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DOE-OPTIMIZED RP-HPLC METHOD FOR SIMULTANEOUS QUANTIFICATION OF EMTRICITABINE AND TENOFOVIR DISOPROXIL FUMARATE IN TABLETS

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Abstract

A robust and efficient RP-HPLC method was developed and validated for the simultaneous quantification of Emtricitabine and Tenofovir Disoproxil Fumarate in both bulk drug and tablet dosage forms. Employing a Design of Experiments (DOE) approach, the mobile phase was optimized using methanol, pH, and flow rate as independent variables. The method achieved retention times of 3.25 min for Tenofovir Disoproxil Fumarate and 4.16 min for Emtricitabine, using a gradient mobile phase composed of 20 mM phosphate buffer and acetonitrile in methanol at a 50:50 v/v ratio (pH 3.0) and a flow rate of 0.7 ml/min, on a Targetsil C18 – 5µm column at room temperature. Detection was performed at an isosbestic wavelength of 238 nm over 10 minutes. Linear responses were observed over concentration ranges of 10-30 µg/ml for Tenofovir and 5-25 µg/ml for Emtricitabine. The limits of detection (LOD) and quantitation (LOQ) for the drugs were 1, 0.5, and 3 µg/ml and 1.5 µg/ml, respectively. The method met the ICH guideline criteria for linearity, accuracy, precision, specificity, and robustness, demonstrating its suitability for the simultaneous estimation of these drugs in combined dosage forms. This validated method addresses the critical need for effective analytical techniques in pharmaceutical quality control, particularly for HIV medications.

Keywords: RP-HPLC, Emtricitabine, Tenofovir Disoproxil Fumarate, DOE, Pharmaceutical Analysis

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Introduction

Tenofovir disoproxil fumarate (TDF) belongs to the adenine class; its chemical structure is illustrated in Figure 1. Its molecular formula is C19H30N5O10P.C4H4O4 with a molecular weight of 635.51 g/mol (Bhavsar et al., 2012; Karunakaran et al., 2012; Monks et al., 2012; Devrukhakar et al., 2013; Viswanath et al., 2013; Manojkumar et al., 2020). The compound was available as a white to pale yellow crystalline powder with a slight odor, was soluble in distilled water, and was freely soluble in methanol. TDF serves as a prodrug for tenofovir, a potent nucleotide analog with reverse transcriptase inhibitory activity against the human immunodeficiency virus (HIV) and hepatitis B virus (Rozet et al., 2012; Nethercote & Ermer, 2012; Lionberger et al., 2008; Garg et al., 2013). Emtricitabine is a synthetic fluoro derivative of thiacytidine that has potent antiviral activity. Its chemical name is 5-fluoro-1-(2R,5S)-{2-(hydroxymethyl)-1,3-oxathiolan-5-yl} cytosine with a molecular weight of 247.248 g/mol (Beg et al., 2012; Imam et al., 2013; Kurmi et al., 2014; Sandhu et al., 2016; Manwar et al., 2017; Suryawanshi et al., 2019; Saha & Pandey, 2022). Emtricitabine is soluble in water and methanol and is a nucleoside reverse transcriptase inhibitor (NRTI) that is effective against both human immunodeficiency virus (HIV) and hepatitis B virus (Yu et al., 2014; Mostafa et al., 2023; Yu & He, 2017).

Despite the necessity for simultaneous quantification methods for these drugs in combination therapies, there is a scarcity of effective analytical procedures available in the literature. This study is the first to report the use of a validated stability-indicating RP-HPLC method for pharmaceutical dosage form analysis that takes less time.

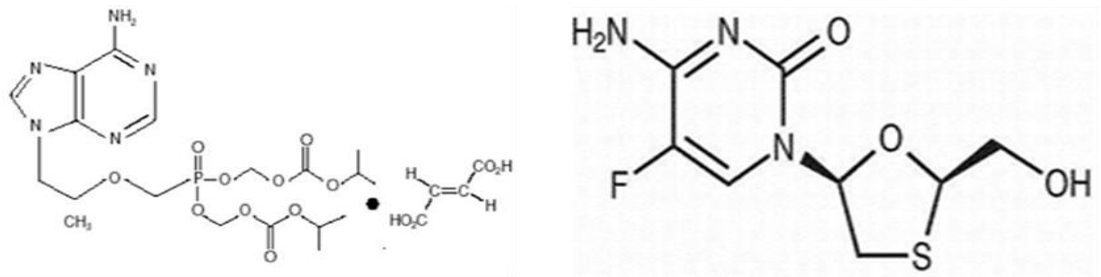

Figure 1 Chemical Structures of Tenofovir Disoproxil Fumarate and Emtricitabine

Although studies on the simultaneous quantification of emtricitabine and other medications in both single and combination forms are limited, methods for the simultaneous analytical determination of these medications are scarce. This study aimed to develop and verify a simple, rapid, cost-effective, and accurate stability-indicating RP-HPLC technique for the measurement of tenofovir disoproxil fumarate and Emtricitabine under ICH criteria. This study is the first to report the use of a validated stability-indicating RP-HPLC method for pharmaceutical dosage form analysis that takes less time.

Recently, the use of the analytical design of experiments (DOE) has gained popularity in the development of analytical methods (Yu & He, 2017; Czyrski & Sznura, 2019). This analytical DOE approach provides a risk-based understanding of the factors affecting the performance of an analytical method (Attimarad et al., 2022). Adhering to the principles of DOE enables a comprehensive understanding of potential risks and their interactions among method variables (Srinubabu et al., 2007; Krishna et al., 2016; Zafar et al., 2019; Attimarad et al., 2020). This involves employing experimental designs for screening and optimizing the method, utilizing the response surface methodology to establish the analytical design space, and implementing a control strategy for continuous improvement (Rozet et al., 2015; Fukuda et al., 2018). Several studies have demonstrated the effectiveness of the DOE approach in developing efficient and cost-effective liquid chromatography (LC) methods for the estimation of analytes in bulk drugs

and pharmaceutical formulations, both separately and in combination (International Council for Harmonisation, 2005; Sangshetti et al., 2017; Moolakkadath et al., 2020).
We aimed to create a straightforward, quick, sensitive, sturdy, effective, and affordable HPLC method using the DOE approach to estimate TDF and EMT in bulk drug and pharmaceutical formulations. To achieve this, we employed an experimental design to optimize the mobile phase, considering methanol, pH, and flow rate as variables. The effects of these variables were observed at the retention times of both compounds, resulting in a reliable assay method for combination drug products containing TDF and EMT.

Resources and Procedures

Equipment

Agilent Technologies 1220 Infinity LC with UV detector, UV-VIS spectrophotometer (Lab India 3092), sonicator (Loba Life), and analytical balance (Shimadzu) are some of the equipment used in the laboratory.

Materials

A pharmaceutical-grade sample of tenofovir disoproxil fumarate and emtricitabine was manufactured by Allvarie Life Sciences, Delhi, India. Potassium dihydrogen orthophosphate, HPLC-grade methanol, water, and acetonitrile were procured from Thermo Fisher Scientific India Pvt. Ltd. (Mumbai, India). Additionally, the marketed tablet formulation used in this research was [Trilavir L], containing [Tenofovir Disoproxil Fumarate (300mg) and Emtricitabine (300mg)], manufactured by [Allvarie Life Sciences, Delhi]. The batch number of the tablets was [7206151], and they were procured from [Seven Hills Pharmacy, Guntur, Andhra Pradesh, India].

Preparation Test Solution

Ten tablets were weighed and ground to a fine powder. An amount of powder equivalent to 250 mg of Emtricitabine and 25 mg of Tenofovir (DESCOVY tablets) was weighed accurately and transferred into a 100 mL volumetric flask containing 25 mL of mobile phase. The mixture was sonicated for 20 min with intermediate shaking to ensure complete extraction of the drugs and then diluted to 100 mL with mobile phase. The solution was filtered through a 0.45 µm membrane filter, and 5 mL of filtrate was taken into a 50 mL volumetric flask and made up to the volume with mobile phase, then injected into HPLC. The results were depicted in Table 1.

Table 1 Analysis of Commercial Formulation

Tablet	Label claimed (mg)		Conc.found (mg)		% Assay	
DESCOVY	Emtricitabine	Tenofovir	Emtricitabine	Tenofovir	Emtricitabine	Tenofovir
Tablets	200	25	201.04	250.17	100.13	101.09

Choice of Wavelength and Isosbestic Point Selection

The optimal wavelength for RP-HPLC analysis was determined by capturing the UV spectrum in the range of 200–400 nm for the individual drug solutions of tenofovir disoproxil fumarate and emtricitabine. The isosbestic point was chosen.

Chromatography Specifications

Tenofovir disoproxil fumarate and emtricitabine were separated using RP-HPLC on a Targetsil C18 column (250 x 4.6 mm, 5µm). Gradient elution was used, with a mobile phase composed of acetonitrile and methanol mixed with phosphate buffer (50:50). The injection volume was 10µL, and the flow rate was set at 0.7 ml/min. Detection was performed at 238 nm. Prior to use, the mobile phase was degassed and filtered through a 0.45µm pore size membrane filter.

Table 2 Design Matrix Used for Optimization of Mobile Phase Conditions with Obtained Responses

	Factor 1	Factor 2	Response 1	Response 2	Response 3
Run	**A: Buffer pH**	**B: flow rate**	**Tailing factor**	**Theoretical plate**	**Resolution**
1	3	0.4	0.91	2617	1.6
2	4	0.4	1.26	2879	1.8
3	5	0.4	1.6	3392	1.9
4	3	0.65	0.78	2198	1.56
5	4	0.65	1.1	2101	1.72
6	5	0.65	1.4	3187	1.8
7	3	0.9	0.66	2063	1.2
8	4	0.9	0.98	2432	1.49
9	5	0.9	1.2	3011	1.46

Formulation of Standard Reference Solution

Tenofovir disoproxil fumarate and emtricitabine were precisely measured at 10 mg each and were subsequently transferred into two separate 10-milliliter volumetric flasks. The samples were then dissolved and diluted with methanol.

Formulation of Working Standard Solutions

100 microliters of tenofovir disoproxil fumarate and emtricitabine solution were withdrawn from the stock solution and transferred into 10-milliliter volumetric flasks. The solutions were prepared by mixing the mobile phase to achieve a final concentration of 10 µg/mL.

Formulation of 20mm Phosphate Buffer

A total of 1.36 grams of potassium dihydrogen orthophosphate was precisely measured and added to a 500-milliliter volumetric flask. The flask was then filled with high-performance liquid chromatography (HPLC)-grade water. To eliminate any air bubbles in the mixture, the flask was placed under vacuum and filtered through a 0.45-micrometer filter. Finally, the mixture was sonicated for 25 min to ensure complete removal of any gas.

Enhancement of Method Parameters

A method for simultaneous RP-HPLC estimation of TDF and EMT was developed using DOE. The Box-Behnken full factorial design was employed to select the range values of the parameters. The mobile phase condition was optimized using a three-factor three-level Box-Behnken design (BBD) with Design-Expert 11 software (Stat-Ease Inc., Minneapolis, USA). The flow rate (X2) and pH (X3) were selected as the independent variables, and the tailing factor (Y1), theoretical plates (Y2), and resolution (Y3) were selected. Response surface analyses were performed to determine the effects of the different independent variables on the observed responses. Table 2 lists the nine experimental runs obtained from the Box-Behnken design (BBD) with their observed and predicted responses. The responses were statistically evaluated using ANOVA. Optimum conditions were selected through a numerical optimization procedure using the desirability function. BBD has the advantage of optimizing experiments by using a 3k-factorial design (where k = 1, 2, 3) with at least three dependent variables or factors and more than one response, compared to other experimental designs such as central composite design (CCD) and fractional factorial design (FFD) (Srinubabu et al., 2007; Zafar et al., 2019; Attimarad et al., 2020; Attimarad et al., 2022). The general polynomial equation quadratic model is as follows:

$$Y = \beta 0 + \beta 1\ X1 + \beta 2\ X2 + \beta 3\ X3 + \beta 12\ X1X2 + \beta 13\ X1X3 + \beta 23\ X2X3 + \beta 11\ X1^2 + \beta 22\ X2^2 + \beta 33\ X3^2 + \cdots$$

The measured response Y was connected to each combination of factor levels in a specific manner. β0 is a fixed value, whereas β1, β2, and β3 are linear coefficients. β12, β13, and β23 are the interaction coefficients of the three factors. β11, β22, and β33 are quadratic coefficients

derived from the observed experimental values of Y in the experimental runs. A, B, and C are the coded levels of the independent variables: high (+), low (−), and center point (0). The terms AB and A2 represent the interaction and quadratic terms, respectively.

After inputting the data into the Design Expert software, the fit summary was applied to the data, resulting in the suggestion of a "quadratic model" by the software. This model provides a polynomial equation in coded terms, which can be used to predict the response for the specified levels of each factor. The equation in terms of coded factors is beneficial for determining the relative influence of factors by comparing the coefficients of each factor. By default, the high levels of the factors were coded as +1, and the low levels were coded as -1. The coded equation is useful for identifying the relative impact of these factors.

DOE Optimization Findings

A response surface modelling technique was used to perform optimization through numerical and graphical methods. The desirability function, which ranges from zero outside the limits to one at the goal, is employed as the objective function. Using numerical optimization, the maximum desirability function was identified. The equation, which is represented by coded factors, is useful in predicting the response for a given set of factor levels. The high levels of the factors were coded as +1, and the low levels were coded as -1 by default. The coded equation was beneficial for determining the relative impact of each factor by comparing the coefficients.

Linear Response: The ability of an analytical method to exhibit a directly proportional relationship between the quantitative response and a specific concentration of an analyte within a defined range of concentrations is referred to as linearity. The linearity of TDF and EMT was assessed by serially diluting their respective stock solutions using appropriate aliquots, resulting in calibration curves covering the concentration ranges of 10-30 μg/ml and 5-25 μg/ml. To establish calibration curves, three replicate analyses were conducted for each concentration.

Accuracy: The accuracy of the method was assessed by injecting five known concentrations of both drugs prepared from fresh stock solutions. The measured concentrations of these samples were interpolated from a specifically created calibration curve to determine the accuracy of the method.

Reproducibility: The precision of the proposed method was assessed through five separate experiments for the TDF and EMT across the concentration ranges examined. To determine intermediate precision, a different analyst operated another instrument to analyze the samples. The %RSD values for all assays were obtained and calculated.

Recovery: The method was established by adding 50%, 100%, and 150% standard solutions to the sample at three different levels. Both compounds were analyzed using the new method, and experiments were carried out. The recoveries and % RSD were calculated based on the analysis of the mixtures.

Specificity: To assess the selectivity of the proposed method, a combination of TDF and EMT was prepared as a tablet formulation. An area comparison of the mixture with the standard solution was conducted, along with the determination of the percentage recovery for both analytes.

Quantitation limit (LOQ) and Detection limit (LOD): The LOD and LOQ were calculated using a method based on the standard deviation (SD) of the response and the slope (S) of the calibration curve at levels approximating the LOD and LOQ. The LOD and LOQ were determined based on the signal-to-noise ratio, and the SD and S were used to calculate the values.

Ruggedness: According to the International Council for Harmonization (ICH), the term "robustness" in the context of an analytical procedure signifies its ability to remain stable and unaffected by slight fluctuations in method parameters, such as modifications in mobile phase

composition by ±5%, alterations in wavelength by ±2 nm, or changes in the use of liquid chromatography (LC) columns from different batches.

System Suitability Assessment: System performance was evaluated by measuring the system suitability parameters. The precision of the system was determined using six replicate injections of standard preparations. Crucial characteristics such as the tailing factor and theoretical plate number were also measured. The method used to determine the content of dosage forms was carried out by first weighing and recording 20 tablets. The average tablet weight was then calculated. A single tablet powder weight equivalent was then weighed and added to a 10 ml volumetric flask, with 5 ml of the mobile phase added and sonicated for 25 min. The volume was increased to 10 ml using a diluent, and the solution was then filtered through a 0.45µ filter. The sample was then filtered, with 0.5µl of the resulting solution being injected. The average tablet content was determined using the appropriate regression equations.

Results and Analysis

The effectiveness of the mobile phase mixture, flow rate, and pH was assessed based on linearity, sensitivity, system compatibility, selectivity, and shorter analysis time (low retention time). Among the several options provided by BBD, the phosphate buffer mobile phase combination demonstrated efficient chromatographic separation of TDF (20µg/ml) and EMT (15µg/ml) with retention times of 3.16 and 4.25 minutes, respectively, as illustrated in Figure 2.

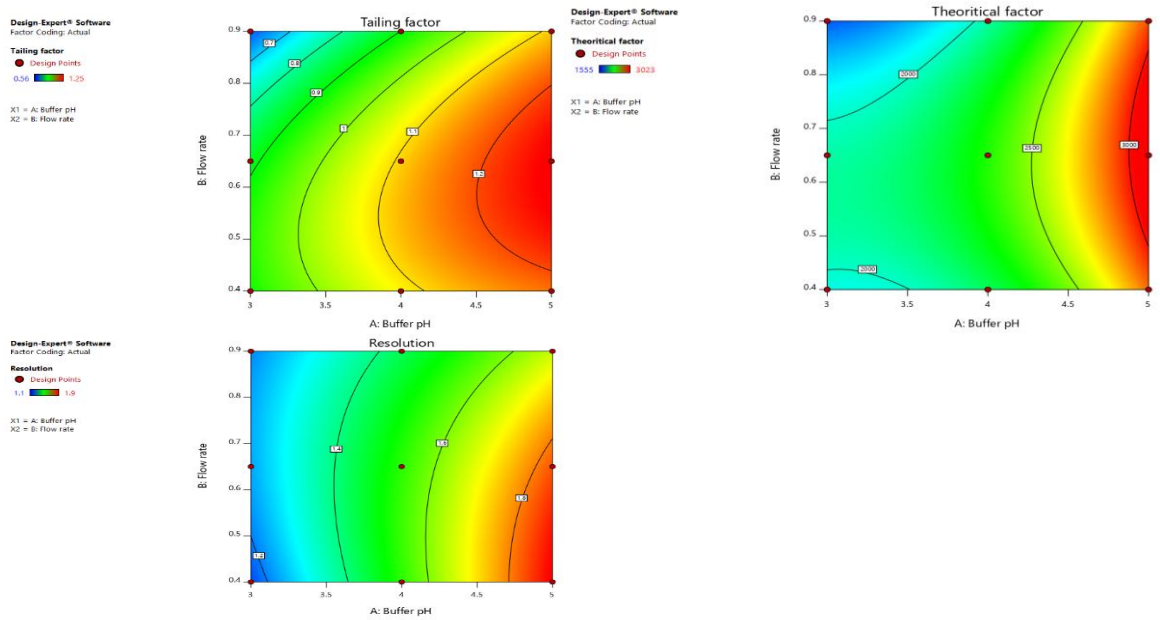

Figure 2 2D Contour Plots Showing the Effects of Buffer pH and Flow Rate on Peak Tailing, Theoretical Plates, and Resolution of Emtricitabine and Tenofovir Disoproxil Fumarate

Refinement of Mobile Phase

Fifteen compositions were prepared according to the experimental design to determine the peak and retention times for both drugs, as shown in Table 1. A response surface analysis was conducted to understand the impact of the selected independent variables on the observed responses. Mathematical relationships were established, and second-order polynomial equations were generated for the retention times of the EMT and TDF. The coefficients of the polynomials fitted well with the data, with R2 values ranging between 0.9958 and 0.9997 for TDF and 0.9914 and 0.9969 for EMT ($P < 0.05$). The response surface plots indicate a relationship between mobile phase composition, flow rate, and retention time. Specifically, the

plots suggest that increasing methanol concentration leads to an increase in retention time, while a gradual decrease in flow rate at intermediate flow rates also affects retention. Similarly, the pH of the mobile phase influences the system suitability parameters for both drugs. The results revealed that increasing the pH of the mobile phase did not significantly affect the suitability parameters. All response surfaces were fitted with quadratic polynomial models and were able to predict the interaction effects. Finally, the ANOVA results, presented in Table 3, show that the model terms for the main and interaction effects were statistically significant ($P < 0.05$). The optimized mobile phase conditions, selected using a numerical point prediction optimization method from software with a desirability value of 1, were methanol (60%), flow rate (0.7 ml/min), and pH (3).

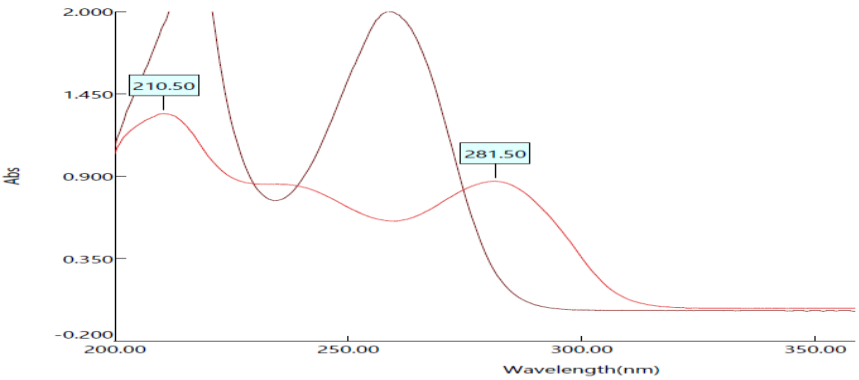

Figure 3 UV Spectrum Showing the Isosbestic Point for Emtricitabine and Tenofovir Disoproxil Fumarate

Table 3 Summary of ANOVA Results and Final Equation in Terms of Coded Factors

ANOVA parameters	Tailing Factor	Theoretical Plates	Resolution
Adjusted R2	0.9970	0.8523	0.9543
Predicted R2	0.9871	0.5743	0.8147
P value	0.0001	0.0421	0.0075
R2 value	0.9989	0.9946	0.9829
Suggested model	Quadratic	Quadratic	Quadratic

Y1(Tailing Factor) = 1.10778 + 0.308333A + -0.155B + -0.0375AB + -0.0216667A2 + 0.00833333B2
Y2(Theoretical plates) = 2312.67 + 452A + -230.333B + 43.25AB + 274A2 + 237B2
Y3(Resolution) = 1.74889 + 0.133333A + -0.191667B + -0.01AB + -0.0833333A2 + -0.118333B2.

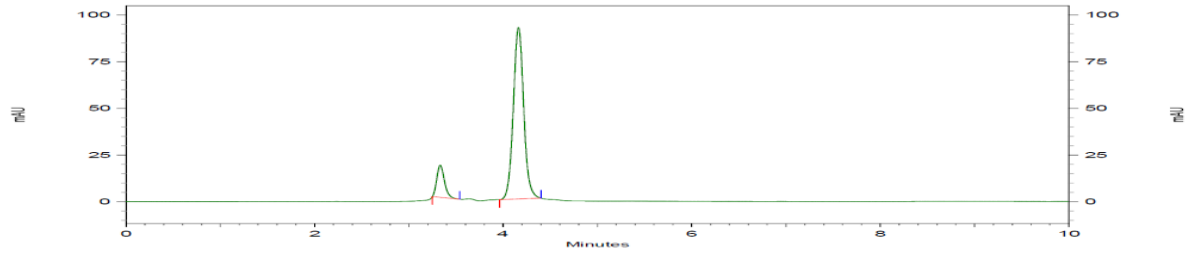

Figure 4 Representative Chromatograms of Emtricitabine (EMT) and Tenofovir Disoproxil Fumarate (TDF) Standard Solutions

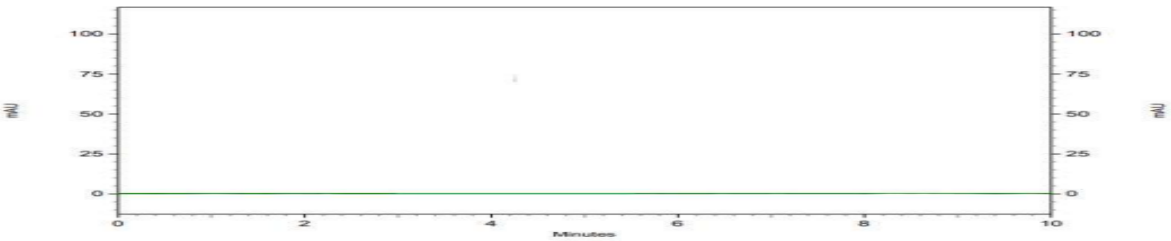

Figure 5 Chromatogram of Blank Solution

Method Verification and Validation

The validity of the proposed analysis approach was confirmed by employing ICH guidelines for evaluation criteria, such as linearity, precision, specificity, accuracy, robustness, limit of detection (LOD), and limit of quantification (LOQ).

System Suitability and Appropriateness

A solution containing Tenofovir Disoproxil Fumarate and Emtricitabine was incorporated into the HPLC system. Chromatograms for standards were utilized to assess the system performance, including retention time, tailing factor, and theoretical plates. The peak areas for the six injections were within the specified range, confirming the system's precision. The optimized chromatographic conditions, including mobile phase composition, flow rate, and column temperature, were selected to achieve optimal separation and resolution. Peaks were obtained for the mixture of standard and sample drug solutions at the working concentration. In contrast, the blank solution without the drug did not produce any peaks at the retention time of tenofovir disoproxil fumarate and emtricitabine. Based on these results, it can be concluded that the proposed method is specific. This specificity confirms the reliability of the method for the determination of these compounds in pharmaceutical formulations.

Table 4 Design Matrix Used to Optimize Mobile Phase Conditions with Obtained Responses for Emtricitabine

	Factor 1	Factor 2	Response 1	Response 2	Response 3
Run	**A: Buffer pH**	**B: Flow rate**	**Tailing factor**	**Theoretical factor**	**Resolution**
1	3	0.4	0.91	2003	1.1
2	4	0.4	1.06	1979	1.6
3	5	0.4	1.2	3006	1.9
4	3	0.65	0.95	2135	1.3
5	4	0.65	1.05	2350	1.5
6	5	0.65	1.25	3023	1.8
7	3	0.9	0.56	1555	1.2
8	4	0.9	0.98	2216	1.4
9	5	0.9	1.09	2878	1.7

Table 5 Optimized Chromatographic Conditions

Parameters	**Conditions**
Column	Targetsil C18 column (250 x 4.6 mm, 5µm)
Mobile phase	Acetonitrile-methanol and phosphate buffer (50:50)
UV detection wavelength	238nm
Flow rate	0.7 ml/min
Injected volume	10 µL
Temperature	Ambient temperature
Runtime	10 mins

Table 6 System Suitability Parameters

Parameters	**Tenofovir disoproxil fumarate**	**Emtricitabine**
Retention time	3.33	4.16
Tailing factor	1.26823	1.06962
Theoretical plates	7958	6991

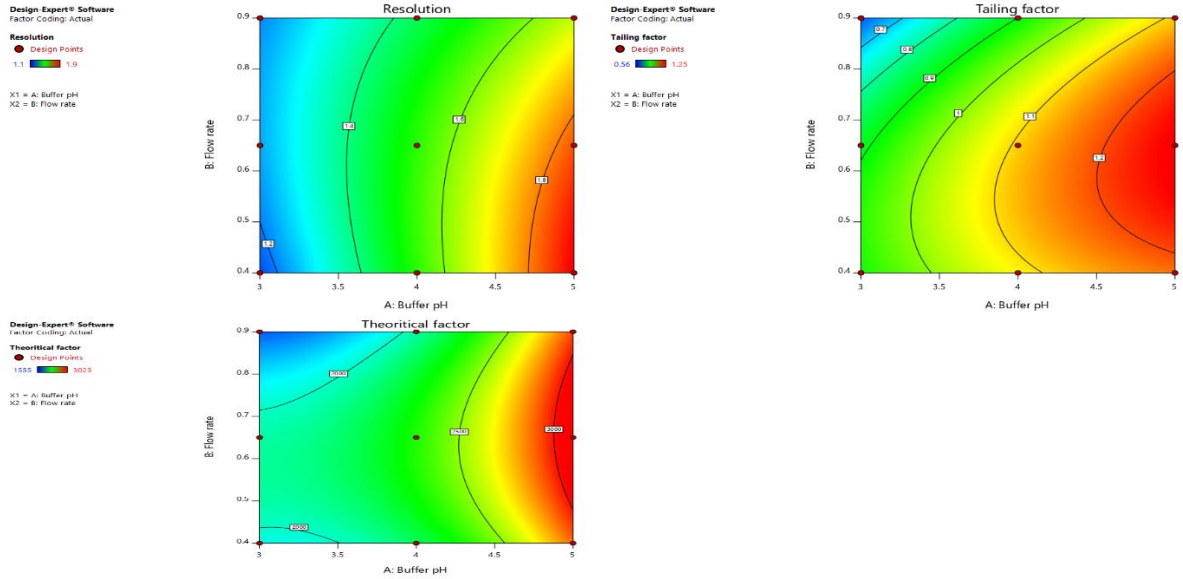

Figure 6 3D Response Surface Plots Illustrating the Effects of Buffer pH and Flow Rate on Tailing Factor, Theoretical Plates, and Resolution for Tenofovir Disoproxil Fumarate

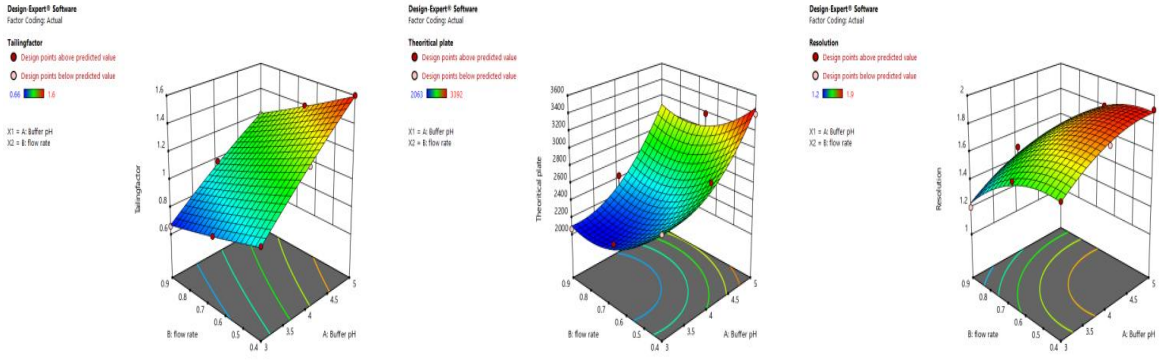

Figure 7 3D Response Surface Plots Illustrating the Effects of Buffer pH and Flow Rate on Tailing Factor, Theoretical Plates, and Resolution for Emtricitabine

Linearity

Different concentrations (50, 75, 100, 125, and 150%) of tenofovir disoproxil fumarate and emtricitabine standard solutions were prepared. Calibration curves were obtained by plotting the graph between the concentration level and the matching mean peak area. From the graph, a strong association between the mean peak area and drug concentration can be determined for tenofovir disoproxil fumarate (10–30 µg/ml) and emtricitabine (5–25 µg/ml).

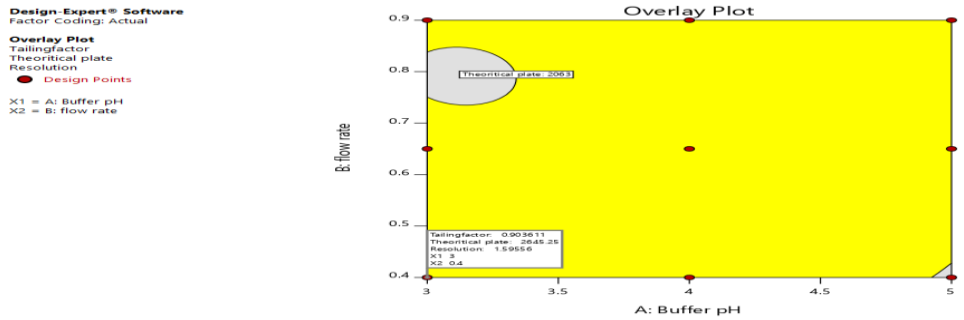

Figure 8 Overlay Plot for Optimization of Tenofovir Disoproxil Fumarate Analysis

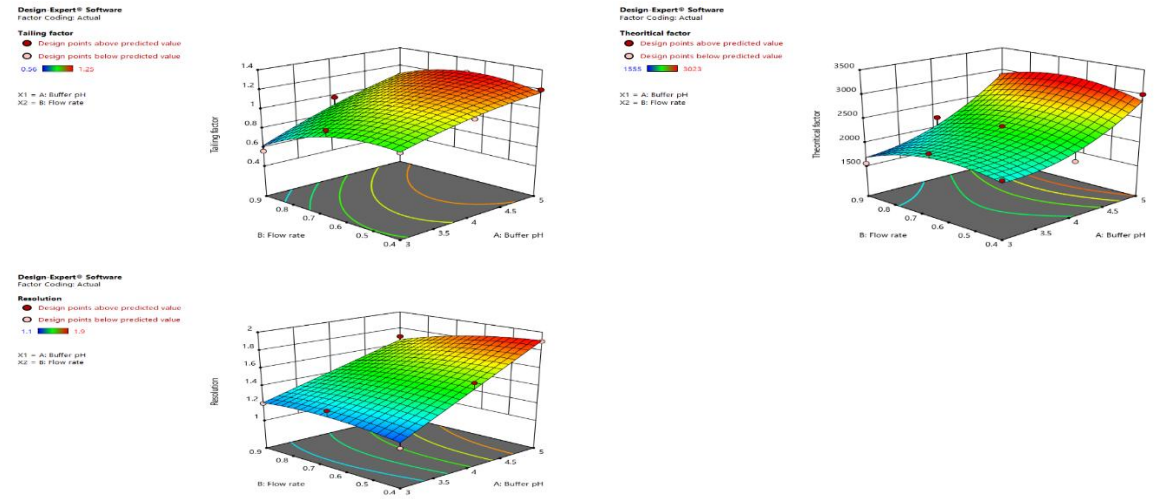

Figure 9 3D Response Surfaces Showing the Effect of Factors on Emtricitabine

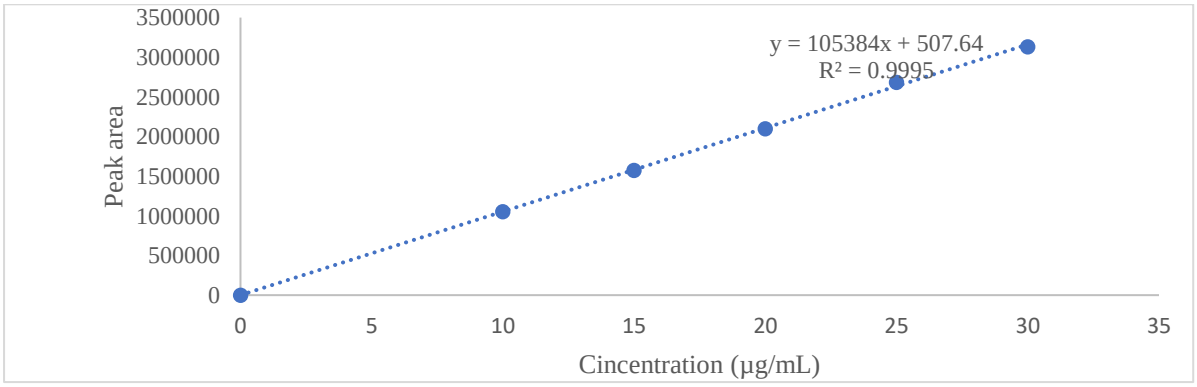

Figure 10 Calibration Curve for Tenofovir Disoproxil Fumarate

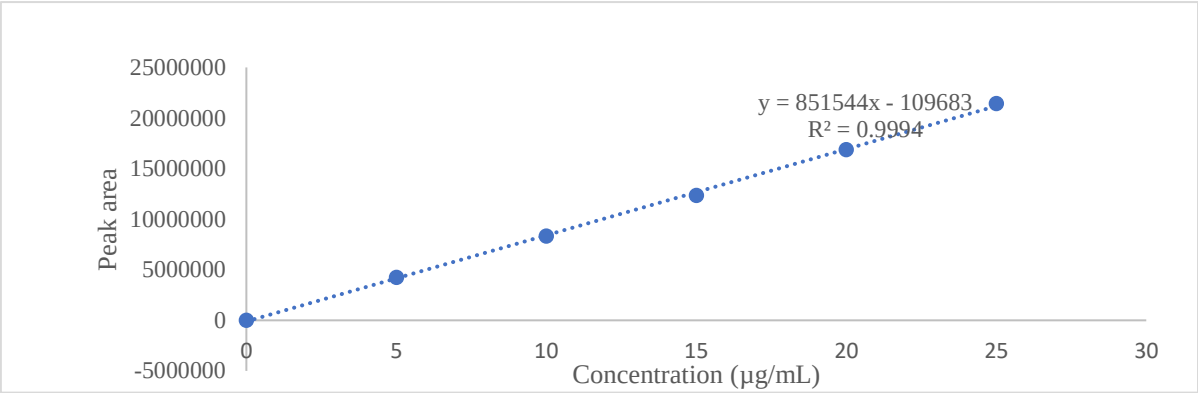

Figure 11 Calibration Curve for Emtricitabine

System Precision

Replicate injections of the standard solution mixture at the target concentration of 100% were performed six times, resulting in a percentage RSD of less than 2. This indicated that the repeatability of the process was acceptable. The precision observations of the system are presented in Table 6. The precision observations further emphasize the method's robustness and reliability for pharmaceutical analysis, a necessary quality for routine use in laboratory settings.

Table 7 Linearity Data for Emtricitabine and Tenofovir Disoproxil Fumarate

Tenofovir disoproxil fumarate		**Emtricitabine**	
Concentration (µg/ml)	**Peak area**	**Concentration (µg/ml)**	**Peak area**
10	1052856	5	4236289
15	1575456	10	8332595
20	2097718	15	12348892
25	2682568	20	16868456
30	3132897	25	21421487

Accuracy

Accuracy was achieved by utilizing varying concentrations of the sample at different levels and conducting three separate measurements (50%, 100%, and 150%) based on the label claim. The acceptable range for mean recovery was between 98% and 102%, and all observed data were within this range, demonstrating satisfactory recovery rates.

Table 8 System Precision and Method Precision for Tenofovir Disoproxil Fumarate and Emtricitabine

% level	**Standard peak area**	**Sample peak area**	**% recovery**	**Average % recovery**	**Mean % recovery**
50	12390222	6215456	99.83	100.05	100.26
	12390222	6242586	100.21		
	12390222	6233789	100.11		
100	12390222	12490222	100.30	100.26	
	12390222	12481215	100.20		
	12390222	12485145	100.28		
150	12390222	18742848	100.38	100.49	
	12390222	18725846	100.28		
	12390222	18825612	100.83		

Table 9 System Precision Data for Tenofovir Disoproxil Fumarate and Emtricitabine

S. No	Peak area of Tenofovir disoproxil fumarate	Peak area of Emtricitabine
1	2132421	12154261
2	2131256	12114482
3	2131121	12103652
4	2129651	12164112
5	2153894	12157154
6	2144128	12117456
Mean	2137079	12135186
S.D	9784.783	26155.55
% RSD	0.46	0.22

Table 10 Accuracy of Tenofovir Disoproxil Fumarate

S.NO	Peak area of Tenofovir disoproxil fumarate	Peak area of Emtricitabine
1	2124225	12123561
2	2124427	12142588
3	2124158	12125696
4	2123448	12158013
5	2121812	12152102
6	2114512	12117875
Mean	2122097	12136639
S.D	3837.803	16578.38
% RSD	0.18	0.14

Table 11 Accuracy of Emtricitabine

% level	Standard peak area	Sample peak area	% recovery	Average % recovery	Mean % recovery
50	2170872	1225865	100.20	100.70	100.48
	2170872	1235562	100.93		
	2170872	1235689	100.98		
100	2170872	2472872	101.05	100.60	
	2170872	2445897	99.92		
	2170872	2466894	100.83		
150	2170872	3688981	100.53	100.16	
	2170872	3662589	99.81		
	2170872	3674589	100.15		

Limit of Detection and Limit of Quantification

The limit of detection (LOD) was calculated and confirmed to be 1µg/ml for tenofovir disoproxil fumarate and 0.5µg/ml for emtricitabine. The limit of quantification (LOQ) was 3µg/ml for tenofovir disoproxil fumarate and 1.5µg/ml for emtricitabine. These results demonstrate that the proposed method is highly sensitive and suitable for pharmaceutical studies, even when detecting small concentrations in the nanogram range.

Table 12 Limit of Detection (LOD) and Limit of Quantification (LOQ)

DRUG	LOD	LOQ
Tenofovir disoproxil fumarate	1 µg/ml	3 µg/ml
Emtricitabine	0.5 µg/ml	1.5 µg/ml

Robustness

The robustness of an analytical technique is a measure of its ability to remain unaffected by minor but deliberate variations in method parameters, and it indicates its reliability during routine use. Table 13 demonstrates that the approach is robust, with a percentage RSD of less than 2, even after making purposeful adjustments to the flow velocity (±0.1), organic phase (±5), and detector (±5).

Table 13 Data of Robustness

S. No	Condition	% RSD of Tenofovir disoproxil fumarate	% RSD of Emtricitabine
1	Flow rate (-) 0.5 ml/min	0.06	1.19
2	Flow rate (+) 0.9mL/min	0.01	1.09
3	Mobile phase ratio change (55:45v/v)	0.01	1.10
4	Mobile phase ratio change (45:55v/v)	0.04	1.09
5	Detector (233nm)	0.13	1.10
6	Detector (242nm)	0.13	1.10

Conclusion and Discussion

In comparing our method to previously published approaches, we observed enhanced precision and a quicker analysis time utilizing RP-HPLC. While our method demonstrated robustness, it is essential to acknowledge limitations such as potential interferences from excipients. Future applications could include routine quality control in pharmaceutical laboratories, ensuring effective monitoring of drug quality.

This study successfully developed a reliable RP-HPLC method for the simultaneous determination of Tenofovir Disoproxil Fumarate (TDF) and Emtricitabine (EMT) using a mobile phase of 20 mM phosphate buffer and acetonitrile in a 50:50 (v/v) ratio at pH 3.0. Optimization was performed through a structured Design-of-Experiments (DOE) methodology, which included screening varied mobile phase compositions and employing a Box-Behnken response surface design to assess interactions between critical parameters, such as buffer pH and flow rate. The established method demonstrated significant robustness and sensitivity, meeting ICH guidelines for quantitative analysis in pharmaceutical dosage forms. The RP-HPLC method also effectively separated TDF and EMT from their degradation products, confirming its reliability for routine quality control. In conclusion, this method is recommended for efficient and cost-effective analysis of both drugs in pharmaceutical applications.

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