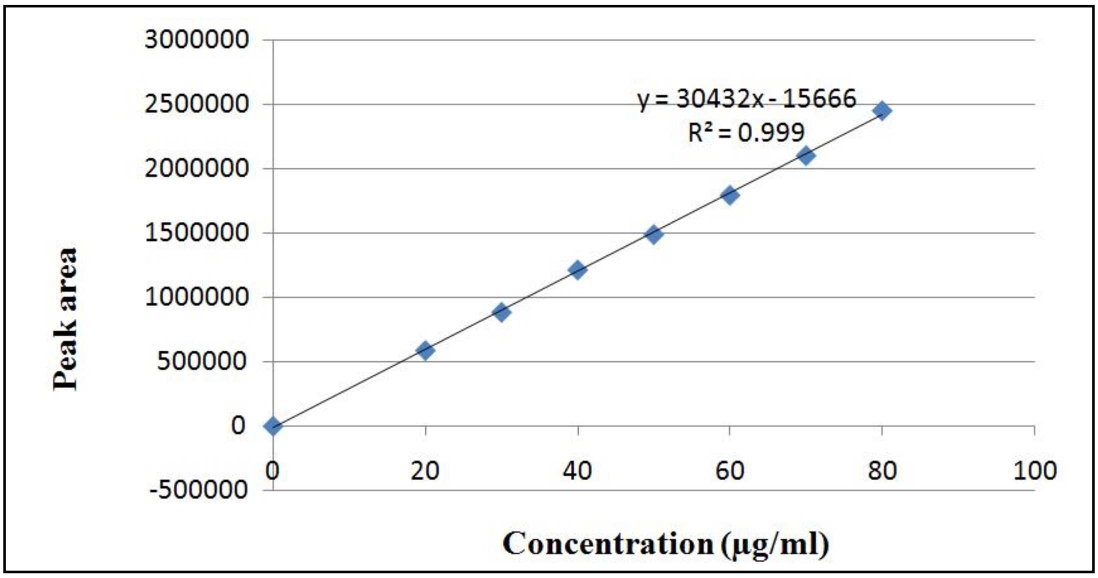

Figure 3: Calibration curve of emtricitabine.

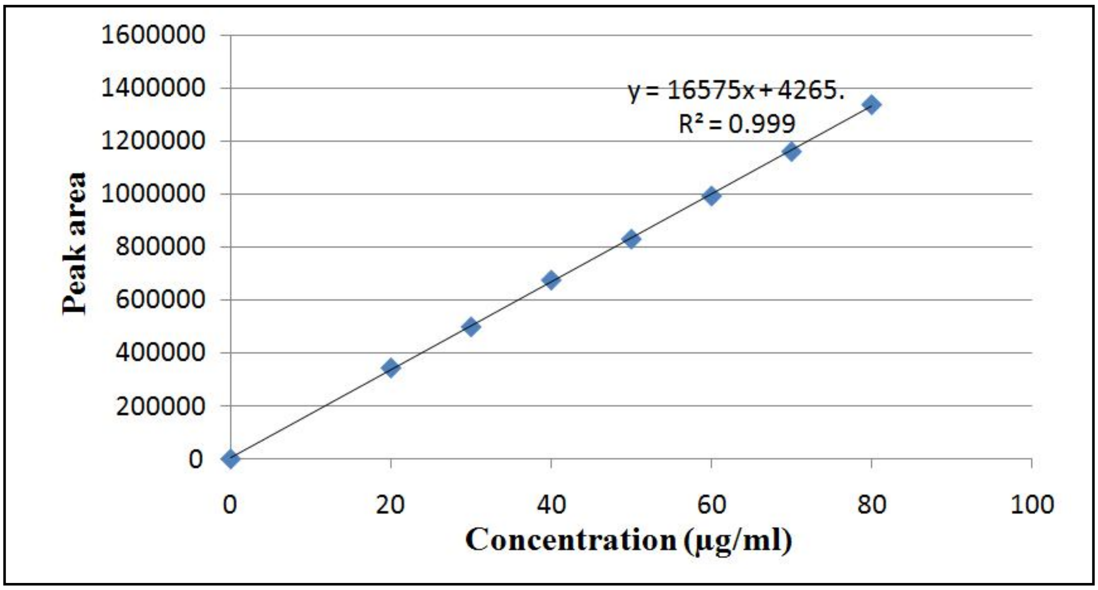

Figure 4: Calibration curve of tenofovir.

3.4 LOD and LOQ

It was done by linearity curve method. Emtricitabine's limits of quantitation (LOQ) and detection (LOD) were determined to be 0.69 and 0.22 µg/ml, respectively. The LOD and LOQ for tenofovir were found to be 0.25 µg/ml and 0.75 µg/ml, respectively.

3.4.1 For emtricitabine

$$LOD = \frac{3.3 \times 2125.583}{30712} = 0.22$$

$$LOQ = \frac{10 \times 2125.583}{30712} = 0.69$$

3.4.2 For tenofovir

$$LOD = \frac{3.3 \times 1252.993}{16499} = 0.25$$

$$LOQ = \frac{10 \times 1252.993}{16499} = 0.75$$

3.5 Precision

3.5.1 System precision

System precision is an essential element of analytical method validation, guaranteeing the consistency and dependability of the analytical system. In this study, the system's precision was assessed by analyzing five replicates of emtricitabine and tenofovir under the same conditions. The results showed excellent repeatability, with an average assay value of 99.91% for emtricitabine and a % RSD of 0.35% for the peak area, indicating high precision within the acceptable limit of 2.0%. Similarly, tenofovir displayed a mean assay value of 100%, with a % RSD of 1.10 for the peak area across five replicates, further validating the reliability of the method. The small % RSD values for both compounds confirm the reliability and reproducibility of the analytical method, highlighting its robustness and compatibility for precise quantification in pharmaceutical quality control. Table 2 discloses results of system precision.

3.5.2 Method precision

For six replicates, the peak area % RSD was 0.35 for emtricitabine and 0.83 for tenofovir, showing exceptional precision. The mean assay results for method precision were 98.48% for emtricitabine and 99.28% for tenofovir. Method precision results are disclosed in Table 3.

Table 2: Data of system precision for emtricitabine and tenofovir

S.No.	Peak areas of emtricitabine	% Assay	Peak areas of tenofovir disoproxil fumarate	% Assay
1	1239704	99.95	676488	98.66
2	1246846	100.24	683935	99.30
3	1252530	100.06	686924	101.53
4	1261073	99.30	687698	100.53
5	1266667	100.00	694665	99.98
Mean	1253364	99.91	685942	100.00
SD	10795.53	0.35819	6586.819	1.107678
% RSD	0.861324	0.35	0.960259	1.10

Table 3: Data of method precision for emtricitabine and tenofovir

S.No.	Peak areas of emtricitabine	% Assay	Peak areas of tenofovir disoproxil fumarate	% Assay
1	1214943	98.6	674665	98.55
2	1220150	99.02	672015	98.88
3	1220212	98.12	672211	99.40
4	1219505	98.31	677612	99.30
5	1265543	98.81	689531	100.53
6	1220150	98.36	672015	98.28
Mean	1226751	98.48	676341.5	99.28
SD	19113.65	0.352647	6824.749	0.827236
% RSD	1.558071	0.35	1.009068	0.83

3.6 Accuracy

The accuracy of emtricitabine and tenofovir were evaluated at levels ranging from 50%, 100%, and 150% with % RSD used to measure precision. The mean accuracy for emtricitabine was 99.62%, 99.83%, and 99.97%, with %RSD values of 0.92, 0.41, and 0.31, respectively. Similarly, tenofovir exhibited mean accuracy values of 99.95%, 100.28%, and 100.45%, with %RSD values of 1.26, 0.22 and 0.85, respectively. Tables 4 and 5 are indicating results of accuracy for emtricitabine and tenofovir, respectively.

3.7 Robustness

The robustness of the approach was assessed by modifying the the velocity of flow by ± 0.2 ml/min from the 1.0 ml/min of standard flow rate and examining % RSD values of six determinations for emtricitabine and tenofovir. For emtricitabine, the % RSD values for six determinations were 1.72% at 0.80 ml/min, 0.19% at 1 ml/min, and 1.59% at 1.2 ml/min. For tenofovir, the % RSD values for six determinations were 1.07% at 0.8 ml/min, 0.96% at 1 ml/min, and 0.03% at 1.2 ml/min. These findings demonstrate that the method remains robust despite variations in flow rate, with the % RSD values staying within acceptable and consistent ranges across the different flow rates.

3.8 Ruggedness

The results of two distinct analysts operating under identical experimental conditions were compared in order to assess the method's ruggedness. For emtricitabine, the % RSD values were 1.56% for analyst 1 and 1.48% for analyst 2, while for tenofovir, the % RSD results were 1.01% and 1.42%, respectively. Mean assay for emtricitabine were 100.19% for analyst 1 and 99.48 analyst 2, while for tenofovir, the mean assay values were 99.28% for analyst 1 and 100.37 % for analyst 2. These results show that the method is rugged, as it yielded consistent and dependable outcomes across different analysts with minimal variation. Results of ruggedness displayed in Table 6.

Table 4: Accuracy results of emtricitabine

Analyte	Level of accuracy	Amount added (µg/ml)	Amount found (µg/ml)	Mean % accuracy	% RSD
Emtricitabine	50	20	20.15	99.62	0.92
	50	20	19.86		
	50	20	19.80		
	100	40	39.88	99.83	0.41
	100	40	40.12		
	100	40	39.80		
	150	60	60.12	99.97	0.31
	150	60	59.76		
	150	60	60.06		

Table 5: Accuracy results of tenofovir

Analyte	% Level of accuracy	Amount added (µg/ml)	Amount found (µg/ml)	Mean % accuracy	% RSD
Tenofovir	50	20	19.95	99.95	1.26
	50	20	20.14		
	50	20	19.64		
	100	40	39.95	100.28	0.22
	100	40	40.12		
	100	40	40.03		
	150	60	59.84	100.45	0.85
	150	60	60.84		
	150	60	60.14		

Table 6: Results of ruggedness parameter

Parameter	Emtricitabine		Tenofivir	
	Analyst 1	Analyst 2	Analyst 1	Analyst 2
% RSD	1.56	1.48	1.01	1.42
% Mean assay	100.19	98.48	99.28	100.37

4. Discussion

To optimize chromatographic separation with adequate resolution, peak symmetry, and system suitability, various mobile phase combinations including water : acetonitrile, acetonitrile : methanol and water : methanol in various ratios were systematically evaluated. Multiple reversed-phase C-18 columns were tested, including kromasil C-18 and inertsil C-18 column. An inertsil octadecyl silane (C-18) column with a mobile phase made up of methanol and 0.05 M potassium dihydrogen phosphate buffer in a 70:30 (v/v) ratio achieved the best separation. The flow rate was maintained at 1 ml/min, and the wavelength of detection was 247 nm, utilizing a 10 min overall runtime, with emtricitabine and tenofovir disoproxil fumarate eluting at retention times of 3.049 and 4.317 min, respectively. Precisely weighed amounts of tenofovir disoproxil fumarate (25.0 mg) and emtricitabine (20.0 mg) were individually placed into two distinct 100.0 ml volumetric flasks. Each substance was diluted to the appropriate level using a mobile phase composing of a 70:30 v/v ratio of methanol and potassium dihydrogen phosphate buffer (pH 3.4), resulting in stock solutions with concentrations of 250 µg/ml for tenofovir disoproxil fumarate and 200 µg/ml for emtricitabine. From these stock solutions, a series of dilutions were prepared to obtain concentrations of 20, 30, 40, 50, 60, 70 and 80 µg/ml for linearity studies. Chromatographic measurement had been carried out with a 1.0 ml/min flow rate, where linearity was assessed by finding correlation coefficients (r^2) 0.9995 for emtricitabine and 0.9998 for tenofovir disoproxil fumarate by graphing concentration against peak area, demonstrating excellent linearity, while accuracy was evaluated through recovery studies at the target concentration of 50%, 100%, and 150%, respectively, Then, the per cent mean recovery of emtricitabine was found to be 99.62%, 99.83%, and 99.97%, respectively, while for tenofovir disoproxil fumarate, the recoveries were 99.95%, 100.28%, and 100.45%, indicating the method is accurate. Sensitivity was assessed by calculating the limits of detection and quantification using the slope of the calibration curve and the standard deviation of the response. Tenofovir disoproxil fumarate showed 0.25 µg/ml and 0.75 µg/ml of LOD and LOQ, respectively, whereas emtricitabine showed 0.22 µg/ml and 0.69 µg/ml, supporting the excellent sensitivity of the method. Precision was evaluated at both system and method levels, with % RSD values below 2% in each case, confirming the method's reliability and consistency. Ruggedness was assessed by comparing results from two different analysts, with % RSD values of 1.56% (analyst 1) and 1.48% (analyst 2) for emtricitabine, and 1.01% and 1.42% for tenofovir disoproxil fumarate, respectively, demonstrating good inter-analyst reproducibility. Robustness was studied by performing a total of sixreplicate injections of the standard solution under varying conditions, including a decline in flow rate to 0.2 ml/min. The % RSD results stayed below 2%, showing the method's robustness to tiny purposeful alterations.

5. Conclusion

Emtricitabine and tenofovir disoproxil fumarate were effectively determined simultaneously using an efficient, specific, sensitive, accurate, precise, and cost-effective RP-HPLC technique. As per our literature review, our method was found to be highly sensitive and serves as a suitable alternative to existing methods. Emtricitabine showed maximum absorbance at 247 nm and tenofovir at 275 nm, with 247 nm chosen as the optimal detection wavelength. The best peak shape was established by using an inertsil ODS C-18 column. Excellent resolution and symmetrical peaks were obtained at a flow rate of 1.0 ml/min with a methanol:buffer 70:30 v/v ratio.The approach showed linearity and accuracy within the range of 20-80 µg/ml and 98-102%, respectively. The detection limits for emtricitabine and tenofovir disoproxil fumarate were both found to be below 1, confirming the high sensitivity of the developed method. Linearity was observed within concentration ranges from 20 to 80 µg/ml, demonstrating consistent precision and accuracy. Robustness was validated by regulate the flow rate by ± 0.2 ml/min. with %RSD values staying within acceptable limits despite minor changes in method parameters. The consistently low relative standard deviation further confirmed the the approach's reliability, stability, and suitability for regular evaluation.

Conflict of interest

The authors declare no conflicts of interest relevant to this article.

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