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DEVELOPMENT AND VALIDATION OF COST EFFECTIVE STABILITY INDICATING RP-HPLC METHOD FOR ESTIMATION OF LAMIVUDINE AND TENOFOVIR

Pranali Hemraj Walthare*, Snehal R. Karmankar
Maharashtra Institute of Pharmacy, Betala, Bramhapuri (M.H)

***Correspondence Info:**
Pranali Hemraj Walthare
Maharashtra Institute of Pharmacy, Betala, Bramhapuri
Email:
pranaliwalthare11@gmail.com

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ABSTRACT

A simple, precise, and accurate reverse-phase high-performance liquid chromatography (RP-HPLC) method has been developed and validated for the simultaneous determination of LAMI (Lamivudine) and TFDF (Tenofovir) in tablet formulations. The chromatographic separation was achieved using a C18 column with a mobile phase consisting of a mixture of methanol and water (pH adjusted to 3.0 with phosphoric acid), and the detection was performed at 265 nm. The method showed excellent linearity for both drugs, with correlation coefficients (r^2) of 0.999 for LAMI and TFDF. The validation parameters, including recovery studies, repeatability, and robustness, demonstrated high precision and accuracy for both drugs. The forced degradation studies indicated the stability of the drugs under normal conditions but revealed susceptibility to degradation under extreme stress conditions. The method was applied successfully to the assay of tablet formulations, yielding results within the acceptable range. The limit of detection (LOD) and limit of quantification (LOQ) values for LAMI and TFDF were found to be sensitive enough for routine pharmaceutical analysis. This method offers a reliable approach for the quality control of LAMI and TFDF in pharmaceutical formulations.

Keywords: LAMI, TFDF, RP-HPLC, Validation, Linearity, Forced Degradation, Recovery, Repeatability, Tablet Formulation, Assay, Stability Studies, Limit of Detection (LOD), Limit of Quantification (LOQ), Pharmaceutical Analysis.

INTRODUCTION

Lamivudine and Tenofovir are two commonly prescribed antiretroviral drugs used in the management of HIV/AIDS and chronic hepatitis B. Lamivudine (LMV) is a nucleoside reverse transcriptase inhibitor (NRTI), while Tenofovir disoproxil fumarate (TDF) is a nucleotide reverse transcriptase inhibitor (NRTI). Both drugs have been extensively studied and are considered essential in combination antiretroviral therapy (cART) due to their effectiveness, safety profile, and high bioavailability. Accurate and reliable estimation of these drugs is crucial for quality control and regulatory compliance during the manufacturing process and clinical administration (Sharma *et al.,* 2017). The stability of pharmaceutical formulations is of paramount importance to ensure their safety, efficacy, and quality throughout their shelf life. A stability-indicating method (SIM) is defined as a validated analytical method capable of detecting changes in the potency and quality of a drug under stress conditions, such as temperature, humidity, light, and oxidative stress.

For effective quality control of LMV and TDF, there is a need for a robust, cost-effective, and efficient analytical technique that can withstand the various conditions that may affect the integrity of the drug formulation. Reverse-phase high-performance liquid chromatography (RP-HPLC) has emerged as a widely employed method for the analysis of pharmaceuticals, offering high resolution, sensitivity, and accuracy (Verma *et al.,* 2016). The RP-HPLC method has been used to estimate various drugs, including antiretroviral agents like lamivudine and tenofovir, due to its reliability, precision, and ability to separate the drugs from excipients and degradation products (Patel *et al.,* 2018).

This study aims to develop and validate a cost-effective, stability-indicating RP-HPLC method for the simultaneous estimation of lamivudine and tenofovir in their formulations and bulk drug samples. The validation will include parameters such as selectivity, linearity, accuracy, precision, robustness, and system suitability, as per the International Conference on Harmonisation (ICH) guidelines (ICH, 2005). Moreover, the method will be subjected to various stress conditions (such as acidic, alkaline, oxidative, and thermal degradation) to evaluate its ability to detect degradation products and establish the stability of the formulation. This validated method will provide a reliable tool for quality control in the production and stability testing of lamivudine and tenofovir formulations.

MATERIALS AND METHODS

Selection of Mobile Phase

Initially, to estimate Lamivudine and Tenofovir Disoproxil Fumarate in a fixed dosage form, various mobile phase compositions were tested. The selection criteria included system suitability parameters such as retention time (RT), tailing factor, number of theoretical plates, and HETP (height equivalent to a theoretical plate) (Kokkirala and Suryakala, 2020). Among the tested compositions, the most suitable mobile phase for analysis was determined to be Acetonitrile: Methanol in a ratio of 60:40 v/v. To ensure purity, the mobile phase was filtered through a 0.45µm filter paper to remove any particulate matter and subsequently degassed by sonication. The flow rate employed for analysis was set at 1.0 ml/min.

Selection of Diluent

The diluent chosen for the preparation of samples was compatible with the mobile phase, ensuring that it did not significantly affect the retention time and resolution of the analytes. Following several trials, methanol was identified as the most suitable diluent. This choice was made to maintain consistency with the mobile phase composition and to ensure accurate and reliable analysis of Lamivudine and Tenofovir Disoproxil Fumarate in the fixed dosage form.

Preparation of Stock Solution

To prepare the stock solutions of Lamivudine (LAMI) and Tenofovir Disoproxil Fumarate (TDF)**,** accurately weigh 10 mg of each drug and transfer them separately into 10 mL volumetric flasks. Then, add 5 mL of methanol as the diluent to each flask. Sonicate the flasks for 20 minutes to ensure complete dissolution of the drugs. Finally, make up the volume of each flask to 10 mL with methanol, resulting in a stock concentration of 1000

µg/mL for both Lamivudine and Tenofovir. This preparation forms the Stock-A solution for each drug, which will be used for further dilution and analysis (Kancherla *et al.,* 2016).

Preparation of Sub Stock Solution

To prepare the working solutions, transfer 5 mL of the Stock-A solution of each drug (Lamivudine and Tenofovir Disoproxil Fumarate) into separate 50 mL volumetric flasks. Then, dilute each solution up to the 50 mL mark with methanol. This dilution results in a concentration of 100µg/mL for both Lamivudine (LAMI) and Tenofovir Disoproxil Fumarate (TDF), referred to as Stock-B for each drug. These Stock-B solutions will be used for further preparation of calibration standards and analysis.

Preparation of Different Solutions

Aliquots of 0.5 mL, 1.0 mL, 1.5 mL, 2.0 mL, and 2.5 mL were taken separately from the Stock-B solutions of Lamivudine (LAMI) and Tenofovir Disoproxil Fumarate (TDF). Each aliquot was transferred into separate 10 mL volumetric flasks. The volume of each flask was then made up to 10 mL with methanol. This process resulted in solutions with concentrations of 5 µg/mL, 10 µg/mL, 15 µg/mL, 20 µg/mL, and 25 µg/mL for both Lamivudine (LAMI) and Tenofovir Disoproxil Fumarate (TDF). These solutions were used to prepare calibration standards for the stability-indicating RP-HPLC method.

Linearity and Calibration Graph

To establish the linearity of the analytical method, a series of dilutions ranging from 5 µg/mL to 25 µg/mL were prepared for both Lamivudine (LAMI) and Tenofovir Disoproxil Fumarate (TDF). Each solution was filtered through a 0.45 µm membrane filter to remove any particulate matter and then injected into the chromatograph. The chromatograms were recorded at a wavelength of 280.0 nm, and the process was repeated five times for consistency. A calibration graph was plotted by correlating the mean peak area with the respective concentrations for both LAMI and TFDF. From this calibration graph, the regression equation was derived, which allowed for the quantification of the drugs in future analyses.

System Suitability Parameters

The separation variables were configured, and the mobile phase was allowed to saturate the column at a flow rate of 1.00 ml/min. Once the column was completely saturated, six replicates of a working standard solution containing 10 mg/ml of LAMI and 10 mg/ml of TFDF were separately injected. Peak reports and column performance reports were recorded for all chromatograms.

Validation of developed Method

Linearity

The linearity of an analytical procedure refers to its capacity, within a specified range, to produce test results that are directly proportional to the area of the analyte in the sample. To assess linearity, a calibration plot was constructed following the analysis of five different concentrations (ranging from 5 to 25µg/ml for LAMI and TFDF). The areas for each concentration were recorded three times, and the mean area was calculated. The response ratio, or response factor, was determined by dividing the area under the curve (AUC) by the respective concentration (Dongala *et al.,* 2019).

Specificity

The method's specificity was evaluated to confirm the presence of the analyte without interference from expected components like impurities, degradation products, and matrix components (Patil *et al.,* 2016).

Accuracy

Recovery studies were conducted to assess the accuracy of the developed method by adding specific concentrations of standard drug (80%, 100%, and 120%) to pre-analyzed sample solutions and analyzing their recovery.

Precision

Repeatability

Repeatability was determined by analyzing five replicates at five concentrations within the linearity range (5, 10, 15, 20, and 25 µg/ml for LAMI and 1, 2, 3, 4, and 5 µg/ml for TFDF), demonstrating precision under consistent operating conditions over a short time interval.

Intermediate Precision

a) Day-to-Day Precision: Intermediate precision was assessed by conducting analyses on different days and by different analysts, with five replicates at five concentrations. Results for day-to-day intermediate precision for LAMI and TFDF are presented (Shete *et al.,* 2014).

Robustness

According to ICH norms, deliberate variations in the concentration of the mobile phase were introduced to determine the method's resilience. The mobile phase ratio was changed from 20mM KH2PO4: Methanol (20:80 %v/v) to (15:85% v/v).

Detection Limit and Quantitation Limit

The LOD and LOQ of the developed method were calculated based on the standard deviation of response and the slope of the linearity curve.

Analysis of Tablet Sample

Twenty tablets were accurately weighed, and their mean weight was determined. After grinding them to a fine powder, an accurately weighed quantity equivalent to 10 mg of LAMI and 10mg of TFDF was transferred to a 10 ml volumetric flask containing methanol. The solution was sonicated for 25 minutes, and the final volume was adjusted with the mobile phase. After filtration through a 0.45 µm filter, the stock solution was further diluted with methanol to obtain a sample solution with drug concentrations of 10µg/mL for LAMI and 10µg/mL for TFDF. The amounts of LAMI and TFDF in the tablet formulation were determined by extrapolating the values of the area from the calibration curve. The analysis procedure was repeated six times for each formulation.

Forced degradation studies

To assess whether the method is stability indicating, forced degradation studies were conducted on the drug powder, and the analysis was performed using HPLC with a UV detector. 20 µl of each forced degradation sample was injected (ICH, 2003).

Acid Degradation: In separate 50 ml round bottom flasks, 50 mg of both drugs samples were taken. 50 ml of 0.1 M HCl solution was added to each flask, and the contents were thoroughly mixed. The flasks were then kept under constant stirring for 8 hours at 80ºC. Samples were withdrawn and diluted to

achieve a concentration of 10 µg/ml. These samples were subjected to HPLC analysis, and the percentage degradation was calculated using the calibration curve of the drugs.

Alkaline Hydrolysis: Similarly, in separate 50 ml round bottom flasks, 50 mg of the both drugs sample was taken. 50 ml of 0.1 M NaOH solution was added to each flask, and the contents were well mixed. The flasks were then kept under constant stirring for 8 hours at 80ºC. Samples were withdrawn, diluted to a concentration of 10 µg/ml, and analyzed using HPLC. The percentage degradation was calculated using the calibration curve of the drugs.

Oxidative Degradation: In separate 50 ml round bottom flasks, 50 mg of the both drugs sample was taken. 50 ml of 3% hydrogen peroxide solution was added to each flask, and the contents were mixed thoroughly. The flasks were then kept under constant stirring for 24 hours at room temperature. Samples were withdrawn, diluted to a concentration of 10 µg/ml, and subjected to HPLC analysis. The percentage degradation was calculated using the calibration curve of the drugs.

Thermal Degradation: In this study, 50 mg of the both drugs sample was placed in a petri dish and kept in an oven at 50ºC for 4 weeks. Samples were periodically withdrawn, diluted to a concentration of 10 µg/ml, and analyzed using HPLC. The percentage degradation was calculated using the calibration curve of the drugs.

RESULTS AND DISCUSSION

The correlation coefficient (r^2) for both LAMI and TFDF was found to be 0.999**,** indicating an excellent linear relationship between concentration and response for both drugs. This suggests that both drugs exhibit a highly consistent response over the tested concentration range, ensuring accurate quantification in subsequent analyses. The slopes for LAMI and TFDF were 71.67 and 94.49, respectively, showing that TFDF has a higher response per unit concentration, indicating greater sensitivity under the experimental conditions. The intercepts for LAMI and TFDF were 0.565 and -17.59, respectively, with TFDF showing a slight negative intercept, which is likely due to the nature of the chromatographic method used.

The system suitability results confirm the robustness and efficiency of the chromatographic method used for both drugs. Retention times (RT) for LAMI and TFDF were 4.4638 ± 0.0084 minutes and **7**.4702 ± 0.0165 minutes, respectively. The Area under the curve (AUC) for LAMI was 714.5017 ± 5.8126 and for TFDF, it was 919.8905 ± 3.0910, reflecting the drug's overall response and efficiency in the system. The number of theoretical plates for both drugs was high (LAMI: 2610.1667 ± 61.3267, TFDF: 2671.3333 ± 21.5747), indicating good column performance and separation of the drug from other components. The Tailing factor for LAMI (1.2667 ± 0.1193) was higher than that for TFDF (1.0517 ± 0.0194)**,** suggesting that the TFDF chromatographic peak is more symmetrical and has better column efficiency.

The recovery studies for LAMI and TFDF showed good accuracy across different concentrations, with mean recoveries close to 100%. The repeatability**,** day-to-day variation, and analyst-to-analyst variation

studies for both drugs indicate minimal variation, suggesting that the method is precise and reliable. LAMI showed slightly higher variation in % RSD for recovery at 80% concentration (0.821%) compared to TFDF (1.515%), but both drugs demonstrated excellent repeatability and precision across different conditions. The robustness studies confirmed the stability of both drugs under slight changes in experimental conditions.

The Limit of Detection (LOD) for LAMI was found to be 0.15 µg/ml, and the Limit of Quantification (LOQ) was 0.45 µg/ml. For TFDF, the LOD was 0.20 µg/ml, and the LOQ was 0.75 µg/ml**.** These values indicate that both methods are sensitive enough to detect low concentrations of the drugs in formulation or plasma samples.

The % assay results for both drugs in tablet formulations were 99.80% for LAMI and 98.21% for TFDF, which are within acceptable limits for pharmaceutical formulations. The low % RSD values (0.125 for LAMI and 0.225 for TFDF) further confirm the precision of the assay method.

The forced degradation studies for LAMI and TFDF under various stress conditions (acidic, alkaline, oxidative, and photolytic degradation) revealed that both drugs maintained good stability, with less than 13% decomposition in most conditions. LAMI showed better stability compared to TFDF in alkaline hydrolysis, with only 9.97% decomposition versus 14.4% decomposition for TFDF.

Table 1: Separation Variable

Variable	Condition
Column	
Dimension.	250mm x 4.60mm
Particle Size	5µ
Bonded Phase	Octadecylsilane (C_{18})
Mobile Phase	
Methanol	40
Acetonitrile	60
Diluent	Methanol
Flow rate	1.0 ml/min
Temperature	Ambient
Sample Size	20 µl
Detection wavelength	254nm
Retention time	
Lamivudine	4.458 ± 0.02min.
Tenofovir Disoproxil Fumarate	7.458 ± 0.02min.

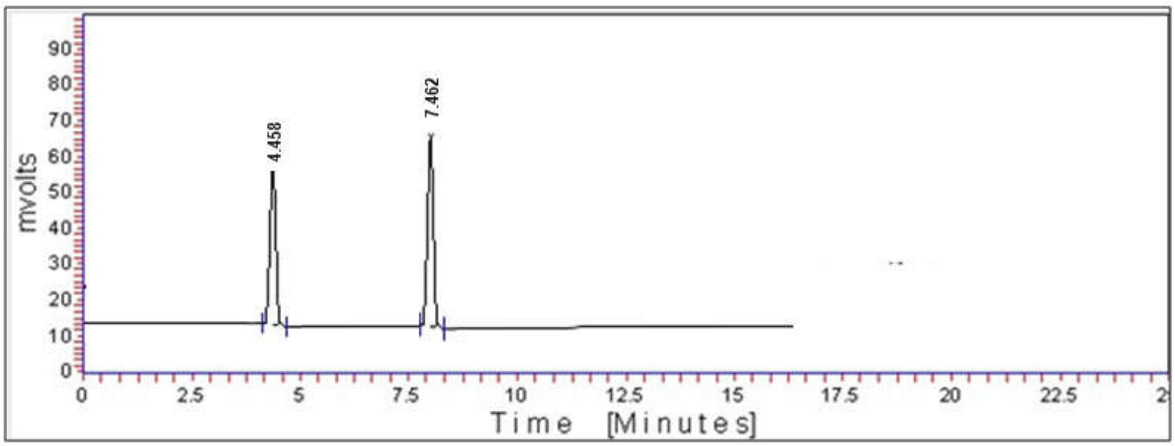

Figure 1: Chromatogram of Both the drug

Table 2: Results of Linearity

Parameter	LAMI	TFDF
Correlation Coefficient (r^2)	0.999	0.999
Slope (m)	71.67	94.49
Intercept (c)	0.565	-17.59

Table 3: Results of System Suitability Parameter

System Suitability Parameter	LAMI	TFDF
Retention Time (RT)	4.4638 ± 0.0084	7.4702 ± 0.0165
Area under Curve (AUC)	714.5017 ± 5.8126	919.8905 ± 3.0910
Number of Theoretical Plates	2610.1667 ± 61.3267	2671.3333 ± 21.5747
Tailing Factor	1.2667 ± 0.1193	1.0517 ± 0.0194

Table 4: Results of Validation parameters

Study/Parameter	Level	Concentration Found (%)	Mean (%)	SD	% RSD
Recovery Study of LAMI	80%	96.75, 98.75, 98.75	98.08	0.802	0.821
	100%	93.00, 97.60, 99.00	96.53	1.011	1.037
	120%	95.67, 99.33, 99.33	98.37	0.975	0.991
Recovery Study of TFDF	80%	93.50, 98.00, 99.80	96.68	1.465	1.515
	100%	97.60, 99.00, 99.67	98.65	0.336	0.341
	120%	97.50, 99.33, 99.83	98.76	0.263	0.266

Repeatability of LAMI	-	97.32 - 99.23	98.32	0.105	0.1094 - 0.1369
Repeatability of TFDF	-	96.6 - 99.10	98.33	0.130	0.1391 - 0.1612
Day-to-Day Variation of LAMI	-	96.07 - 98.99	98.03	0.130	0.101 - 0.173
Analyst-to-Analyst Variation of LAMI	-	98.43 - 99.58	98.81	0.109	0.072 - 0.165
Analyst-to-Analyst Variation of TFDF	-	97.10 - 99.00	98.47	0.120	0.094 - 0.153
Robustness of LAMI	-	97.4 - 99.22	98.40	0.110	0.0921 - 0.1368
Robustness of TFDF	-	97.44 - 98.91	98.29	0.108	0.1055 - 0.1418

Table 5: LOD and LOQ of LAMI and TFDF

Name	LOD (µg/ml)	LOQ (µg/ml)
LAMI	0.15	0.45
TFDF	0.20	0.75

Table 6: Result of assay of tablet formulation

	LAMI*	TFDF*
Label Claim (mg)	**300mg**	**300mg**
% Found (mg)	299.4	294.65
% Assay	99.80	98.21
% RSD	0.125	0.225

*Average of three determination

Table 7: Results of Forced degradation studies of LAMI

Stress conditions	Drug recovered (%)	Drug decomposed (%)
Standard drug	99.95	0
Acidic hydrolysis	87.85	12.1
Alkaline hydrolysis	89.98	9.97
Oxidative degradation	93.32	6.63
Photolytic degradation	91.14	8.81

Table 8: Results of Forced degradation studies of TFDF

Stress conditions	Drug recovered (%)	Drug decomposed (%)
Standard drug	99.85	0
Acidic hydrolysis	92.23	7.62
Alkaline hydrolysis	85.45	14.4
Oxidative degradation	91.45	8.4
Photolytic degradation	90.25	9.6

CONCLUSION

Overall, both LAMI and TFDF demonstrate good linearity, precision, stability, and accuracy in their analysis, making them suitable for routine pharmaceutical quality control. The forced degradation studies indicate that both drugs are stable under normal conditions but may degrade under more extreme conditions, highlighting the importance of proper storage and handling. The data supports the reliability of the proposed chromatographic methods for the quantification and quality control of these drugs in formulations.

DECLARATION OF INTEREST

The authors declare no conflicts of interests. The authors alone are responsible for the content and writing of this article.

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