RESEARCH ARTICLE

Stability Indicating HPTLC Method for Estimation of Tenofovir Disoproxil Fumarate in Bulk and Tablet Dosage Form

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Abstract

Objective: A simple, precise, accurate, and rapid validated stability indicating high-performance thin-layer chromatography (HPTLC) method for estimation of Tenofovir Disproxil Fumerate was successfully developed. **Materials and Methods:** The method is based on HPTLC separation followed by densitometric measurements of their spots at 266 nm. This method is based on HPTLC separation followed by UV detection at 266 nm. The separation was carried out on merckTLC aluminum sheets precoated with silica gel 60F254 using a camag Linomat 5. **Results and Discussion:** The mobile consists of Butanol:Ethyl acetate: Acetic acid (4.5:0.5:0.3 v/v). Calibration curves were linear in the range of 300–1800 ng/band,respectively Tenofovir disoproxil fumarate gave sharp and well-defined peaks at Rf value are 0.51, respectively,stress degradation study shows that sample degraded with acid and base hydrolysis, under oxidation, thermal, and photolytic stress conditions. No chromatographic interferences from the tablet excipients were found. The method was validated in accordance with the requirements of International Conference on Harmonization guidelines **Conclusion:** Proposed method is precise, selective and accurate for the estimation of tenofovir disoproxil fumarate in the bulk and dosage form.

Key words: Forced degradation, high-performance thin-layer chromatography, tenofovir disoproxil fumarate, validation

INTRODUCTION

Tenofovir disoproxilis is chemically "9[(R2[[bis[[(iso propoxy carbonyl)oxy] methoxy] phospo phenyl]methoxy] propyl] adenine fumarate is a nucleotide analog reverse-transcriptase inhibitor.[1,2] It selectively inhibits viral reverse transcriptase, a crucial enzyme in retroviruses such as human immunodeficiency virus (HIV), Tenofovir disoproxil, is a medication used to treat chronic hepatitis B and to prevent and treat HIV/AIDS.[1] It is generally recommended for use with other antiretrovirals.[3,4]

The chemical structures of Tenofovir dioproxil are shown in Figure 1.

The literature survey makes known that very few analytical methods have been conveyed for the estimation of Tenofovir disoproxil fumarate (TDF).[5-9] Hence, considering inherent advantage of high-performance thin-layer chromatography (HPTLC) over HPLC, the aim and objective of the present work were to develop and validate a stability indicating simple, precise, sensitive high-performance thin-layer chromatographic method for estimation of TDF in its bulk and tablet dosage form[10-12] and validate as per International Conference on Harmonization (ICH) Q2 (R2) guidelines.[13,14]

EXPERIMENTAL

Materials and reagents

TDF was kindly supplied as a gift sample by Spectrum Research Labs, Hyderabad. India. All chemicals and

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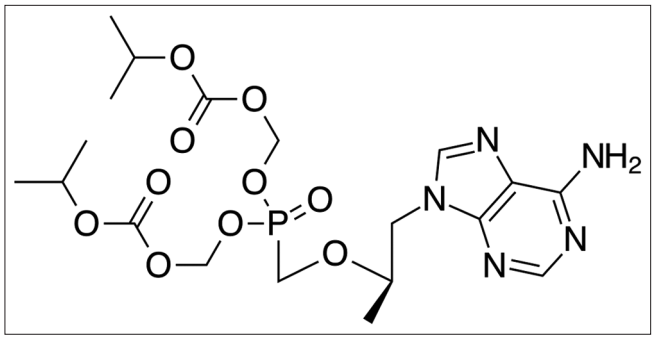

Figure 1: Chemical structure of Ritonavir

Figure 2: Instrumentation of high-performance thin-layer chromatography

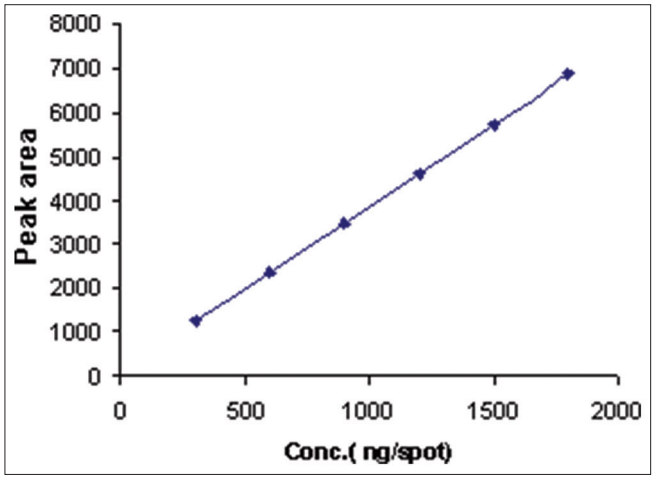

Figure 3: Calibration curve of tenofovir disoproxil fumarate, Y=3.7603 X +105.85; where, Slope =3.7603, Intercept=105.85, Correlation coefficient =0.9999

reagents employed were of analytical grade and were purchased from Rankem, Avantor Performance Material India Limited, Mumbai and used without further purification.[15]

Instrumentation and chromatographic condition

The HPTLC system (CAMAG, Switzerland) consisted of Linomat V auto sampler connected to a nitrogen cylinder, a twin trough chamber (20 × 10 cm), a derivatization chamber, a plate heater, TLC Scanner IV (Camag Muttenz, Switzerland), UV lamps, and UV cabinet with dual wave length and win CATS software was used for chromatographic study. Electronic analytical balance

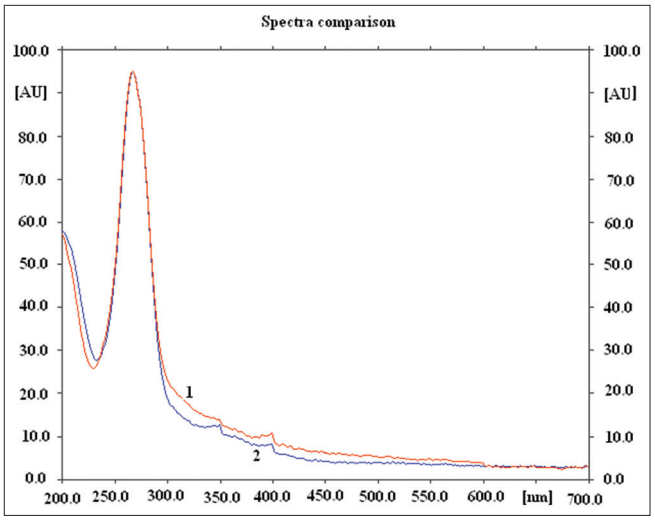

Figure 4: Absorption spectra of TDF from (1) standard TDF and (2) TDF extracted from tablet solution

Table 1: Linearity study of TDF

S. No.	Concentration (ng/spot)	Peak area (mean±SD; *n*=5)	% RSD
1	300	1257.08±22.09	1.76
2	600	2323.00±37.30	1.61
3	900	3494.14±13.32	0.38
4	1200	4622.34±79.01	1.71
5	1500	5725.86±64.84	1.13
6	1800	6892.26±38.14	0.55

TDF: Tenofovir disoproxil fumarate

Table 2: Results of bulk TDF

Drug	Amount taken (ng/spot)	Amount found (ng/spot)	Amount found (%)
	600	599.75	99.95
	600	594.69	99.11
TDF	600	600.11	100.01
	600	597.97	99.66
	600	597.86	99.64
	Mean	598.76	99.67
	SD	2.15	0.36
	% RSD	0.36	0.36

(ShimadzuAUX-220) was used for all the weighing purpose[15].[Figure 2].

The HPTLC analysis was performed on Pre-coated SilicaGel 60 F_{254} HPTLC plates (20 × 10 cm, layer thickness 0.2 mm (E. Merck KGaA, Darmstadt, Germany). HPTLC plates were pre-washed with 10 mL of methanol and activated at 80ºC for 5 min before application of sample. The standard and formulation samples of TDF were spotted using a Linomat 5 auto sampler fitted with a 100 µL Hamilton

syringe (CAMAG, Muttenz, Switzerland) and operated with settings of a band length of 3.5 mm; band distance of 7.2 mm; distance from the side of plate of 10 mm; and distance from the bottom of the plate of 10 mm. The plates were developed to a distance of 70 mm in a mobile phase consisting of Butanol: Ethyl acetate: Acetic acid (4.5:0.5:0.3 *v*/*v*) and development was approved out in twin trough chamber (20 × 10 cm) pre-saturated with the mobile phase. The developed HPTLC plates were air dried and densitometric scanning was completed on CAMAGTLC scanner III in absorbance mode equipped with WINCATS planar chromatography manager (version 1.4.6) software. The spots were analyzed at a wavelength of 260 nm. The scanning of the spots was done at a rate of 20 mm/s. Evaluation was performed using linear regression analysis through peak areas.[10,16]

- Stationary phase: Precoated silicagel $60F_{254}$ HPTLC aluminum plates (20 × 10 cm, 0.2 mm thick)
- Mobilephase: Butanol: Ethyl acetate: Acetic acid (4.5:0.5:0.3 *v*/*v*)
- Saturation time: 20 min
- Wavelength: 266 nm
- Lamp: Deuterium.

Table 3: Results of tablet formulation

Brand Name: TENVIR Batch No.: D70816		Mfg. By: Cipla Ltd., Solan Avg.Wt. : 0.6838 g	
Drug	**Label claim (mg)**	**Amount found (mg)**	**Amount found (%)**
TDF	300	301.18	100.39
	300	295.32	100.01
	300	302.97	100.99
	300	300.96	100.31
	300	301.3	100.43
	Mean±SD	300.34±2.92	100.4±0.98
	%RSD	0.97	0.98

Linearity study

Different volumes in the range 0.3–1.8 µL of TDF standard stock solution were applied on TLC plate by microliter syringe with the help of automatic sample applicator Linomat5 to obtain concentration 300, 600, 900, 1200, 1500, and 1800 ng. The plate was developed and scanned and as described in Section 6. Calibration curve was constructed by plotting the peak area versus corresponding drug concentration. The results are reported in Table 1 and the calibration curve in Figure 3.

APPLICATION OF THE PROPOSED METHOD FOR ESTIMATION OF THE DRUGS IN BULK

Correctly weighed 10 mg of TDF was transferred to 10 mL volumetric flask, dissolved in methanol and bulk was made up to mark. An appropriate volume 0.6 µL, containing 600 ng of TDF was spotted; the plate was developed and scanned. The concentration was determined by a linear regression equation and the results are shown in Table 2.

APPLICATION OF THE PROPOSED METHOD FOR ESTIMATION OF THE DRUGS IN TABLETS

To determine the content of TDF in tablets; 20 tablets were weighed; their average weight was determined and ground to fine powder. A quantity equivalent to 200 mg of TDF was transferred to 100 mL volumetric flask and extracted with methanol for 20 min by shaking mechanically. The solution was diluted to volume with the same solvent and filtered. Appropriate volume 0.3 µL was spotted on TLC plate, developed, and scanned as described in Figure 4.

Table 4: Results of recovery studies

Drug	Initial amount (ng)	Amount added (ng)	Amount recovered SD (*n*=3)	% recovered	%RSD
TDF	600	0	601.30±4.08	100.21	0.678
	600	480	475.94±3.23	99.61	1.065
	600	600	608.20±4.12	101.36	0.607
	600	720	719.26±4.88	99.89	0.750

Table 5: Results of precision studies (intraday and interday)

Drug	Conc. (ng/spot)	Intraday amount found (ng)		Interday amount found (ng)	
		Mean±SD (*n*=3)	%RSD	Mean±SD (*n*=3)	%RSD
TDF	600	605.33±6.43	1.06	606±3.61	0.59
	900	902.33±3.51	0.39	908±3.00	0.33
	1200	1211.66±3.78	0.31	1210±7.21	0.60

Table 6: Results of repeatability studies

S. No.	Application volume (µL)	Area TDF (600 ng/spot)
1	6.0	2364.2
2	6.0	2344.8
3	6.0	2355.4
4	6.0	2348.2
5	6.0	2363.7
6	6.0	2345.6
	Mean	2353.65
	SD	8.81
	% RSD	0.37

Table 7: Results of ruggedness studies

Analyst	%Amount found (*n*=3)	%RSD
I	100.55	0.38
II	100.04	0.32

Table 8: Results of robustness studies

Parameters	SD of peak area (*n*=6)	%RSD
Mobile phase composition		
(Butanol: Ethylacetate: Aceticacid; 4.0:1.0:0.3)	38.45	1.63
(Butanol: Ethylacetate: Aceticacid; 4.8: 0.2:0.3)	41.09	1.74
Mobile phase volume		
5.3 mL	9.51	0.4
10.3 mL	20.08	0.85
Development distance		
7 cm	14.84	0.63
7.5 cm	16.90	0.71
8 cm	26.08	1.10
Relative humidity		
55	15.91	0.67
65	21.72	0.92
Duration of saturation		
20 min	19.11	0.81
25 min	20.81	0.88
30 min	29.40	1.24
Activation of prewashed TLC plates		
8 min	11.06	0.47
10 min	17.04	0.72
12 min	28.91	1.05
Time from spotting to chromatography	13.51	0.57
Time from chromatography to scanning	9.25	0.39

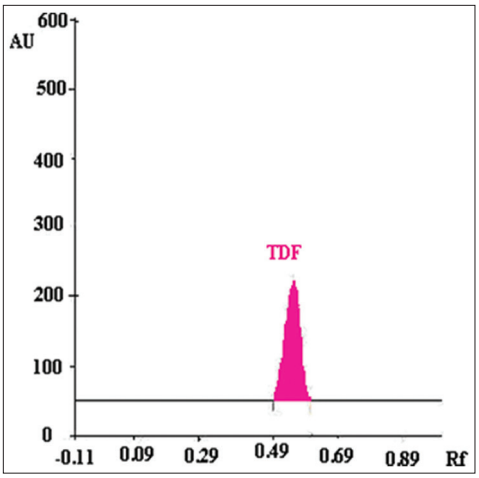

Figure 5: High-performance thin-layer chromatography chromatogram from solution (R_f=0.51)

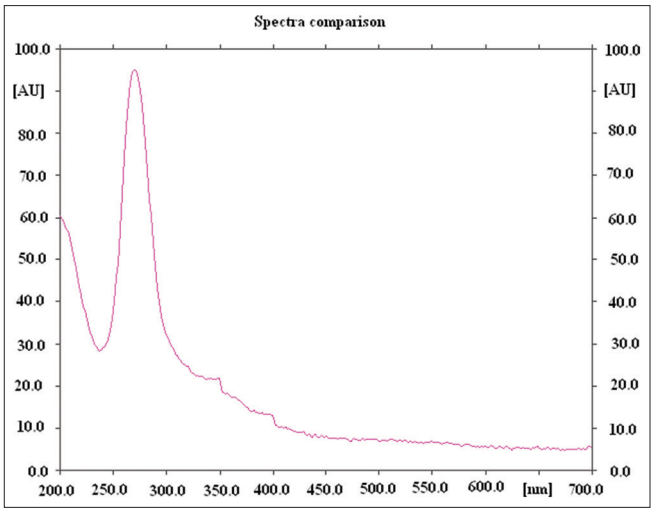

Figure 6: Absorption spectra of Tenofovir disoproxil fumarate standard extracted from tablet formulation

The concentration was determined using a linear regression equation. The results of the assay are shown in Table 3.

ANALYTICAL METHOD VALIDATION

The developed method was validated for different parameters such as linearity, precision, accuracy, specificity, ruggedness, robustness, limit of detection (LOD), and limit of quantitation (LOQ) as per ICH Q2 (R_1) guidelines.

Accuracy

Recovery study was carried out by over spotting 80%, 100%, and 120% of the standard drug solution of TDF and the mixtures were reanalyzed by the proposed method. The experiment was conducted in triplicate. This was done to check the recovery of the drug at different levels in the formulation. The results of % recovery are calculated and shown in Table 4.

Precision (intraday and interday precision)

Precision was assessed as intraday and interday variations. Intraday precision was determined by analyzing three different concentrations 600 ng, 900 ng, and 1200 ng of TDF, for 3 times within a day. Interday precision was assessed using the same concentration of drug (mentioned above) and analyzing it for 3 different days, over a period of week. The results are shown in Table 5.

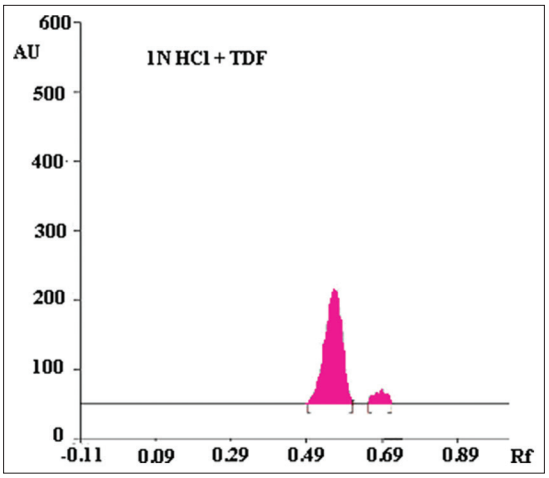

Figure 7: 1 M HCl + TDF

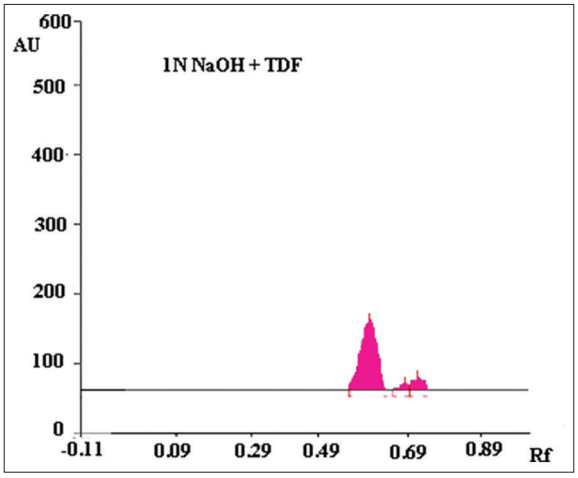

Figure 8: 1 M NaOH + TDF

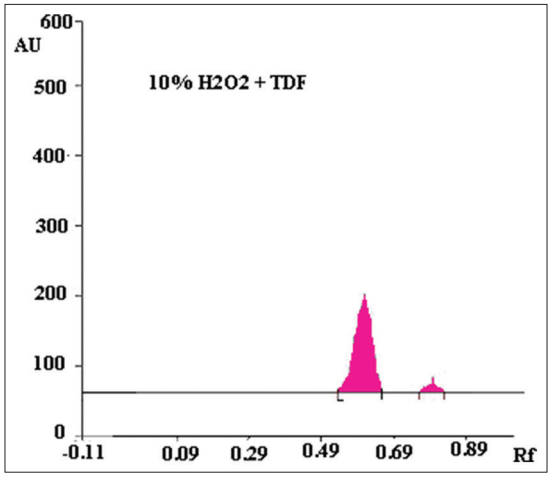

Figure 9: 10% H2O2 + TDF

Repeatability

Repeatability of sample presentation was assessed by spotting 0.6 µL containing 600 ng of TDF 6 times on a TLC plate followed by development and scanning. The results are shown in Table 6.

Sensitivity

Sensitivity of the proposed method was estimated in terms of LOD and LOQ. The LOD and LOQ were calculated by the use of the equation LOD = 3.3 XN/B and LOQ = 10 X N/B; where, "N" is the standard deviation of the peak areas of the drugs (n = 3), taken as a measure of noise, and "B" is the slope of the corresponding calibration curve. Different volume of stock solution in the range 300–600 ng was spotted on TLC plate. The procedure was repeated in triplicate. The linearity equation was found to be Y = 3.7603X +138.71. LOD and LOQ were found to be 29.90 and 90.61, respectively. (Where, N = 34.0706, B = 3.7603).

Specificity

To confirm the specificity of the proposed method, TDF tablet solution was spotted on TLC plate, developed, and scanned as described in Section 6. The mobile phase designed for the method resolved TDF very efficiently Figure 5. Typical absorption spectrum of TDF is shown in Figure 6. The peak purity of TDF was tested by correlating the spectra of TDF extracted from tablets and standard at the peak start (S), peak apex (A), and at the peak end (E) positions Figure 4. Correlation between these spectra indicated purity of TDF peak {correlation r (S, M) =0.998, r (M, E)=0.999}.

Ruggedness

Ruggedness of the proposed method was studied by two dissimilar analysts using the same experimental and environmental conditions. The spot 600 ng TDF was applied on TLC plates. The development and scanning of spots were performed as discussed in Section 6. This procedure was repeated in triplicates; the results are shown in Table 7.

Robustness

Robustness was studied in six replicates at the concentration level of 600 ng/spot. In this study, seven parameters (mobile

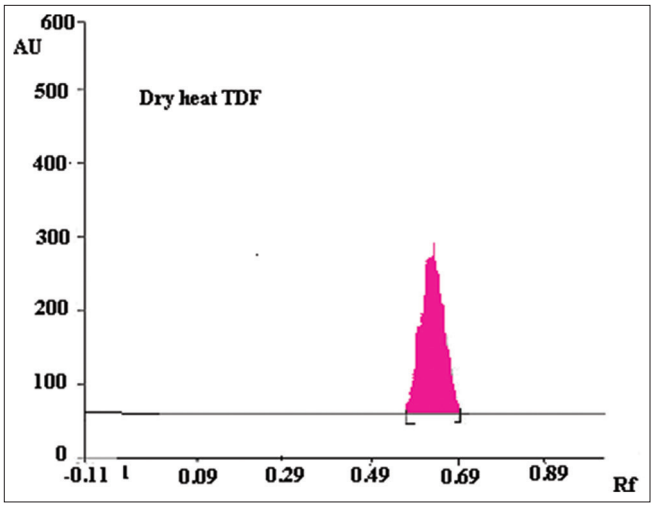

Figure 10: Dryheat Tenofovir disoproxil fumarate

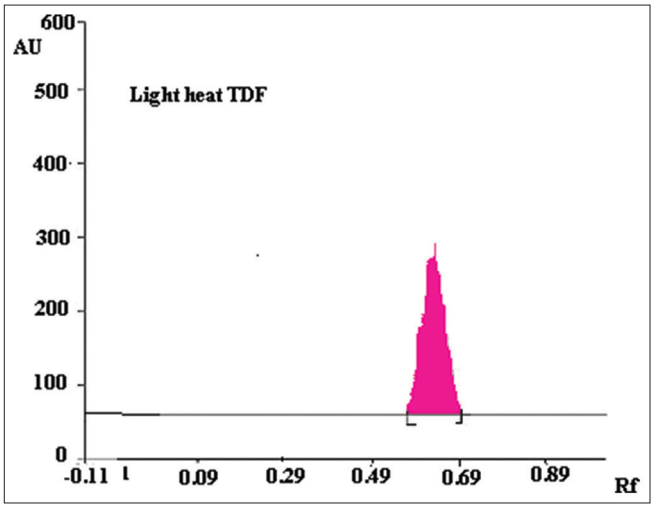

Figure 11: Lightheat Tenofovir disoproxil fumarate

phase volume, mobile phase composition, development distance, relative humidity, duration of saturation, time from spotting to chromatography, and chromatography to spotting) were studied and the effects on the results were examined. The results of the studies are shown in Table 8.

Forced degradation of TDF

Degradation studies of all and the average peak area of TDF after application (600 ng/spot) of six replicates were obtained. The plate was developed and scanned in above established chromatographic conditions. Peak area was recorded for each concentration of the degraded drug.

Acid and base induced degradation

The 10 mg of TDF was separately dissolved in 10ml methanolic solution of 1 MHCl and 1 M NaOH. These solutions were kept for 8 h at room temperature in the dark to exclude the possible degradative effect of light. The 1 mL of above solution was taken, neutralized, and diluted up to 10 mL with methanol.

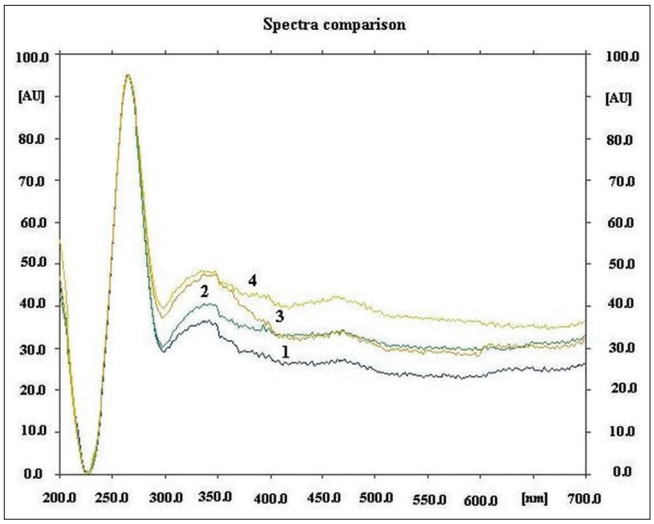

Figure 12: Peak purity spectra of degradation of tenofovir disoproxil fumarate; (1) Std TDF {correlationr (S,M)=0.998,r(M,E) = 0.999}; (2) 1NHCl + TDF {correlationr (S, M) = 0.998, r (M, E) = 0.995}; (3) 1NNaOH + TDF {correlation r (S, M) = 0.994, r (M,E) =0.997}; (4) 10% H_2O_2 + TDF {correlation r(S, M) =0.996, r (M,E) =0.999}

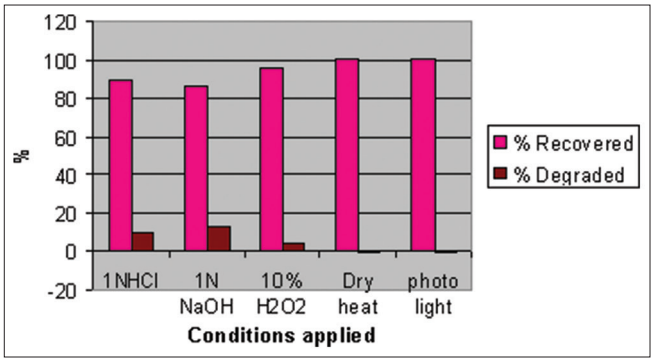

Figure 13: Force degradation for tenofovir disoproxil fumarate

Appropriate volume 6 µL containing 600 ng/spot of TDF was applied on TLC plate in six replicates. The plate was developed and scanned as described in Figures 7 and 8.

Hydrogen peroxide-induced degradation

The 10 mg of TDF was separately dissolved in 10 mL of methanolic solution of hydrogen peroxide (10%, *v/v*). The solution was kept for 8 h at room temperature in the dark to exclude the possible degradative effect of light. The 1 mL of above solution was taken and diluted up to 10 mL with methanol. Appropriate volume 6 µL containing 600 ng/spot of TDF was applied on TLC plate in six replicates. The plate was developed and scanned as described in Figure 9.

Dry heat degradation products

The 10 mg of TDF was stored at 55°C for 3 h in oven. It was transferred to 10 mL volumetric flask containing methanol and volume was made up to the mark. Appropriate volume 0.6 µL containing 600 ng/spot of TDF was applied on TLC

Table 9: Force degradation of TDF

Sample exposure condition	Number of degradation products (R_f values)	TDF remained (600 ng/spot)	SD	Recovery (%)
1M HCl, 8h, RT	1 (0.69)	538.3	12.98	89.71
1M NaOH, 8h, RT[a]	2 (0.61 0.69)	522.83	10.40	87.13
10%H_2O_2,8h, RT[a]	1 (0.65)	576.45	7.40	96.07
Heat, 3h, 55°C	No degradation	601.75	4.98	100.29
Photo, 8 h	No degradation	600.82	2.56	100.13

[a]RT: Room temperature

Table 10: Comparison of developed and reported method by Student's *t*-test

S. No	Reported method	Developed method
1	99.34	100.39
2	99.56	100.01
3	100.03	100.99
4	99.45	100.31
5	99.61	100.43
T test: Paired two sample for means		
	Variable 1	**Variable 2**
Mean	99.598	100.426
Variance	0.06917	0.12648
Observations	5	5
Pearson Correlation	0.764320452	
Hypothesized Mean Difference	0	
Df	4	
t Stat	–8.067401653	
P (T<=t) one-tail	0.000641151	
T Criticalone-tail	2.131846782	
P (T<=t) two-tail	0.001282301	
T Critical two-tail	2.776445105	

plate in six replicates. The plate was developed and scanned as described in Figure 10. No degradation was observed in dry heat.

Light heat degradation products

The 10 mg of TDF was dissolved in 10 mL of methanol. The solution was kept in the sunlight for 8 h. The 1 mL of above solution was taken and diluted up to 10 mL with methanol. Appropriate volume 6 µL containing 600 ng was applied on TLC in six replicates. The plate was developed and scanned as described in Section 6. No degradation was observed in light heat.

Degradation of TDF in acid, base, H_2O_2, dry heat, and light heat is as shown in Figure 10 TDF recovered after forced degradation in acid, alkali, and H_2O_2 solution were compared for peak purity with that of standard EFA. The purity of these spectra was tested at peak start, peak apex, and peak end position, as shown in Figure 11.

Graphical representation of force degradation for TDF is shown in Figures 12 and 13.

The summary of degradation study is shown in Table 9.

Statistical analysis

To test means of developed and reported method a paired Student's t-test was applied. From the Student's *t*-test, t statistics was found signifying that there is no significant difference between the means; results are shown in Table 10.

T statistical

T Statistical analysis of Paired two sample for means are 99.598 for Variable1 and 100.426 for Variable2.

SUMMARY AND CONCLUSION

TDF belongs to a class of antiretroviral drugs known as nucleotide analog reverse transcriptase inhibitors. TDF is rapidly converted to tenofovir, which is metabolized intracellularly to its active anabolite tenofovir diphosphate, which is a competitive inhibitor of HIV-1 reverse transcriptase and terminates the growing DNA chain. Stability – Indicating HPTLC Determination of TDF in Bulk and Tablet Dosage Form is developed. The method employed TLC aluminum plates precoated with silicagel 60F-254 as the stationary phase. The solvents system consisted of butanol: ethylacetate: aceticacid (4.5:0.5:0.3*v/v*) and densitometric analysis was carried out in the absorbance mode at 266 nm. The system was found to give a compact spot for TDF (Rf value of 0.51 ± 0.02). The linear regression analysis data for the calibration plots showed a good relationship with r^2=0.9999±0.0001 in the concentration range 300–1800 ng/spot. TDF was subjected to acid and alkali hydrolysis, oxidation, thermal degradation, and photodegradation. There was no interference of the excipients on the determination of the active pharmaceutical ingredients. Proposed method is

precise, selective, and accurate for the estimation of TDF in the bulk and dosage form.

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