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Method Development and Validation for Estimation of Tinofovir Disoproxil Fumarate in Pharmaceutical Preparation by UV-Spectroscopy and HPLC with Force Degradation Study

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Abstract

Tinofovir Disoproxil Fumarate (TDF) is an acyclic nucleotide diester analog of adenosine monophosphate from the antiviral category utilized in the AIDS and hepatitis B treatment. In the present study, two analytical methods, i.e. UV and HPLC were developed for the TDF's estimation in pharmaceutical preparation. In the UV method, the mobile phase used was Methanol and water in 60:40 ratio for estimation of the drug at 260 nm and an assay of the (TDF) obtained was 99.53%. The method's validation was carried out as per ICH Q2 R1 guidelines in which linearity was detected from 10-50 µg/ml range with a regression value of 0.999 Percent RSD value of accuracy, precision and robustness were below 2. In the HPLC method estimation of (TDF) was evaluated on Cosmosil C-18 (250mm×4.6ID, Particle size: 5 µ) column utilizing Methanol: Water (60:40), 0.9 ml/min of flow rate, detection wavelength was 260 nm and the time of retention observed was around 4.63 minutes with the assay value 99.07%. HPLC method was also validated according to ICH guidelines, where linearity was detected in the 10-50 µg/ml of range with the regression coefficient value 0.999. The % RSD of precision, accuracy, and robustness was below 2%. The study of forced degradation was also performed by the HPLC method utilizing methanol: water (60:40) at 260nm. From this study, it can be concluded that the developed methods for estimation of (TDF) drug in pharmaceutical preparation are simple, accurate, precise, and can be utilized in the routine analysis for quantification of the drug in a dosage form.

Keywords: Force Degradation Study, HPLC, Tinofovir Disoproxil Fumarate, UV, Validation

1. Introduction

Tenofovir Disoproxil Fumarate (TDF) (2E)-but-2-enedioic acid; bis({[(propan-2-yloxy) carbonyl]oxy} methyl){[(2R)-1-(6-amino-9H-purin-9-yl)propan-2-yl] oxy} methanephosphonate (Figure 1) is a bis-alkoxy ester prodrug of tenofovir a nucleoside monophosphate (nucleotide) analog belongs to a class of antiretroviral drugs known as nucleotide analogue Reverse Transcriptase Inhibitors (nRTIs), used to treat HIV/AIDS and is in clinical preliminaries for treatment of hepatitis B disease. It's molecular formula is C19H30N5O10P and molecular weight is 635.52[1]. TDF is less absorbed when administered orally due to the existence of two negative charges among its structure which limits its cellular penetration, its passive diffusion across cellular membranes and intestinal mucosa hindering its availability[2]. Following absorption from the gut, TDF is converted to tenofovir rapidly, which

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is metabolized within the cells to active anabolite tenofovir, which is an inhibitor of HIV-1 reverse transcriptase and DNA growing chain is terminated. In a range of cells, along with resting cells, TDF exerts an antiviral effect[3]. The objective of the present work is to develop and validate UV and HPLC methods for estimation of TDF in the pharmaceutical product as per the Q1R2 guidelines of ICH. Further the force degradation study is carried out by HPLC.

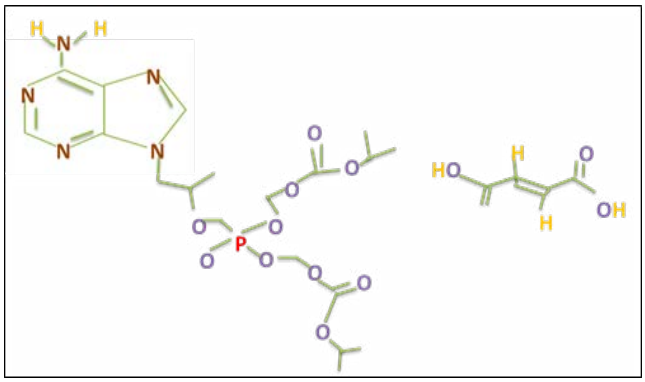

Figure 1. Chemical structure of TDF disoproxil fumarate[3].

TDF is excreted in the urine as an unchanged form, with a significant component of active tubular secretion[4]. Long term utilization of TDF disoproxil is related to nephrotoxicity and bone misfortune. The introduction of nephrotoxicity can show up as Fanconi disorder, intense kidney damage, or decay of Glomerular Filtration Rate (GFR)[5]. TDF interacts with did anosine and HIV-1 protease inhibitors. TDF increases didanosine concentrations and can bring about unfriendly impacts, for example, pancreatitis and neuropathy[6]. TDF associates with HIV-1 protease inhibitors, for example, atazanavir, by diminishing atazanavir concentrations while increasing TDF concentrations[7]. Literature survey revealed that few analytical methods like RP-HPLC, UV, LC-MS, HPTLC have been reported for simultaneous determination of other drugs with TDF disoproxil fumarate in pharmaceutical preparation[8-20]. Hence an effort has been taken in the present study to develop UV and HPLC methods for estimation of (TDF) in pharmaceutical preparation and also the force degradation study of the (TDF) was performed by HPLC method utilizing Cosmosil C-18 (250mm×4.6ID, Particle size: 5µ) column and methanol: water (60:40) as mobile phase.

2. Materials and Methods

2.1 Chemicals and Reagents Used

A pure standard reference TDF was obtained as a gift sample from Dr. Reddy's Lab Ltd. Hyderabad, India. Methanol and water of HPLC grade from Thermo Fisher Scientific and TDF tablets manufactured by Cipla Ltd. were bought from the local marketplace.

2.2 Instruments

2.2.1 HPLC

HPLC Analysis was executed on HPLC-3000 series (Binary gradient framework, Analytical technologies Ltd.) manufactured by Analytical Technologies Ltd., comprising of a siphon with the loop limit of 20 µl, UV-Vis (model no. UV2012) detector, waters column (250 mm x 4.6 mm, 5 µ).

2.2.2 UV-Visible Spectrophotometer

The absorbance was recorded on a UV-Visible spectro photometer (UV-3000-M) manufactured by Analytical Technologies Ltd., with a spectral bandwidth 2 cm furnished with 1 mm quartz cell at medium scan speed.

2.2.3 Weighing Balance and Sonicator

All the weighing was executed on wensar high accuracy electronic balance PGB 100 and sonication was performed on ultra sonicator WUC-4L manufactured by wensar. The Borosilicate 25 µl glass syringe was utilized for testing. Mdi 0.2 µm membrane filter paper was utilized.

2.3 Identification of Drug

2.3.1 Melting Point Determination

The melting point of TDF was estimated by utilizing a capillary strategy on a melting point device[21].

2.3.2 FTIR Spectroscopy of Drugs

Fourier Transform Infrared (FT-IR Alpha Brucker, Germany) was utilized to recognize the characteristic functional group blank reading was taken by Potassium

Bromate (KBr). The small quantity of the sample was combined with standard at the point of examination from 4000-400cm^{-1}[22].

2.3.3 Solubility Studies

The solubility investigation of TDF was investigated by utilizing various solvents, like methanol, water, and dichloromethane[23].

2.4 Selection and Optimization of UV-Visible Spectrophotometric Method[24]

2.4.1 Selection of Mobile Phase

As per the solubility of the drug in water, methanol and dichloromethane, the different combination of these solvents with different ratios was tried and based on the results obtained, the mobile phase was selected.

2.4.2 Preparation of Standard Stock Solution

10mg of (TDF) was precisely weighed and shifted into a 10ml volumetric flask, adda adequate volume of mobile phase till the mark to obtain a standard solution of the strength of 1000 µg/ml.

2.4.3 Preparation of Working Solution

1 ml of the solution of the standard was pipette and transferred into a volumetric flask of 10 ml and the mobile phase was added till the mark to make a 100 µg/ml of solution.

2.4.4 Selection of Detection Wavelength (λ_{max})

To acquire a wavelength of maximum absorption (λ_{max}) of (TDF) the 1ml working solution was diluted to make 10 µg/ml of the solution by the mobile phase and scanned for the entire UV range (200-400 nm) in UV spectrophotometer on the spectrum mode.

2.4.5 Establishment of the Calibration Curve

From the working solution of different aliquots were made by diluting 0.1, 0.2, 0.3, 0.4, 0.5 ml of solution till 10 ml in a volumetric flask separately with the mobile phase, to get solutions of strength from 10-50 µg/ml of range. Blank reading was taken with methanol: water (60:40). The absorbance of the working standard was recorded at 260 nm. The standard calibration curve of drug concentration versus absorbance was plotted.

2.4.6 Assay of (TDF) Tablet

The average weight of TDF tablets determined by measuring the weight of 10 tablets independently, Assay of TDF tablet was performed by dissolving 12.01 mg tablet powder which is equal to 10 mg of the standard drug in 10 ml of mobile phase in a volumetric flask to get 1000 µg/ml of solution. To dissolve the tablet contents completely sonication of the solution was done for 10 mins. The solution was sifted through 0.22 µ membrane filter. After chromatographic development peak zone was estimated at 260 nm, and (TDF) present in the sample was evaluated from the region of peak % Assay was evaluated by utilizing the following equation:

$$\% \text{ Assay} = \frac{\text{Concentation of drug in } \frac{\mu g}{ml} \text{ X Dilution Factor} \times 100 \times \text{Average weight of the sample}}{\text{Label Chain} \times \text{Weight of the sample taken}}$$

2.4.7 Validation of UV-Visible Spectrophotometric Method[25-27]

The optimized method was validated according to ICH Q2A (R1) parameters.

2.4.8 Accuracy (Recovery Study)

The method of standard addition was utilized to perform accuracy studies in which top re-analyzed samples and an amount is known of the standard drug was combined and exposed to the method. At three different levels, this study was carried out i.e., (50%, 100%, and 150%). The recovery studies were executed for each level, then % recovery and standard deviation was calculated is represented in the Table 1.

2.4.8.1 Precision

Intermediate precision (intra-day and intra day) was achieved by utilizing an investigation of 30 µg/ml of concentration of (TDF) drug on the same day multiple times. Inter day precision of the strategy was checked by rehashing a similar examination on the two distinct days. The estimation of absorbance for the test was communicated regarding % relative standard deviation (% RSD).

Table 1. Composition of sample solution for accuracy by UV method

Sr. No.	% Recovery	Concentration of Tablet	The concentration of Standard added	Final Concentration
1.	50%	20ppm	10ppm	30ppm
2.	100%	20ppm	20ppm	40ppm
3.	150%	20ppm	30ppm	50ppm

2.4.8.2 Linearity

The sample solution of TDF (1mg/ml) was made by dissolving 12.01 mg of tablet powder in 10 ml of a volumetric flask in a sufficient mobile phase to dissolve the tablet content and further volume made upto 10 ml to obtain a final concentration of 1000 µg/ml. From the above sample solution 0.1, 0.2, 0.3, 0.4, 0.5 ml was pipetted out and moved to 10 ml volumetric flask separately and diluted up to 10 ml with the mobile phase, calibration curve of drug concentration versus absorbance was plotted and regression coefficient was determined from the graph.

2.4.8.3 Robustness

To assess the developed method's strength, some parameters purposely fluctuated. These parameters like stream rate (±1) or wavelength of determination (±1) were varied within the specified range. The robustness of the technique was done at the 30 µg/ml concentration. For the assurance of strategy's robustness, parameters like wavelength were varied in a reasonable range and the quantitative impact of the factor was resolved.

2.5 Selection and Optimization of Chromatographic Condition (HPLC)[28-30]

2.5.1 Selection and Optimization of Mobile Phase

After trying different solvents in different ratios, methanol: water gave sharp peaks and good resolution. This mobile phase was made by mixing methanol: water in 60:40v/v ratios the following filtration through a membrane filter of 0.45µ, which was then degassed using an ultra sonicator.

2.5.2 Selection of Wavelength by UV-Vis Spectrophotometric Method

The standard solution of TDF (1 mg/ml) was made by dissolving 10 mg of unadulterated TDF standard in 10 ml of the volumetric flask, to it, 5 ml of the mobile phase was combined and to dissolve the drug completely in the solution, it was sonicated and further volume was made till the mark for acquiring solution strength of 1000 µg/ml. From this solution, 0.1 ml was diluted up to 10 ml in a volumetric flask. The working standard solution of 10µg/ml was scanned for the whole UV range (200-400 nm) in a 1 cm quartz cell with the usage of methanol as diluent. The working standard arrangement of 10 µg/ml was examined for the whole UV go (200-400 nm) in a 1 cm quartz cell using methanol as a diluents.

2.5.3 Preparation of Standard Stock Solution

Accurately weigh 10 mg of TDF of standard reference and transfer it to a 10 ml volumetric flask, to it 5 ml of the mobile phase was combined and sonicated for 10 minutes for the drug to dissolve completely, further volume was made up till the mark with diluent to get solution strength of 1000 µg/ml. Working standards were prepared by diluting the standard stock solution.

2.5.4 Establishment of Calibration Curve by HPLC Method

From standard stocks 0.1, 0.2, 0.3, 0.4, and 0.5 ml was diluted till 10 ml in a volumetric flask separately to get 10, 20, 30, 40 and 50 µg/ml of solutions. Each solution's absorbance was taken at 260 nm and the assessment of the correlation coefficient was determined individually.

2.5.5 Assay

10 tablets were weighed separately to determine the average weight. Weight equivalent to 12.02 mg TDF tablet power (equal to 10 mg of standard TDF) was moved to 10 ml volumetric flask, to it 5 ml diluent was added, and for dissolving the tablet content the solution was sonicated for 10 min to and then the volume was made till the mark to make a solution of strength 1000µg/ml. The solution was moved to a falcon tube and centrifuged for 30 minutes; the supernatant was sifted through 0.22µ membrane filter. After chromatographic advancement peak region

was estimated at 260 nm and the amount of drug present in the test sample was estimated from the area under the peak. % Assay was calculated by the following formula:

$$\% \text{ Assay} = \frac{\text{Concentation of drug in}\mu g / ml \text{ X Dilution Factor} \times 100 \times \text{Average weight of the sample}}{\text{Label Chaim} \times \text{Weight of the sample taken}}$$

2.6 Validation of HPLC Method[31 - 33]

According to the International Council on Harmonization (ICH) Q2 (R1) and guidelines, the optimized chromatographic method was validated for specificity, accuracy, linearity, LOD and LOQ, specificity.

2.6.1 Specificity

The optimized method's specificity was evaluated by relating the chromatograms of marketed preparation, mobile phase and diluent to detect obstructions of excipients, mobile phase and diluent.

2.6.2 Accuracy (Recovery)

For recovery studies, the method of standard addition was utilized for checking the accuracy of optimized methods in which to a pre-analyzed sample, the amount known of the standard drug was combined and exposed to the method. These studies were carried out at different three levels, i.e. (50%, 100%, and 150%). The recovery studies were carried out, the % recovery and standard deviation were calculated. The solutions were set up by technique of standard addition is given in Table 2.

2.6.3 Precision

The analytical technique's precision is the level of agreement between the results of single tests when the method is applied again and again to different samples of a homogeneous sample. The analytical strategy's accuracy is normally reported as the standard deviation or the relative standard deviation. Precision is the proportion to the level of repeatability of an analytical technique under typical activity.

2.6.3.1 Repeatability

The method's repeatability is assessed by examining the same sample solution 6 times and the relative standard deviation is determined.

2.6.3.2 Intermediate Precision

i) *Intraday Precision*

The intraday precision is evaluated by testing the sample solution 3 times each on the same day with a time interval of 4 hours, based on the results relative standard deviation was determined.

ii) *Inter day Precision*

The inter-day precision is evaluated by examining the sample solution 3 times a day for 2 days and the relative standard deviation is determined.

2.6.4 Linearity

The method's linearity was evaluated at 5 different concentrations by diluting the standard stock solution to make 10-50µg/ml of solutions. The linearity graph was plotted with the peak zone of drug v/s drug concentration. The data of linearity are given in table 21.

2.6.5 Preparation of Standard Solution

From the stock solution 0.1, 0.2, 0.3, 0.4, 0.5 ml was pipette out in 10ml volumetric flask separately, diluent was combined to make the volume till the mark to get solutions of strength 10, 20, 30, 40, 50 µg/ml.

2.6.6 Limit of Detection (LOD) and Limit of Quantitation (LOQ)

The LOD and LOQ were determined utilizing the following conditions, as stated in ICH rules.

$LOD = 3.3 \times \sigma/S$

$LOQ = 10 \times \sigma/S$

Where σ is the standard deviation of the response and S is the slope of the calibration curve

Table 2. Data for accuracy

Sr. No.	% Recovery	The concentration of drug in Tablet	The concentration of Standard drug added	Final Concentration
1.	50%	20ppm	10ppm	30ppm
2.	100%	20ppm	20ppm	40ppm
3.	150%	20ppm	30ppm	50ppm

2.6.7 Robustness

It is the measure of the method's capacity to stay unaffected by little yet intentional variety in strategy parameter and gives a sign of its dependability under typical utilization. The strength of an analytical technique is controlled by the investigation of aliquots from homogenous portions by varying physical parameters that may however are still inside the predetermined parameters of the assay. For instance, change in physical parameters like pH of the mobile stage and its proportion. Standard and test samples were evaluated in triplicate. The various chromatographic conditions under which standard and sample solution were evaluated are given in Table 3.

Table 3. Parameters varied to check the robustness

Sr. No.	Change in flow rate	Different wavelength
1.	0.8 ml/min	256 nm
2.	1.0 ml/min	258 nm
3.	0.9 ml/min	262 nm

2.6.8 System Suitability Study

System suitability is a pharmacopoeial prerequisite and is utilized to confirm, regardless of whether the reproducibility of the chromatography system is satisfactory for the investigation to be finished. The tests were executed by gathering information from five replicate infusion of the standard solution.

2.7 Forced Degradation Studies[34-36]

Force degradation is the deterioration of medicated substances or its product by exposing it to conditions more severe than accelerated degradation conditions. This investigation shows the chemical conduct of the particles which thus helps in the development of formulation and packaging of the product. Therefore oxidative, hydrolytic, photolytic and heat degradation for (TDF) standard solutions have been investigated.

2.7.1 Oxidative Degradation

Pipette 1ml of standard stocks in a volumetric flask, add1ml of 3% hydrogen peroxide (H_2O_2), and this solution was kept for 24 hours at room temperature. The resulting solutions were diluted to get a 50µg/ml solution with methanol: water (60:40) and 20µl was injected in the system and from the recorded chromatogram at 260nm, oxidative stability of the test sample was determined.

2.7.2 Acid Degradation

Pipette 1ml of the stock solution into a volumetric flask, to its1ml of 0.1N Hydrochloric Acid was combined and reflexed for 2 hours and 30 minutes at 60˚C. This solution was diluted to acquire 50µg/ml with methanol:water (60:40) of it 20µl was injected into the system and the chromatogram obtained at 260nm was utilized for acid stability evaluation.

2.7.3 Alkaline Degradation

To 1ml of stock solution, 1ml of 0.1N sodium hydroxide was combined and reflexed for 2 hours. 1ml of 0.1N sodium hydroxide with 1 ml stock was reflex for 30 minutes at 60˚C. The subsequent solution was diluted to get a 50µg/ml solution with methanol:water (60:40) and 20µl were injected into the system and the alkaline stability evaluation was done by its chromatogram at 260 nm.

2.7.4 Photolytic Degradation

The photochemical stability was additionally examined by uncovering the stocks in UV light by keeping the beaker at room temperature for 24 hours in a photostability chamber. The resulting solution was diluted to acquire 50µg/ml of solution with methanol:water (60:40), of it, 20µl was injected into the system and the chromatogram was recorded at 260 nm to assess the photolytic stability.

2.7.5 Thermal Degradation

The standard Drug solution was kept in an oven 60°C for 24 hours then diluted to get 50µg/ml of solution with methanol:water (60:40) from this 20µl was injected into the system and the thermal stability was determined by chromatogram recorded at 260 nm.

3. Results and Discussion

3.1 Identification of Drug

3.1.1 Melting Point Determination

The slim technique was utilized to decide the melting point by using the thieles tube. The Melting point of (TDF) was seen as 278˚C, while its ideal melting point range is from 277 to 279˚C.

3.1.2 FTIR Spectrum

To identify the functional group of the drug, its FTIR spectrum was recorded. The spectrum showed a strong, broad absorption band extending across the region 2500-3500cm^{-1} (Table 4 & Figure 2).

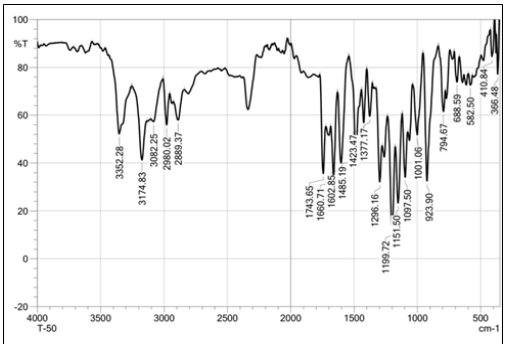

Figure 2. FTIR spectrum of TDF.=

Table 4. IR data of TDF

Wave number (cm1)	Functional group
3352.28	N-H
3082.25	Aromatic C-H
2980.02	Aliphatic C-H
1660.71	C=C/C=N
1423.47	Aliphatic C-H
1296.16	C-O-C
1097.50/1001.06	C-O-C

3.1.3 Solubility Studies

Assessment of solubility of the drug was observed in 3 different solvents and it was observed that the drug was slightly soluble in water, very slightly soluble in dichloromethane, and freely soluble in methanol.

3.2 Development of UV Visible Spectrophotometric Method for TDF

3.2.1 Selection and Optimization of Mobile Phase

Based on the solubility of the drug, methanol and water were selected as the solvents of the mobile phase. Both the solvents were run in different ratios and from the acquired chromatograms it was observed that methanol: water in (60:40) ratio gave good results.

3.2.2 Selection of Detection of Wavelength

Solution strength 10µg/ml of the drug was scanned for the entire UV range, i.e. from 200 to 400 nm, on spectrum mode in UV spectrophotometer. Maximum absorption was observed at 260nm (Figure 3).

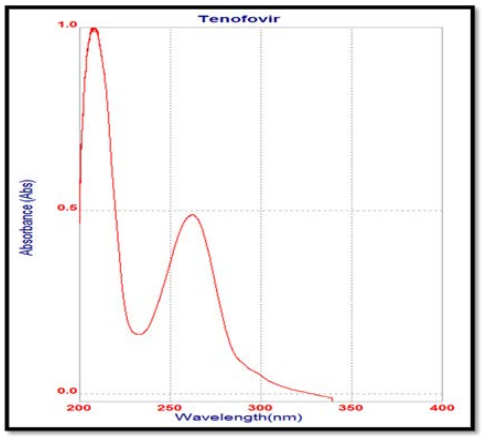

Figure 3. UV spectrum of TDF.

3.2.3 Establishment of Calibration Curve by UV Method

The absorbance of each concentration was estimated at the observed analytical wavelength. The standard calibration curves of absorbance versus drug concentration were plotted to utilize the mean of the five different concentrations. The range concentration over which the drug obeyed Beer-Lambert's law was observed between 10-60µg/ml. In the investigation of the calibration curve, a straight line with the regression coefficient 0.999 was obtained that is shown in Figure 4 and Table 5.

Table 5. Data for calibration curve for TDF

Sr. No.	Conc. (µg/ml)	Absorbance
1.	10	0.2795
2.	20	0.4692
3.	30	0.6594
4.	40	0.8606
5.	50	1.0582

3.2.4 Assay

Optimized experimental condition for quantification of marketed formulation given as a % of label claim was acceptable with the label claim, thereby proposing that there is no interference of any excipients. The assay of

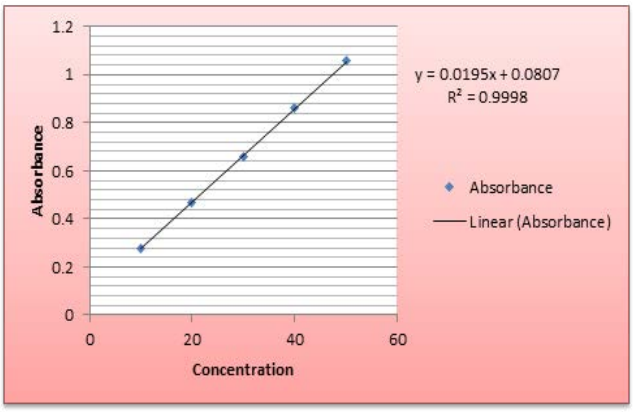

Figure 4. Calibration curve of TDF.

the drug observed was 99.53% and the standard range of (TDF) is NLT 98.0% and NMT 102.0% (Table 6).

Table 6. Assay of TDF

Sr. No.	Conc. (µg/ml)	Abs. of sample	% Assay	Mean % Assay	S.D. (±)	% RSD
1.	30	0.659	99.19	99.53	0.294618	0.296009
2.	30	0.6598	99.71			
3.	30	0.6594	99.69			

3.3 Validation of UV Method

3.3.1 Accuracy (Recovery Studies)

The optimized condition was utilized for the estimation of the drug in bulk and pharmaceutical products in the way of spiking extra (TDF), and the % recovery observed was 99.64% with % RSD of 0.56% (Table 7&8).

3.3.2 Precision

Intermediate precision anticipated was performed by utilizing examination of each test solution containing 30µg/ml in triplicate. The % RSD was seen at 0.04763% for intraday precision 0.06578% for inter-day precision. Therefore, the proposed UV technique was seen to be precise (Table 9&10).

3.3.3 Linearity

For assessing linearity, the calibration graph, i.e. concentration versus absorbance was plotted. It was observed that the optimized method, obey beer- lambert's law as the graph of calibration obtained was linear. The absorbance of each concentration is given in the (Table 11 and Figure 5).

3.3.4 Robustness

For the assurance of the technique's robustness, parameter like wavelength was varied within a practical range and the quantitative impact of the factors was resolved. The robustness of the strategy was assessed at a concentration level of 30µg/ml in triplicate (n=3) (Table 12).

3.4 Development of HPLC Method for (TDF)

3.4.1 Selection and Optimization of Mobile Phase

Different mobile phase was run with a different ratio, a sharp peak with minimum tailing was observed with methanol: water in the ratio 60:40. Trial runs on methanol: water is given in (Table 13 and Figure 6&7).

Table 7. Accuracy determination of (TDF) by UV

Sr. No.	Concentration (µg/ml)	Area	Mean	% SD	% RSD
1.	10	0.2795	0.27943333	0.00020817	0.07449598
2.	10	0.2792			
3.	10	0.2796			
4.	30	0.6594	0.65943333	0.00035119	0.0532561
5.	30	0.6598			
6.	30	0.6591			
7.	50	1.0582	1.05846667	0.00030551	0.02886298
8.	50	1.0588			
9.	50	1.0584			

Table 8. Recovery determination of (TDF) by UV

Sr. No.	Concentration	% Added	Absorbance	% Recovery
1.	20+10	50%	0.6589	98.99
2.	20+20	100%	0.8602	99.98
3.	20+30	150%	1.0578	99.96
			Mean	99.6433
			SD	0.56589
			% RSD	0.56792

Table 9. Intra-day precision for TDF

Time (Day)	Conc. (µg/ml)	Absorbance	Mean	% RSD (±)
Morning	30	0.6594	0.65943	0.04763
		0.6598		
		0.6591		
Evening	30	0.9698		
		0.6591		
		0.6594		

Table10. Inter-day precision for TDF

Time (Day)	Conc. (µg/ml)	Absorbance	Mean	% RSD (±)
Day 1	30	0.6594	0.65927	0.06578
		0.6598		
		0.6591		
Day 2	30	0.6599		
		0.6593		
		0.6597		

Table 11. Study of linearity for TDF

Sr. No.	Conc. (µg/ml)	Absorbance
1.	10	0.2795
2.	20	0.4692
3.	30	0.6594
4.	40	0.8606
5.	50	1.0582

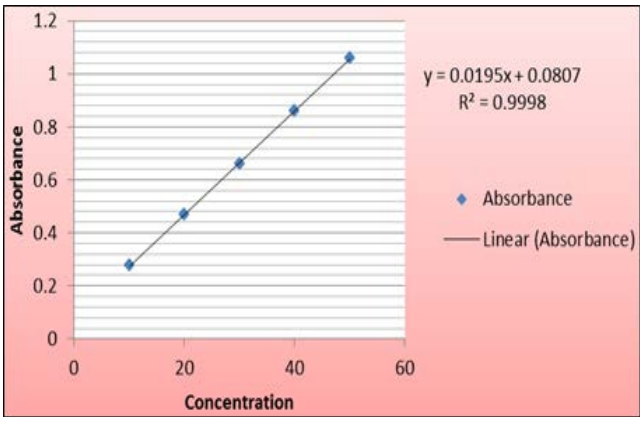

Figure 5. Calibration graph of TDF.

Table 12. Result of robustness at a different wavelength

Sr. No.	Concentration	Wavelength	Absorbance
1.	30	264 nm	0.6598
2.	30	265 nm	0.6591
3.	30	263 nm	0.6593
		Mean	0.6594
		SD	0.00036
		% RSD	0.05468

Table 13. Result of optimization of chromatographic conditions

Trial No.	Column used	Mobile phases (v/v)	Retention time	Observation	Results
1.	CosmosilC18 (250mm × 4.6ID, Particle size: 5µ)	Methanol: Water (60:40)	4.63 min	Sharp peak with minimum tailing	Accepted
2.	CosmosilC18 (250mm × 4.6ID, Particle size: 5µ)	Methanol:Water (60:40)	3.2 min	Broad peak	Rejected

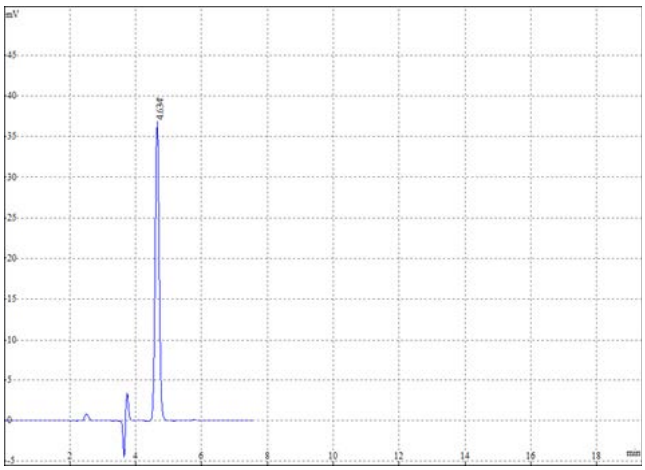

Figure 6. Trial 1-Methanol: Water (60:40) (4.63 minutes of retention time).

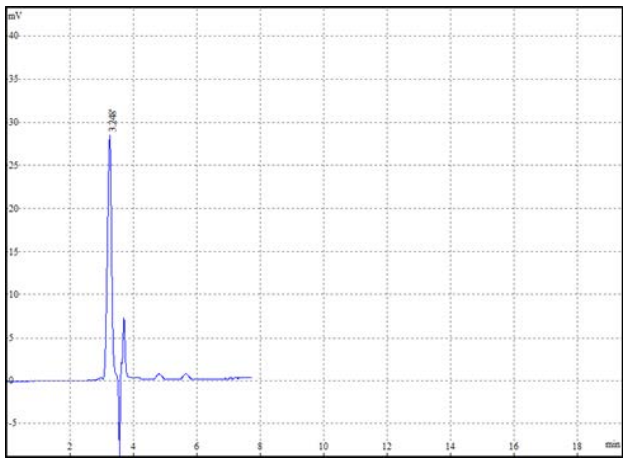

Figure 7. Trial 2-Methanol: Water (60:40) (3.2 minutes of retention time).

3.4.2 *Establishment of Calibration Curve by HPLC Method*

The calibration curve of (TDF) was obtained by plotting a graph of five ascending drug concentrations versus the area of the peak. The regression coefficient acquired was 0.999 with 0.70572x slope (Table 14 and Figure 8).

Table 14. Calibration of (TDF) by HPLC method

Sr. No.	Concentration (µg/ml)	Avg. Area
1.	10	314719
2.	20	879850
3.	30	1555509
4.	40	2133827
5.	50	2785164

3.4.3 *Assay*

The HPLC method developed is sensitive and specific for the quantitative determination of TDF. Tablets of 300 mg strength, manufactured by Cipla were assessed for the content of drug present in the formulation. The assay of the drug was observed to be 99.0716%. The reported

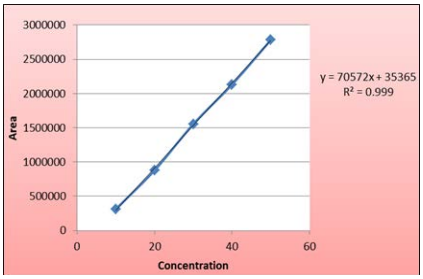

Figure 8. Calibration curve of (TDF).

standard % assay range of (TDF) tablets is NLT 90.00% and NMT 105.0% of Labeled claim. Chromatograms below represent (TDF) in bulk and tablet dosage form (Table 15 and Figure 9&10).

Table 15. Assay of (TDF) tablet

Drug	The concentration of sample (µg/ml)	Area of standard	Area of sample	% Assay
TDF	30	1574389	1559773	99.0716

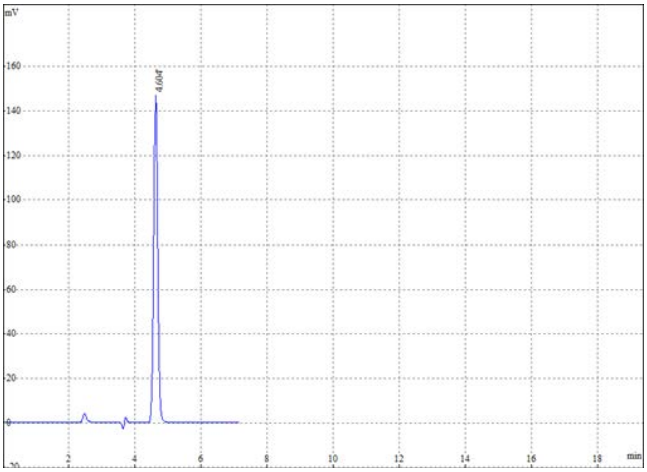

Figure 9. Standard chromatogram of TDF.

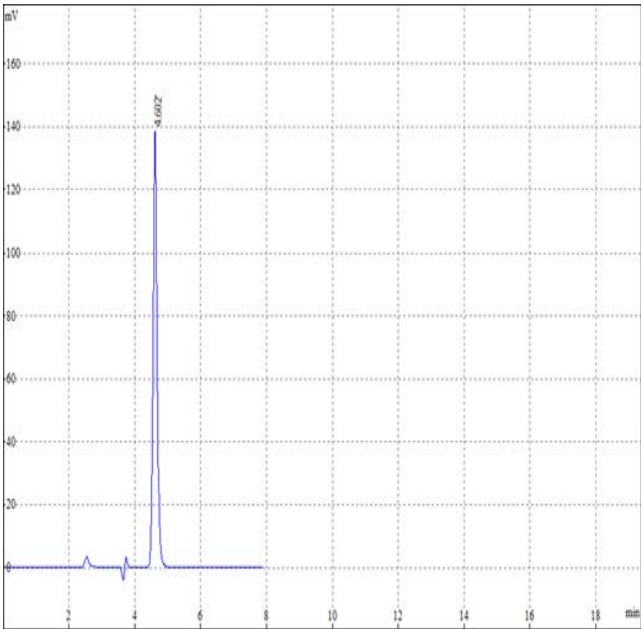

Figure 10. Chromatogram of (TDF) sample.

3.5 HPLC Method Validation

3.5.1 Specificity

The specificity of the optimized method was observed by injecting only the mobile phase and no peaks of it were observed. Another injection of the sample solution was injected in the system to observe that any interference of excipient with the method, but the only peak observed was of the drug. The chromatograms below represent the specificity of the optimized method (Figure 11&12).

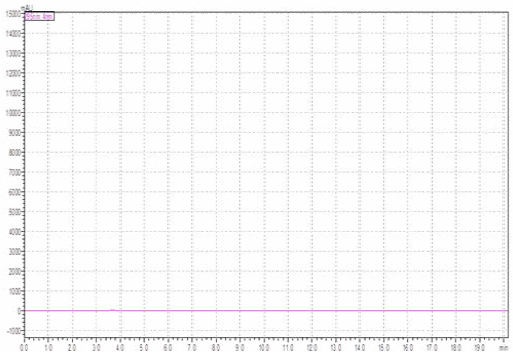

Figure 11. Chromatogram of mobile phase (Methanol: Water (60:40)).

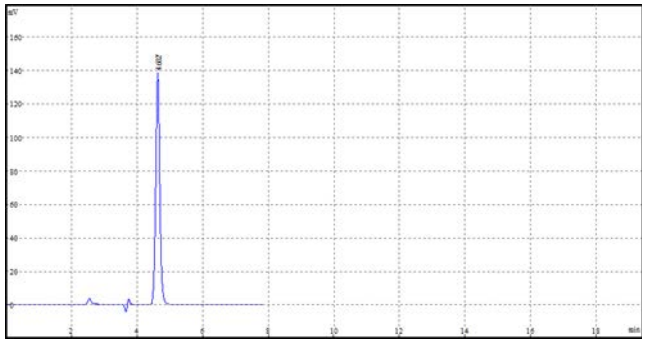

Figure 12. Chromatogram of (TDF) sample for specificity.

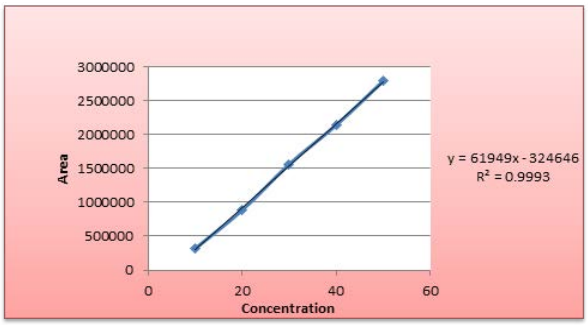

Figure 13. Calibration graph of Tinofovir disproxil fumarate

3.5.2 Accuracy (Recovery Studies)

Three injections per-concentration, i.e. 30, 40, 50 µg/ml were injected. The amount obtained and the amount added was estimated and the mean recovery of individual recovery values was determined which was within the standard range specified i.e. 90-102%. Each concentration's relative standard deviation was below 2.0%.In recovery studies, the optimized method when utilized for the estimation of the drug in pharmaceutical dosage form and bulk, the % recovery was seen at 99.72%. The data obtained for accuracy and recovery study is given in the (Table 16 &17).

3.5.3 Precision

Intermediate precision (Intra-day and Inter-day).

The injection standard solution twice in triplicate on the same as well as on 2 consequent days and the peak area was calculated for all injections. The %RSD for the peak area of triplicate injections was observed to be in the limits specified, i.e. % RSD of intra-day precision was 0.19 and 0.39 for inter-day precision. The results of intra-day and inter-day precision are summarized in the (Tables 18&19).

3.5.4 Linearity

From the linearity of the calibration graph, it was found that the method optimized obeys Beer-Lamberts law in the range of concentration from 10-50µg/ml and the regression coefficient obtained from the graph was 0.9993 with slope 61949x and 32464 intercepts. The peak area of all concentration is given in the (Table 20 and Figure 13).

3.5.5 Limit of Detection and Limit of Quantitation (LOD & LOQ)

LOD and LOQ were resolved based on standard deviation acquired from the calibration curve data. LOD for (TDF)

Table 16. Accuracy determination of (TDF) by HPLC

Sr. No.	Concentration (µg/ml)	Area	Mean	% SD	% RSD
1.	30	314719	314824.6667	0.3760955	0.37609
2.	30	313697			
3.	30	316058			
4.	40	1555509	1557067.667	0.4507214	0.45072
5.	40	1564734			
6.	40	1550960			
7.	50	2785164	2783311.667	0.3007443	0.3007
8.	50	2774170			
9.	50	2790601			

Table 17. Recovery determination of (TDF) by HPLC

Sr. No.	% Recovery level	Area of standard	Area of sample	% Recovery
1.	50%	1555509	1546054	99.39216038
2.	100%	2133827	2123852	99.53253005
3.	150%	2785164	2791908	100.2421401
			Mean	99.72227686
			SD	0.455652593
			% RSD	0.45692157

Table 18. Intra-day Precision for TDF, (n=3)

Time (Day)	Conc. (µg/ml)	Area	Mean	% RSD (±)
Morning	30	1563452		
		1561320		
		1559783	1561209	0.19
Evening	30	1561521		
		1564892		
		1556287		

Table 19. Inter-day Precision for TDF, (n=3)

Time (Day)	Conc. (µg/ml)	Area	Mean	% RSD (±)
Day 1	30	1555509	1554621	0.39
		1564734		
		1550960		
Day 2	30	1566466		
		1560250		
		1554621		

Table 20. Linearity of (TDF) (n=3)

Concentration (µg/ml)	Average Area
10	314719
20	879850
30	1555509
40	2133827
50	2785164

obtained was 0.3738 µg/ml while the LOQ was 1.1328 µg/ml.

3.5.6 Robustness

For the assurance of the technique's robustness, parameters like wavelength and flow rate were changed in a specified range and its impact of the factors was resolved. The technique's strength of the assessed by keeping the constant concentration of 20µg/ml of (TDF) in triplicate and the % RSD was detected to be 0.46% for the flow rate and 0.41% for the wavelength (Table 21&22).

3.5.7 System Suitability Study

System suitability studies demonstrate the proficiency of the column. Several theoretical plates should be more prominent than in 2000. The estimation of asymmetry factor or following variable must be under 2. Results for system suitability studies of 30 µg/ml solution of the drug

Table 21. Result of robustness for (TDF) at a different flow rate

Sr. No.	Concentration (µg/ml)	Flow rate	Area
1.	20	0.8 ml/min	873045
2.	20	1.0 ml/min	880443
3.	20	0.9 ml/min	879856
		Mean	877781
		SD	4112.27
		% RSD	0.468485

Table 22. Result of robustness for (TDF) at a different wavelength

Sr. No.	Concentration (µg/ml)	Wavelength	Area
1.	20	258 nm	875632
2.	20	262 nm	872584
3.	20	260 nm	879850
		Mean	876022
		SD	3648.67
		% RSD	0.41650393

obtained were a time of retention - 4.604 min, theoretical plate- 8036, tailing factor -1.06, several theoretical plates- 8036 with a peak zone of 1555509 (Figure 14).

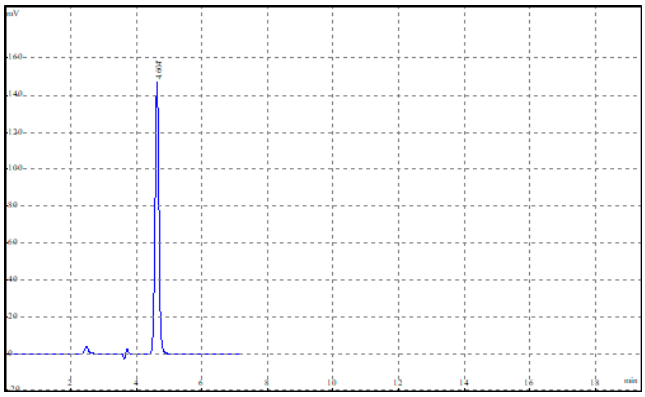

Figure 14. Chromatogram of system suitability of TDF.

3.6 Degradation Studies

Forced degradation studies were performed on (TDF) drugs. The respective chromatograms of the degradation study uncover that all the decomposed constituents were evaluated, which shows that the method can be used for degradation evaluation and for observing the TDF's stability in pharmaceutical products and bulk.

3.6.1 Oxidative Degradation

The mixture of stock and hydrogen peroxide when injected into the HPLC system to evaluate the drug degraded from the peak zone of sample degraded, the % degradation calculated was 20.95%. The chromatogram of oxidative degradation is given (Figure 15).

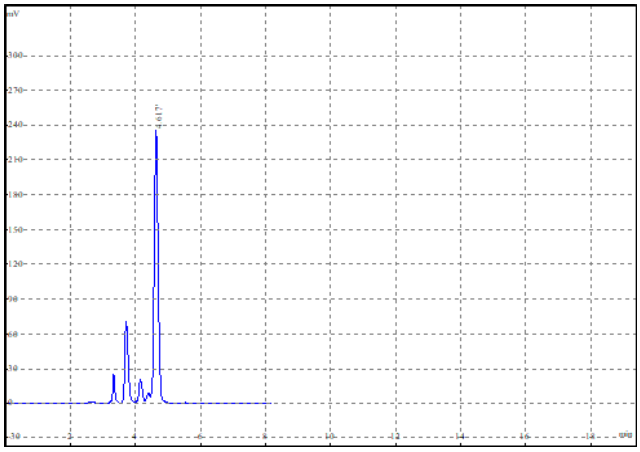

Figure 15. Chromatogram representing oxidative degradation of TDF.

3.6.2 Acid Degradation

A solution containing drug and 0.1NHCl was injected in the system and from the chromatogram obtained it was found that 17.61 % of the drug was degraded due to the presence of acid. The chromatogram of Acid degradation is represented (Figure 16).

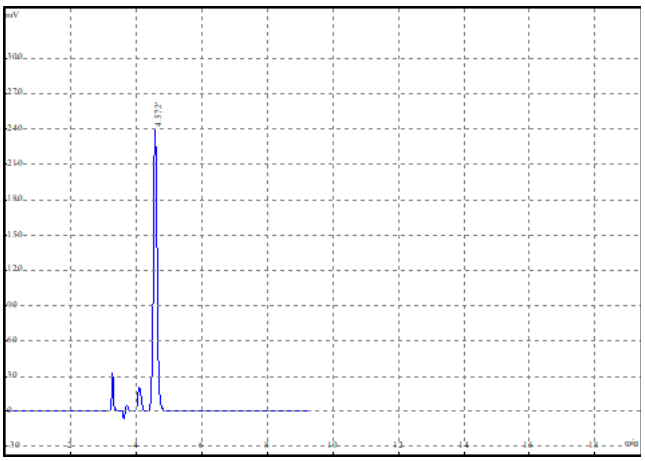

Figure 16. Chromatogram representing acid degradation of TDF.

3.6.3 Alkaline Degradation

A solution of drug and 0.1N NaOH was injected in the system for assessing the alkaline degradation. From the chromatograms, it was evaluated that the drug was 15.08% degraded in the presence of sodium hydroxide. The alkaline degradation chromatogram is given (Figure 17).

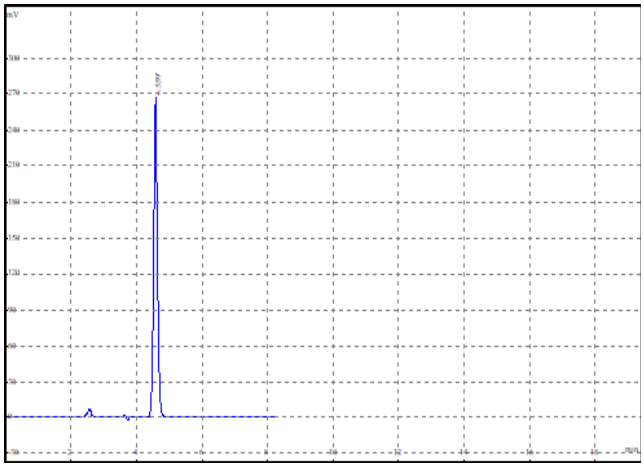

Figure 17. Chromatogram representing alkaline degradation of TDF.

3.6.4 *Photolytic Degradation*

Solution exposed to UV light in a stability chamber for 24 hours was injected in the HPLC system from the chromatogram it was noticed that 0.81% of the dug was degraded (Figure 18).

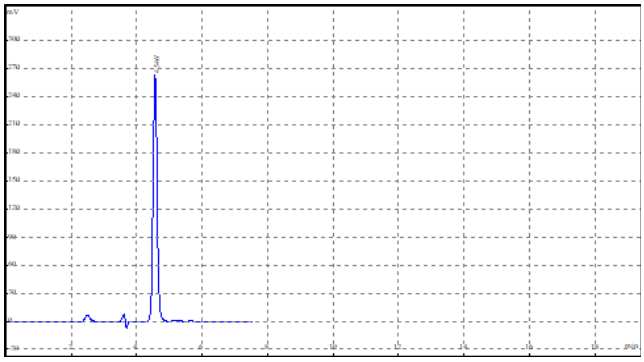

Figure 18. Chromatogram representing photolytic degradation of TDF.

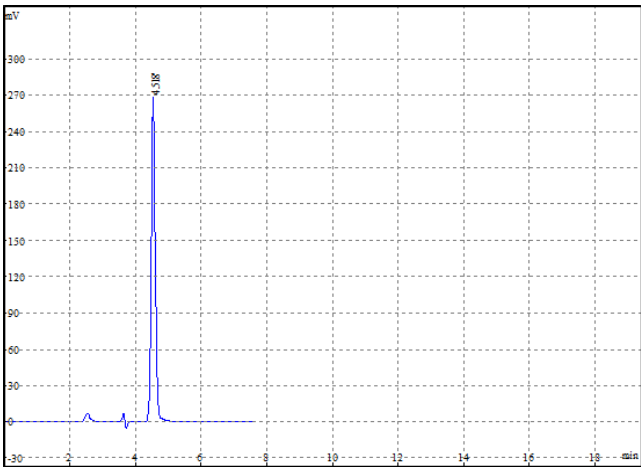

Figure 19. Chromatogram representing thermal degradation of (TDF).

3.6.5 *Thermal Degradation*

To evaluate the degradation due to heat stock solution which was kept in the oven was injected in the system, from the results obtained it was detected that 3.39% of the drug was degraded when exposed to 60°C (Figure 19).

Summarized the force degradation data in (Table 23).

Table 23. Degradation study results of TDF

Sr. No.	Condition	Area of degraded sample	Degraded up to %	Actual % degradation
1.	Oxidative degradation	2201665	79.0497	20.9502
2.	Acidic degradation	2294590	82.3861	17.61382
3.	Alkaline degradation	2364925	84.9115	15.0884
4.	Photolytic degradation	2762422	99.1834	0.81654
5.	Thermal degradation	2690683	96.6077	3.39229

4. Conclusion

In the present work UV and HPLC methods for estimation of (TDF) in the pharmaceutical product were developed and validated as per the guidelines of ICH Q1R2. Methods were developed using methanol:water in 60:40 ratio and detection wavelength determined was 260nm. The assay value obtained was 99.53% and 99.07% of UV and HPLC methods. Linearity was seen in the concentration ranging from 10-50 µg/ml with 0.999 regression coefficient was obtained from UV and HPLC methods. Accuracy, precision and robustness were also evaluated for both the method in which % RSD obtained was under 2%. Study of force degradation was also performed by the HPLC method utilizing methanol:water (60:40) at 260 nm, in which it was detected that the drug is susceptible to oxidation, photolysis, alkaline, acid and thermal degradation. Hence this method proposed can be employed in routine analysis for quantitative estimation and degradation evaluation of (TDF) in pharmaceutical preparation and bulk.

5. Conflict of Interest

Author has no conflict of interest for this research work.

6. Acknowledgement

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