RESEARCH PAPER

Stability-Indicating Analytical Method Development and Validation of a Tenofovir Disoproxil Fumarate in Bulk Drug and Pharmaceutical Dosage Form

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Abstract

The development and validation of a method to assess the stability of Tenofovir Disoproxil Fumarate (TDF) in bulk pharmaceutical and dosage forms have developed. TDF, a nucleotide reverse transcriptase, is used to treat HIV and chronic hepatitis B and is most probably degraded under different stressful conditions hence undermining its performance and safety. In this paper, High performance liquid chromatography (HPLC) is employed to develop a stable mechanism to determine the TDF and also to distinguish it with the products of its degradation. The flow rate of 1.0 mL/min, detection at 260 nm, and 50: 50 (v/v) mix of 0.01 M potassium dihydrogen phosphate buffer (pH 4.0) and acetonitrile optimized the method. Specificity, linearity ($r^2 > 0.999$), accuracy (98-102 % recovery) and precision of the method were confirmed with the help of validation studies (RSD values of less than 0.516 %). The forced degradation experiments showed that the method could be used to test the stability of TDF in acid, alkaline and oxidative conditions, thermal and photolytic conditions. The results suggest that the validated variation of the HPLC process is the suitable one that must be used in the everyday analysis of TDF, which helps to increase the quality regulation and perception of its stability in the pharmaceutical setting.

Keywords: disoproxil fumarate, Tenofovir, RP-HPLC, development of a method, validate, stability indicating method, forced degradation, ICH guidelines.

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1. Introduction

The nucleotide reverse transcriptase inhibitor tenofovir disoproxyl fumarate (TDF), formally named 9-[[(R)-2-[[bis[[(isopropoxycarbonyl)oxy]methoxy]phosphinyl] methoxy]propyl]adenine fumarate, serves as a notable example of this category of pharmaceuticals. has found its way to the limelight of management of human immunodeficiency virus (HIV) infection and chronic hepatitis B virus (HBV) infection (Gallant et al., 2004). A prodrug of tenofovir is all that TDF is; when used orally, it is chemically transformed to its active metabolite, the phosphorylated tenofovir diphosphate, which inhibits viral reverse transcriptase (Kearney et al., 2004). The drug has shown remarkable efficacy and tolerance in clinical settings when used in conjunction with other antiretroviral agents (Pozniak et al., 2006)

In order to facilitate the safety, efficacy, and quality of medications, the United States Pharmacopeia (USP) and other regulatory authorities require validated methods of analytical control of quality of the pharmaceutical products throughout a shelf life (Kalra, 2011). Especially the analytical techniques which reveal the state of stability prove to be important in detecting and establishing the degradation products which may result during manufacturing, storage or use of pharmaceutical products (Bakshi & Singh, 2002). International Conference on harmonisation (ICH), has also prepared specifications in both validation of analytical procedures (ICH Q2(R1)) and stability test of new drug substances and products (ICH Q1A(R2)) in great detail (International Conference on Harmonisation (ICH), 2003, 2005)

Literature Survey reviled that the reported methods of analysis of the TDF in the pharmaceutical formulation include: high-performance liquid chromatography (HPLC). Most of these methods however are limited by time complexity of its analysis, complicated mobile phase compositions or lack of validation of its ability to remain stable. Moreover, the forced degradation studies that show how TDF can be resolved by its products in the case of different stress factors are not commonly performed and reported (Ashenafi et al., 2010a;

Devrukhakar et al., 2013; Havele & Dhaneshwar, 2012; Kalra, 2011; Sumanth et al., 2018).

This paper aimed at developing and establishing a simple, fast, targeted and stability indicative reversed-phase HPLC method in order to determine the TDF content in bulk drugs and tablet. The procedure assessed using ICH Q2(R1) for specificity, linearity, precision, accuracy, robustness, limit detection (LOD) and limit-quantification (LOQ) and solution stability. Comprehensive forced degradation experiments were performed to analyze the degradation profile of TDF under various stress conditions, including acidic, alkaline, oxidative, thermal, and photolytic environments, to determine the stability-indicating characteristics of the method. It will be performed through the working technique of performing routine quality control and stability research of TDF pharmaceutical products (Ashenafi et al., 2010a; NN et al., 2018).

2. Materials and Methods

2.1 Chemicals and Reagents

Tenofovir Disoproxil Fumarate (TDF) reference sample & bulk drug was Purchased from Yarrow Chem Products, Mumbai, India and the identity, assay and purity parameters of the products were certified by certificates of analysis. Local markets purchased Tablet completion of 300mg TDF per tablet (label claim). All the chemicals and solvents were of HPLC-grade, including methanol, acetonitrile, and water that were bought at Spectrochem Pvt. Ltd., Mumbai, India. Analytical-grade acidic, alkaline, oxidative, thermal, and photolytic reagents of SD Fine-Chem Limited, Mumbai, India were taken in the case of forced degradation.

2.2 Chromatographic Equipment and Methods.

While developing the method, the HPLC was used, having the model Number Shimadzu LC-2010C HT (Shimadzu Corporation, Kyoto, Japan) with a quaternary pump, autosampler, column oven and photodiode array (PDA) detector. Data was acquired and processed by use of the LC Solution software (Shimadzu). PH changes have been made by using A Lab India pH meter. The process of the preparation of a sample was assisted by an ultrasonicator of Life Care Instruments Pvt. Ltd. and an analytical balance with the readability of 0.1 mg (Pravadali et al., 2013; Sadaphal & Dhamak, 2022; Zaza et al., 2015).

2.3 The following were the chromatographic conditions.

Agilent C18 (250 mm × 4.6 mm internal diameter, 5 µm particle size) was used as a column to do the chromatographic separation and was stored at ambient temperature. The mobile phase comprised a 0.01 M potassium dihydrogen phosphate buffer (pH adjusted to 4.0 using orthophosphoric acid) and acetonitrile in a 50:50 (v/v) ratio. A 0.45 µm membrane filter was used to filter the mobile phase and ultrasonicated and left to cool down to 15° C. Isocratic elution with a flow rate of 1.0 mL/min was used. This was added 20 µL and it was observed at 260 nm that is the highest absorption wavelength of TDF. The total run time was approximately 10 minutes and TDF eluted at a retention time of approximately 4.98 minutes.

2.4 Preparation of Solutions

2.4.1 Mobile Phase Preparation

A 0.01 M mobile phase buffer was made by dissolving 1.36 g of potassium dihydrogen phosphate in 1000 mL of HPLC-grade water. The pH was adjusted to 4.0 employing orthophosphoric acid. The buffer solution underwent filtration by means of a 0.45 µm membrane filter as well had been then degassed via sonication for a duration of 15 minutes before use. The mobile phase was prepared by mixing 50 mL of the buffer solution with 50 mL of HPLC-grade acetonitrile, achieving a 50:50 v/v ratio (TEJADA et al., 2021).

2.4.2 Diluent

The entire preparation of the sample and standards was performed using methanol (grade HPLC).

2.4.3. Preparation of Standard Solutions.

For preparing the standard stock solution, approximately 30 mg of TDF reference standard was precisely measured and added into a 100 mL volumetric flask. The standard was suspended in methanol and it was sonicated to make it dissolve fully. Methanol was used to adjust the stock solution to the specified volume, achieving a concentration of 300 µg/mL. Stock solution (5.0 mL) was pipeted in a 50 mL volumetric flask and diluted to volume using methanol to produce end standard solutions, which were 30 µg/mL in concentration.

2.4.4 Samples Preparation Solutions.

Bulk Drug Analysis: A pre-weighed amount of appropriate bulk drug of TDF (approximately 30 mg) was added to 100 mL of Volumetric flask and sonicated in methanol before being diluted to the final volume with methanol to achieve 300 ug/mL. Further dilutions were in the same manner.

Tablet Formulation Analysis: 20 tablets were weighed then the average weight was calculated. Using a mortar and pestle, the tablets were broken down into powdered form. The required amount of powder, corresponding to 300 mg of TDF, was accurately measured and placed into a 100 ml volumetric flask. Methanol was added (approximately 70 mL) and the mixture was sonicated after 15 minutes with several shakes to ensure that all the drug was extracted. Methanol was then added to take the volume up to 100 mL and filtered with 0.45 membrane filter. The 1.0 mL was then pipetted into a 100 mL volume flask diluted to volume with methanol to obtain an approximate 30 ug/mL sample solution (Anandakumar et al., 2013a; Bhavsar et al., 2012).

2.4.5 Placebo Preparation

To validate the interference of the excipients, placebo solutions containing all the tablet excipients except the active pharmaceutical ingredient were formulated as per the same procedure as that of preparation of the sample.

2.5 Method Validation

The limits of the designed HPLC procedure were established as per the specifications of ICH Q2(R1), the following parameters were established as specificity, linearity, precision (repeatability and intermediate precision), accuracy, limit of detection, limit of quantification, solution stability and robustness.

2.5.1 Specificity

Specificity was also evaluated by calculation of blank (methanol) and standard and sample solution with the intention of establishing that the diluent and excipients did not affect the retention time of TDF. The PDA detector was used to determine the purity of the peak such that the TDF peak was not contaminated by any other impurities or degradation products that accompanied the process (Anandakumar et al., 2013b; Ashenafi et al., 2010b).

2.5.2 Linearity and Range

A linear relationship was found using the six concentration levels, which ranged from 12.8 to 19.6 µg/mL, or 80 to 120 percent of the working value of 16 µg/mL. The calibration standards were prepared by diluting properly the stock solution. Three injections of individual concentrations were done with an observation of the peak area. The equation of area of the peak (y-axis) and the concentration (x-axis) was plotted, and the linear regression analysis was applied to estimate the slope, intercept and the correlation coefficient (r) to draw a calibration curve (Burrows and Watson, 2015).

2.5.3 Precision

Repeatability (Intra-day Precision): The precision of the system was discovered by using 6 replicate injections of the standard solution (30 µg/mL). The methods were also evaluated by subjecting six sample solutions which were made the same day by the same analyst to determine their precision. The relative standard deviation (RSD) and the percent assay were established.

Intermediate Precision (Inter-day Precision): In this case, the precision test was conducted on a different day and by another analyst with a new prepared mobile phase and with fresh prepared solutions. The results were compared to those of first day and total RSD was determined.

2.5.4 Accuracy (Recovery Study)

The developed method's accuracy was assessed through the standard addition technique at three distinct concentration levels: 80%, 100%, and 120% of the nominal target concentration. A specified amount of Tenofovir Disoproxil Fumarate reference standard was added to the pre-analyzed sample solution. The fortified samples underwent examination, and the percentage recovery was determined by calculating the ratio of the amount recovered to the amount added. Each concentration level underwent analysis in triplicate

(International Conference on Harmonisation (ICH), 2003, 2005).

2.5.5 Limit of detection (LOD) and Limit of quantification (LOQ).

The signal-noise-ratio and the standard deviation of the response and slope of the calibration curve method of ICH Q2(R1) guide have been used to calculate the LOD and the LOQ. LOD and LOQ were estimated using formulae:

$LOD = 3.3 \times \sigma / S$

$LOQ = 10 \times \sigma / S$

and where sigma is the standard deviation of the y-intercept of the regression line in and S is the slope of the calibration curve (International Conference on Harmonisation (ICH), 2003, 2005)

2.5.6 Solution Stability

The stability of TDF in the standard as well as the sample solution was determined by keeping the solutions at RT (25 ± 2°C) and then the analysis was undertaken of the solutions after 0, 8 and 24 hours. The percent discrepancy in the value of assays was determined so as to determine the stability of the solutions.

2.5.7 Robustness

Robustness was assessed by intentionally altering key chromatographic parameters and examining their impact on system suitability and assay outcomes. The parameters that were varied included; detection wavelength (260 ± 5 nm), flow rate (1.0 ± 0.1 mL/min) and composition of the mobile phase (± 5% buffer:acetonitrile). The procedure was considered sound whereby the RSD of percent assay and peak area were all acceptable (below 2.0-percent) and the error between normal assay and test was also below 2.0-percent.

2.6 Forced Degradation Studies

To demonstrate the stability indicating properties of the process forced degradation tests were performed, as well as to find out the inherent stability of TDF under varying stress conditions, as required by the ICH Q1A(R2) requirements (International Conference on Harmonisation (ICH), 2003, 2005).

2.6.1 Acidic Degradation

The 1 M hydrochloric acid (50 mL), was mixed with the TDF sample (300 mg) and heated at 60 °C over a period of 30 minutes. That solution was cooled and neutralized with the help of 1M sodium hydroxide and diluted appropriately with methanol for HPLC analysis.

2.6.2 Alkaline Degradation

TDF sample (synonymous to 300 mg) was mixed in 50 mL of 1 M sodium hydroxide and stirred and heated at 60 °C over a period of 30 minutes. After cooling, the solution was treated with 1 M hydrochloric acid to neutralize it, and then diluted with Methanol. HPLC analysis was subsequently done (International Conference on Harmonisation (ICH), 2003, 2005).

2.6.3 Oxidative Degradation

TDF sample (equivalent to 300 mg) was reacted with 50 mL of 3-percent hydrogen peroxide and it was refluxed in room temperature over a period of 30 minutes. Methanol was used to dilute the solution when necessary, so as to analyze the solution by use of HPLC (International Conference on Harmonisation (ICH), 2003, 2005).

2.6.4 Thermal Degradation

The sample (synonymous with 300 mg) weighing 300 mg was forced to dry under the hot heat (105°C) in a hot air oven and left to keep dry 6 hours. The product was heat-treated and left to cool to room temperature and it was then dissolved in methanol to analyze it using HPLC (International Conference on Harmonisation (ICH), 2003, 2005).

2.6.5 Photolytic Degradation

The sample of TDF (equivalent to 300 mg) was exposed to direct sunlight, which was 7 days in length. The sample was then subjected to light and then it was dissolved in the HPLC using a mixture of methanol (International Conference on Harmonisation (ICH), 2003, 2005).

A control sample (untreated) was prepared and analyzed equally in all stress conditions. The percentage degradation was determined by comparing the peak area of the stressed sample with that of the control sample. The percent assay over percent degradation calculated to ensure that all the degradation products were accounted was known as mass balance (International Conference on Harmonisation (ICH), 2003, 2005).

3. Results and Discussion

3.1 Method Development and Optimization

Good retention, sharpe peak shape and good resolution of TDF of the potential degradation products at an acceptable analysis time was the major objective of method development. The optimum of the methods was performed with various mobile phase combinations, pH and flow rate.

Initial acetonitrile-water mobile phases experiments had poor retention and poor peak shape. The addition of phosphate made the peaks more symmetrical and it resulted in greater control of the retention time. The appropriate pH of the buffer was determined to be 4.0 because this produced the optimum shape of the peak and retention. Blending 0.01 M potassium dihydrogen phosphate buffer (pH 4.0) and acetonitrile (50:50, v/v) has been determined to provide the most desirable mobile phase composition that results in TDF having a retention time of approximately 4.98 minutes and a great deal of symmetry in the peak.

The wavelength at which it was detected (260 nm) was selected since the UV absorption spectrum of TDF exhibits the greatest absorption in the 260 nm. The rate at which the flow was adjusted was 1.0 mL/min since this rate provided sufficient resolution and analysis time that was reasonable without creating too much column back pressure. The reason why methanol was selected as the diluent is that it provided a complete solution of TDF as well as TDF was not soluble in solvents that would not permit the separation of the chromatographic system.

3.2 System Suitability

System suitability parameters were also taken into consideration in determining the suitability of the chromatographic system in the analysis which was required. Parameters that were measured included; retention time, theoretical plates, tailing factor and relative standard deviation of repeat injections. Based on the chromatography results and the comparison with published TDF HPLC methods, the parameters of appropriateness of the system were the following: theoretical number of plates (N) = 5,000, tailing factor (T) = 1.1, and the RSD of six repeat injections area = 2.0% (Manikya Rao et al., 2013; Bhairav and Chavan, 2021). The validity of the chromatographic system was authenticated by making sure that the requirements of system suitability were achieved during validation study.

Table 1. System Suitability Parameters

Parameter	Observed Value	Acceptance Criteria	Status
Retention time (tR)	4.98 ± 0.01 min	RSD ≤ 2.0%	Pass
Theoretical plates (N)	> 5,000	> 2,000	Pass
Tailing factor (T)	≤ 1.1	≤ 2.0	Pass
Peak area RSD (n=6)	0.27–0.32%	≤ 2.0%	Pass

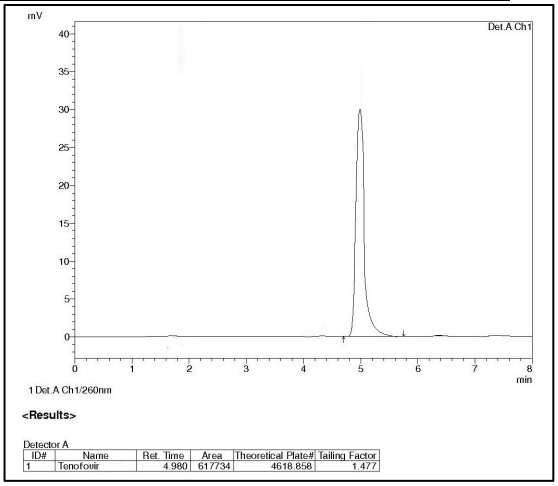

Figure 1: *Chromatogram of TDF standard solution showing retention time of 4.980 minutes*

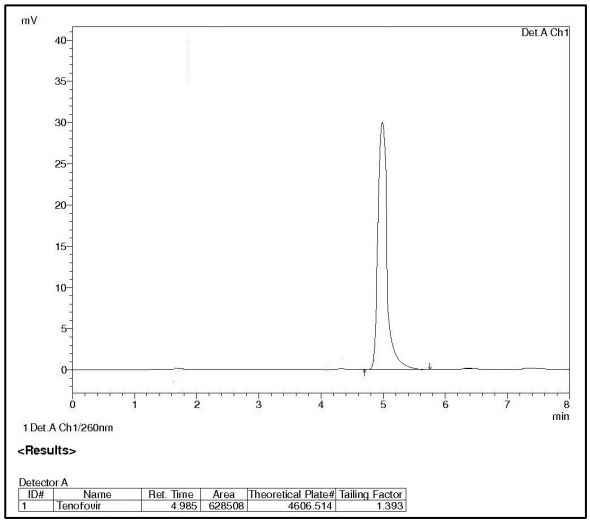

Figure 2: *Chromatogram of TDF sample solution showing retention time of 4.985 minutes*

3.3 Specificity

Specificity was found within the constraints of all the probable sample constituents, such as impurities and damage products, and excipients. The purity of the method was proven using reference to blank (methanol), standard solution, and sample solution. The effect of diluent and excipients interference on the retention time of TDF was not observed (4.98 minutes). Figure 2 showed a peak of the TDF of the sample solution as 4.985 minutes, no other excipient peaks were visible, but there was a peak that was well resolved with the maximum value of 4.980 minutes (Figure 1) that was observed with the standard solution. In order to ensure that TDF peak was homogenous and free of any co-eluting compound, the PDA detector was used to perform the PDA detector analysis.

The results are provided in table 2. The RSD of peak area for six replicate injections of the standard solution was 0.27%, well within the acceptance criterion of ≤ 2.0 %, confirming excellent system precision.

Table 2. Specificity Study Results

Injection	Peak Area	Retention Time (min)
1	616,539	4.980
2	618,660	4.980
3	617,734	4.980
4	616,575	4.980
5	618,402	4.980
6	616,539	4.980
Mean	**617,408**	**4.980**
SD	**1,667**	**0.000**
RSD (%)	**0.27**	**0.00**

The procedure proved to be highly specific as there was no blank interference or excipients at the retention time of TDF.

3.4 Linearity and Range

The linearity of the method in 12.8-19.6 µg/mL concentration range between the 80-120 % working concentration range was tested. The concentration had six levels and these concentrations were determined in triplicates and calibration curve was plotted between the maximum area and the concentration.

The correlation coefficient (r) of the calibration curve was determined to be highly linear with the value of 0.9995 that is much higher than the accepted value of 0.999 and above. The regression equation was 38459.32x +2706.77, with y representing the area of the peaks and x representing the concentration in 2 µg/mL (Figure 3). The correlation coefficient is high and the y-intercept is low which means there is high level of linearity and low level of systematic error.

Table 3. Linearity Data

Concentration (µg/mL)	% Level	Mean Peak Area (n=3)
0.0	0	0
12.8	80	497,339
14.4	90	559,507
16.0	100	621,674
17.6	110	683,841
19.6	120	746,009

Regression Statistics: - Slope: 38,459.32 - Intercept: 2,706.77 - Correlation coefficient (r): 0.9995 - Linearity range: 12.8–19.6 µg/mL

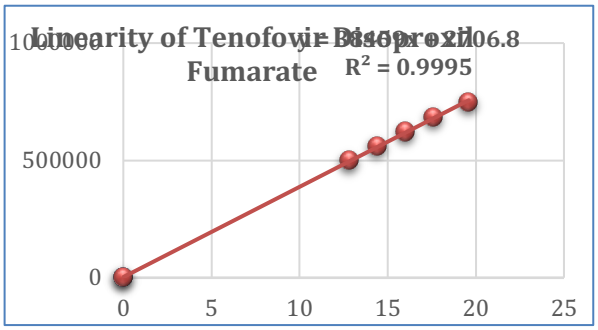

Figure 3: *Calibration curve showing linearity of TDF over the concentration range of 12.8–19.6 µg/mL]*

The high linearity is a hint that the method is suitable in the quantitative analysis of TDF within the specified concentration range.

3.5 Precision

The measurement of precision was done at two levels; repeatability (intra-day precision), and intermediate precision (inter-day precision).

3.5.1. Repeatability (Method Precision)

Repeated injections of the standard solution were used to test the precision of the system 6 times. The peak area RSD was established at 0.30 %, which was a great performance by the system. The determination of the method precision was done by comparing the six solutions of the samples that were prepared on the same day separately. Percent assay was found to be between 99.62-100.85 with the mean of 100.16 % and RSD of 0.63 %. The value of RSD is lower indicating that the method is very repeatable.

Table 4. Precision Study Results

Parameter	System Precision	Method Precision
Number of determinations	6	6
Mean peak area / % assay	619,738	100.16%
Standard deviation	1,831	0.628
RSD (%)	0.30	0.63
Acceptance criteria	≤ 2.0%	≤ 2.0%
Status	Pass	Pass

3.5.2 Intermediate Precision

Intermediate precision was also challenged by subjecting the precision study to another day using a different analyst using freshly prepared mobile phase and solutions. The values of the percent test were between 99.20 - 99.89 with the mean of the values being 99.61 and RSD 0.36. The two values of the upper and lower control limit of the two days differed by 0.55 percent which falls within the acceptability limit of 2.0 percent.

Table 5. Intermediate Precision Results

Parameter	Day 1 (Analyst 1)	Day 2 (Analyst 2)
Number of determinations	6	6
Mean % assay	100.16	99.61
Standard deviation	0.628	0.363
RSD (%)	0.63	0.36
Difference between days	-	0.55%
Acceptance criteria	≤ 2.0%	≤ 2.0%
Status	Pass	Pass

The high precision and intermediate precision values indicate that the method is highly reproducible and routine analysis can be conducted using the method.

3.6 Accuracy

The term accuracy is used to refer to the location of a value in relation to the value that is considered as a conventional true value or accepted reference value. Precision at three target concentration of 80, 100 and 120 percent were determined using the standard addition technique. Sample solution pre-analysis was performed by addition of the known quantity of TDF reference standard and the percent recovery determined(International Conference on Harmonisation (ICH), 2005).

The percent recovery was 99.26 up to 100.47 percent with mean of 99.85 and RSD of 0.61. All the values of the recovery were in the 98.0-102.0 % acceptance criterion and this demonstrates that the method was highly precise.

Table 6. Accuracy Study Results

Spike Level	Amount Added (mg)	Amount Recovered (mg)	% Recovery
80%	0.024000	0.024114	100.47
100%	0.030000	0.029779	99.26
120%	0.036000	0.035929	99.80
Mean	-	-	**99.85**
SD	-	-	**0.61**
RSD (%)	-	-	**0.61**

The fact that the rates of recovery are high testifies that the approach is right and contains no systematic errors.

3.7 Limit of Detection and Quantification

The LOD and LOQ using the Signal to Noise ratio method and standard deviation of the response and slope method were determined as 0.249 µg/mL and 0.755 µg/mL, respectively. These coincide with those values in the literature on the use of the similar RP-HPLC methods on TDF (Anandakumar et al., 2013b).

Table 7. LOD and LOQ Values

Parameter	Value (µg/mL)	Method
LOD	0.249	3.3 × σ/S
LOQ	0.755	10 × σ/S

The found values demonstrate that the method possesses sufficient sensitivity to measure TDF in pharmaceutical formulations and also in stability studies to identify degradation products.

3.8 Solution Stability

The stability of the solution was also determined for standard and sample solution of TDF at room temperature (25 ± 2 °C). Analyses were done after 0, 8 and 24 hours and percent difference in assay values calculated. The results showed that the TDF solutions had a stability of 24 hours and it had a difference of only 0.13 percent at 8 hours and 0.82 percent at 24 hours which is in the acceptable range of ≤ 2.0%.

Table 8. Solution Stability Results

Time Point	Mean % Assay	% Difference from Initial
Initial (0 h)	100.16	-
8 hours	100.03	0.13
24 hours	99.34	0.82

The solution is good and allows a large number of samples to be analyzed simultaneously without the concerns of a degraded analyte during the entire duration of the analysis.

3.9 Robustness

The robustness is the ability of the method to hardly change when there is a slight change in method parameters but the change is deliberate. The stability of the procedure was attributed by adjusting the detection wavelength (255 and 265 nm), flow rate (0.9 and 1.1 mL/min) and mobile phase composition (47.5:52.5, 52.5:47.5). RSD of the peak area and percent assay had all the changes that were below 2.0 percent and less difference between normal assay and method which means that the method is robust to deliberate changes in chromatographical conditions.

Table 9. Robustness Study Results

Parameter Varied	Variation	RSD (%)	% Difference from Normal	Status
Wavelength	255 nm	< 2.0	< 2.0	Pass
Wavelength	265 nm	< 2.0	< 2.0	Pass
Flow Rate	0.9 mL/min	< 2.0	< 2.0	Pass
Flow Rate	1.1 mL/min	< 2.0	< 2.0	Pass
Mobile phase	47.5:52.5	< 2.0	< 2.0	Pass
Mobile phase	52.5:47.5	< 2.0	< 2.0	Pass

The strength of the approach ensures a stable functionality of the approach in the conditions of normal use and gives confidence in the use of the method in everyday analysis (Bhairav & Chavan, 2021).

3.10 Forced Degradation Studies

The forced degradation tests were carried out in order to establish the stability implying capacity of the technique and also in order to establish the natural stability of TDF under various stressful conditions. The TDF samples were exposed to the stress conditions of acid, alkali, oxidative, thermal and photolytic and degraded samples were analyzed through the developed HPLC methodology (International Conference on Harmonisation (ICH), 2005).

3.10.1 Acidic Degradation

TDF degraded considerably in acidic conditions (1 M HCl, 60 °C, 30 minutes) with a degradation percentage of 36.80. The chromatogram of the acid stressed sample had other peaks that were degradation products and they were well separated with the main TDF peak. The total percentage assay (percent-degradation) was 100.38, which meant that there were no degradation products omitted.

3.10.2 Alkaline Degradation

TDF was degraded by 21.65 % under the influence of alkaline stress (1 M NaOH, 60 °C, 30 minutes). In the chromatogram, the peaks of degradation products were clearly separated with the peak of TDF. The mass balance was 100.37 %.

3.10.3 Oxidative Degradation

TDF underwent degradation of 23.16 percent when exposed to conditions of oxidative stress (3 percent of H_2O_2, room temperature, 30 minutes). The chromatogram indicated the presence of oxidative degradation products that had a good resolution relative to that of the parent drug. The mass balance was 100.38 %.

3.10.4 Thermal Degradation

TDF was not found to be very degraded in thermal stress conditions (105 °C, 6 hours, 4.49 percent), signifying good thermal stability. The mass balance was 100.37 %.

3.10.5 Photolytic Degradation

TDF exhibited low degradation (3.36) under the stress of photolytic degradation (direct sunlight, 7 days), which implies that it was well-stable under photolytic degradation. The mass balance was 100.38 %.

Table 10. Forced Degradation Study Results

Stress Condition	Conditions Applied	Peak Area	% Assay	% Degradation	Mass Balance (%)
Control	No stress	622,304	100.38	0.00	100.38
Acidic	1 M HCl, 60°C, 30 min	394,172	63.58	36.80	100.38
Alkaline	1 M NaOH, 60°C, 30 min	488,055	78.72	21.65	100.37
Oxidative	3% H_2O_2, RT, 30 min	478,722	77.22	23.16	100.38
Thermal	105°C, 6 hours	594,450	95.88	4.49	100.37
Photolytic	Sunlight, 7 days	601,476	97.02	3.36	100.38

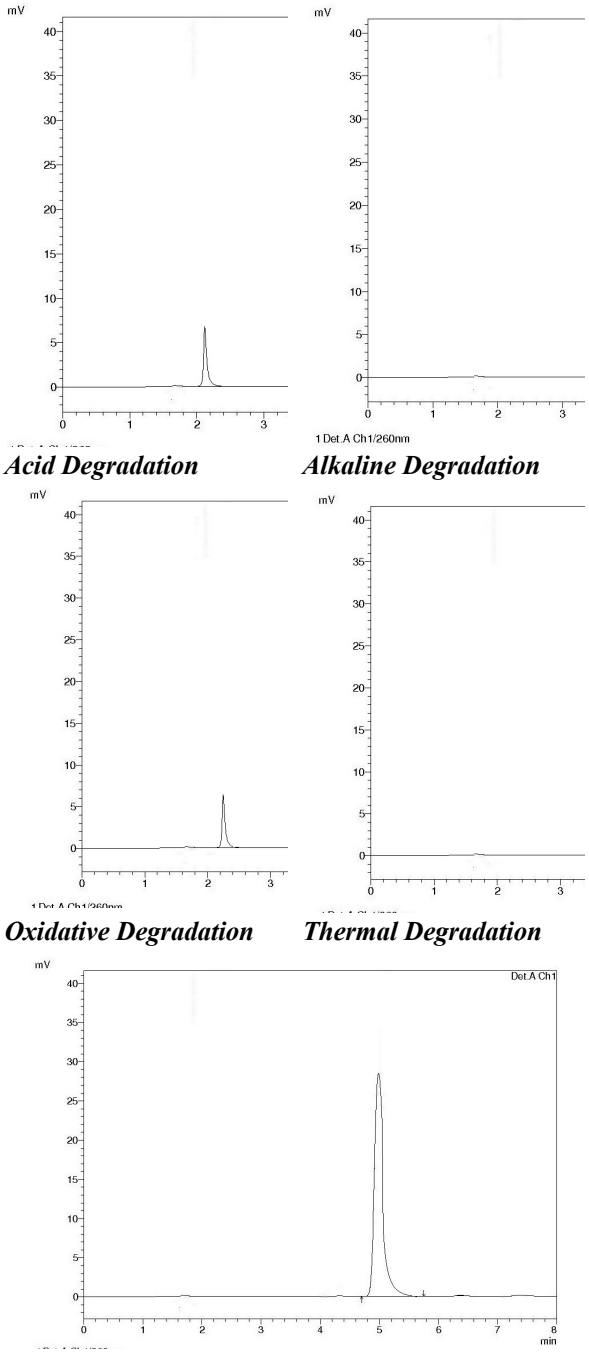

Figure 3: Chromatograms of forced degradation samples showing resolution of TDF from degradation products

The forced degradation experiments showed TDF is most vulnerable to acidic hydrolysis, then oxidative degradation, and alkaline degradation, and resistant to thermal and photolytic degradation stress conditions. The high mass balance (100.37-100.38 %), in all stress conditions proves the stability-indicating character of the technique, and proves that all the products of degradation are sufficiently covered and considered. These observations are in tandem with the established chemical structure of TDF that is composed of ester bonds that are prone to hydrolysis at acidic and alkaline temperatures (International Conference on Harmonisation (ICH), 2005).

3.11 The Assay of Pharmaceutical Formulations

The approved procedure was used to test TDF in bulk drug and two commercial tablet preparations (T-I and T-II) with each having 300 mg of TDF per tablet. It was found that the bulk drug had 99.80 % of laboratory label, whereas T-I and T-II tablet formulations had 99.90 % and 100.35 % laboratory label, respectively. The tested products were of good quality and all assay values fell under the acceptance criteria of 98.0-102.0 percent bulk drug and 90.0-110.0 percent tablet formulations.

Table 11. Assay Results of TDF in Bulk Drug and Tablet Formulations

Sample	Label Claim (mg)	% Assay	Specification	Status
Bulk Drug	-	99.80	98.0–102.0%	Pass
Tablet T-I	300	99.90	90.0–110.0%	Pass
Tablet T-II	300	100.35	90.0–110.0%	Pass

The effectiveness of the technique when applied to pharmaceutical preparations proves the suitability of the technique in everyday quality control examination.

4. Discussion

Analytical methods of drugs to indicate their stability are a necessity requirement to ensure quality of drugs, safety, and efficacy of drugs via the product lifecycle (Bakshi & Singh, 2002). The current paper has succeeding in developing and demonstrating an effortless, rapid and stability uncovering RP-HPLC methodology to find TDF in bulk pharmaceutical dosage type and drug in ICH protocols.

Chromatography parameters yielded the best separation of TDF with a retention time of 4.98 minutes that is comparable with the literature values of the same retention time of TDF using C18 columns and buffer-acetonitrile as mobile phases in a similar RP-HPLC technique. The technique is appropriate because the retention time is relatively short as well as the total run time of 10 minutes, which is optimal in quality control laboratory. The method is also more practical and economical in place of a complex of isocratic mobile phase composition and ambient column temperature(TEJADA et al., 2021).

According to the validation results, the method meets all the specificity, linearity, precision and accuracy requirements of the ICH Q2(R1) specificity, linearity, precision and accuracy, range, LOD, LOQ, solution stability and robustness (ICH, 2005). The excellent linearity (r = 0.9995) of the 80-120 percent working range of the concentration gives the quantification accuracy of the pharmaceutical formulations on the basis of the anticipated range of concentration. The values of RSD of precision (0.63) and intermediate precision (0.36) are small, which is very good reproducibility, which is needed in the routine quality control applications. The arithmetic mean of the recovery of 99.85 and the RSD of 0.61 shows that the method is right and the systematic errors and the matrix effects are absent.

The forced degradation tests were helpful in observing the behavior of TDF in degradation under various stresses. The property of TDF being the most vulnerable to the acidic hydrolysis (36.80 percent degradation) follows the presence of ester bonds in TDF molecule, which is volatile when the environment is acidic (Kearney et al., 2004). The degradation Under the oxidative stress is (23.16 %) and alkaline (21.65 %) shows that the formulations of TDF should be Protected from the Oxidizing agents and the alkaline Environments. The small degradation of thermal (4.49 %) and photolytic (3.36 %) depicts that TDF possesses good inherent stability to heat and light of which is a good product storage and handling indicator.

The remarkable mass balance (100.37-100.38 %), which is observed in all stress conditions is a crucial sign of the nature of the method, it is stable. It demonstrates that the technique is capable of quantifying and determining all the major products of degradation which occur under stress conditions ensuring that no major products of degradation are overlooked. The feature is required in stability research and in the search of the potential flaws in quality in the cycle of developing and producing products (International Conference on Harmonisation (ICH), 2005)..

A comparison of developed method with published HPLC methods of TDF reveal that there are several advantages. Majority of the stated reported methods utilize similar principles of chromatography, though the methodology offers a justifiable tradeoff between ease, speed, and the general validation (Agrahari & Youan, 2012; HoneyDiana et al., 2015; Omoteso et al., 2022; Patel et al., 2021; Runja et al., 2016; Venkatesan et al., 2014) This retention time of 4.98 minutes compares or even falls short of various published techniques as well as the forced degradation experiments that were exhaustive and the mass of the sample was well balanced is a good indicator of the stability signaling ability. The power of the approach to intentional adjustments of the chromatographic conditions is that it can be reliable in other labs and also within other working environments.

The technique has been found to be practically helpful as demonstrated by the effective use of the technique on the analysis of bulk drug and commercial tablet preparations. The results of the assays (99.80 -100.35 percent) obtained demonstrate that the tested products fall within the specification of the quality and, the method can be used in regular analysis of TDF pharmaceutical products.

There are some negative aspects of the study that are welcome. Even though it was a successful method to remove TDF using the products of the degradation, the study did not characterize the products of the degradation (e.g., using mass spectrometry). Such characterization would provide a better insight into the pathways of degradation and perhaps become the object of the future research. In addition, though the process was also found to be valid in tablet preparations, its application in other dosage forms (e.g. oral solutions, combination preparations) would have to be confirmed.

5. Conclusion

The appropriate, quick, accurate, and stability-relevant RP-HPLC method was effectively developed and confirmed to measure tenofovir disoproxil fumarate in large quantities of drug and pharmaceutical dosage. It uses a basic isocratic elution by a C18 column and a mobile phase of buffer and acetonitrile (C2) that yielded a retention time of 4.98 minutes and an overall run time of some 10 minutes. It was proved that the procedure was compliant with ICH Q2(R1) specifications and was shown to be quite effective with all the validation parameters such as specificity, linearity (r = 0.9995), precision (RSD = 0.63%), accuracy (mean recovery = 99.85%), LOD (0.249 µg/mL), LOQ (0.755 µg/mL) and solution stability and robustness.

Experiments on forced degradation carried out in the ICH Q1A(R2) guidelines established that TDF is the most vulnerable to acidic hydrolysis (36.80 % degradation), oxidative (23.16 % degradation), and alkaline (21.65 % degradation) degradation and tends to be well-stabilized to thermal (4.49 % degradation) and photolytic (3.36 % degradation) degradation. The stability-connoting condition of the method indicated by the fact that the mass balance (100.37-100.38) is also excellent in all the stress conditions and the potentiality of finding solutions to degradation products as well as quantitative degradation.

The standard method can be used in normal quality control analysis of TDF in bulk dosage drug and pharmaceutical samples and in stability studies. Its simplicity, speed and the fact that it can be fully validated does make it an attractive option to the less simple or time-consuming methods which may be found in the literature.

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