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Stability indicating RP-HPLC method development and validation of Tenofovir in Bulk and Pharmaceutical formulation

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ABSTRACT

This study was aimed to develop stability indicating RP-HPLC method for analysis of Tenofovir. Successful separation of drug from degradation products formed under stress conditions was achieved by a gradient HPLC method, which was performed by Perkin Elmer HPLC using C_{18}(Phenomenex 100 x 4.6 mm x 5 micron) Column and optimized mobile phase consists of Methanol as Solvent-A,10 mM potassium di hydrogen ortho phosphate buffer of pH -3 as a Solvent-B in the ratio of 30:70 % v/vand UV detection was carried out at 260 nm. The retention time was observed at 7.33 min. The method was validated with respect to system suitability, linearity, precision, accuracy, limit of detection (LOD), limit of quantification (LOQ) and robustness. Linearity studies was performed (20–100 µg/ml), LOD and LOQ was found to be 0.9 and 2.71 µg/ml respectively. The RSD for precision were found to be less than 2 %. The mean percentage recovery was 99.13% to 100.21 %.The forceddegradation studies were carried out by using 0.01N NaOH, 1.0 N HCl, 3 % H_2O_2. Degradation behavior shows that the major degradation was observed at basic condition i.e.NaOH (91.1 %) followed by HCl (48.04%) and H_2O_2 (47.44%).Tenofovir is more susceptible towards acidic, basic and oxidative conditions within 30 min study. The used method is specific for the estimation of Tenofovir in presence of their degradation products and impurities. Thus the method is simple, specific, precise, robust and accurate for the determination of Tenofovir in formulations.

Keywords: Tenofovir, RP-HPLC, Method development,Validation,Forced degradation.

INTRODUCTION

Chemically Tenofovir is 9-[(R)-2-[[(isopropoxcarbonyl)-oxy]methoxy]phosphiny] methoxy]propyl] adeninefumarate. Tenofovir inhibits the activity of HIV reverse transcriptase by competing with the natural substrate deoxyadenosine 5'-triphosphate and after incorporation into DNA, by DNA chain termination, It gets converted into diphosphate intracellularly, which inhibits the DNA synthesis of HIV by competitive inhibition of reverse transcriptase and incorporation into viral DNA. It also inhibits hepatitis B virus polymerase, resulting in inhibition of viral replication. It is used for the treatment of HIV infection and chronic hepatitis B infection. [1,2]

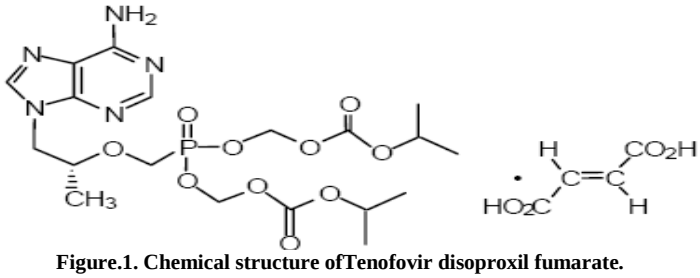

Figure.1. Chemical structure ofTenofovir disoproxil fumarate.

Stability-Indicating Method (SIM) is defined as a validated analytical procedure that accurately and precisely identified active Pharmaceutical ingredients (API), free from interferences like degraded product, process impurities, excipients. SIM is a quantitative analytical method which is used for the detection of decrease in the amount ofAPI present due to degradation by applying various stress conditions. For developing SIM, a forceddegradation study is normally carried out under more severe conditions than those used in accelerated studies.

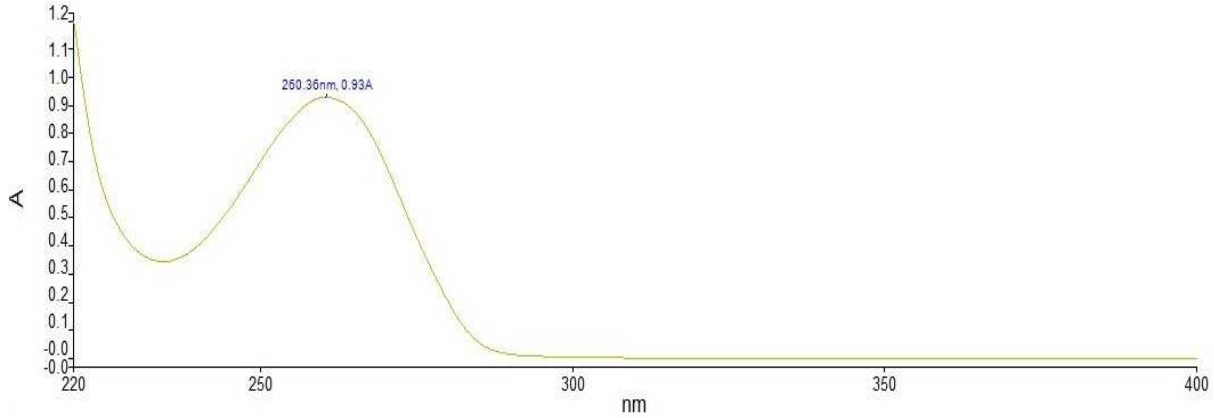

Figure2: Figure: 2 Tenofovir UV spectrum (λ_{max} = 260nm)

Literature survey revealed that many HPLC methods were developed for Tenofovir in bulk and pharmaceutical formulation individually and combination with other drugs[3-8]HPTLC[9] and HPLC[10]stability indicating assay also available, Present developed methods can able to separate the degradation peaksin all conditions. Certain review articles given brief idea that how to go for method development, validation, forced degradation studies and regulatory aspects. [11,12]

The main aim of the present research work is to develop a rapid, precise,economical,alternative stability indicating method for Tenofovir in bulk which was applied for the marketed formulation.

MATERIALS AND METHODS

2.1 Chemicals and Reagents

Tenofovir disoproxil fumarate API procured from Wockhardt Ltd.Methanol HPLC grade was procured from Merck India. GR grade potassium di-hydrogen orthophosphate from Molychem, o-phosphoric acid, hydrochloric acid (HCl), Hydrogen peroxide (H_2O_2) 3% w/v were procured from Merck India, SQ grade sodium hydroxide (NaOH) pellets were procured from RFCL limited. HPLC grade water was obtained through milli Q water purification system. All chemicals were of analytical grade and used as received. TAVIN tabletsprocured from Emcure pharmaceuticals.

2.2 Instrumentation

Perkin Elmer (USA) HPLC instrument was used for the analysis purpose,Series 200 UV–visible detector using total chrome navigator software for data handling using $C_{18(100\ x\ 4.6\ mm,\ 5\ \mu m)}$ column. Samples were injected through a Rheodyne injector valve with 20-µL sample loop

2.3 Chromatographic Conditions

The chromatographic separation was carried out at room temperature on a Phenomenex C_{18} (100 mm × 4.6 mm × 5 micron) analytical column under RP-HPLC conditions.Mobile phase was Methanol as Solvent-A ,10 mM potassium di hydrogen ortho phosphate buffer of PH -3 as a Solvent-B in the ratio of 30:70 %v/v.The mobile phase was filtered through 0.45 micron membrane filter which was degassed by ultrasonic bath. Flow rate was 1 mL/min. The column temperature maintained at 25°Cand the detection Wavelength was 260 nm. The injection volume was 20-µL.

2.3.1 Preparation of standard solution

Accurately weighed 10 mg of Tenofovir disoproxil fumarate was transferred to a 10 ml volumetric flask, sufficient amount of Methanol was added to dissolve it and volume was made up to 10 ml (Stock A; 1000 µg/ml). Aliquots of stock A were further diluted with diluent (Methanol: potassium dihydrogen orthophosphate buffer 70:30) up to 10 ml to get concentration of 20, 40, 60, 80, and 100 µg/ml for the linearity study.

2.3.2 Selection and Preparation of mobile phase

The mobile phase consists of two solvents: Solvent A, Methanol and Solvent B, Buffer having pH 3was prepared by using 0.680 gm of potassium di-hydrogen phosphate dissolved in 500 ml of water). Before proceeding for analysis the mobile phase was degassed by use of a sonicator (Oscar) and filtered through a 0.45 µm HPLC filtration assembly. The diluents used to attain the final concentration consist of a mixture of Methanol: buffer. Flow rate was 1 ml/min, injected volume 20µL, wavelength of detection was 260 nm. The system was equilibrated before each injection. Gradient elution system was performed which consist of solvent A and Solvent B. The program of gradient elution is shown in Table 1.Based on the optimised chromatographic conditions the chromatogram is shown in figure no 3.

2.3.3 Method Validation

The proposed method was validated according to the ICH guidelines.[13-16]

2.3.3.1 System Suitability:

The stock solution of the drug was prepared at strength of 100 µg/ml. It was diluted to prepare solutions containing 40, 60 and 100 µg/ml of the drug Tenofovir and 3 replicate injection of each concentration were injected into the HPLC system.

2.3.3.2 Linearity Curve (Calibration Curve):

Aliquots of standard Tenofovir stock solution were taken in five different volumetric flasks and diluted up to the mark with the diluents (Methanol: Buffer) such that the final concentrations of Tenofovir was 20, 40, 60, 80 and 100 µg/ml. Each of these drug solutions was injected 20µl into the injector and recorded the peak area.

2.3.3.3 Accuracy:

The accuracy of the method was determined by calculating percentage recovery of Tenofovir. Recovery studies were carried out by applying the spiking method in which known amount of Tenofovir corresponding to 80, 100 and 120% was added (standard addition method) to the TAVIN sample. At each level of the amount three determinations were performed and fitting these values to the linear equation of the calibration curve.

2.3.3.4 Precision:

Precision study of sample (Tenofovir)was carried out on Intraday and Interday by estimating corresponding responses 3 times each for 40, 60 and 80 ppm concentration.

2.3.3.5 LOD and LOQ:

The limit of detection (LOD) is defined as the smallest quantities of Tenofovir clearly distinguishable from baseline, which has a signal to noise ratio of 3:1. LOD was calculated using following formula, LOD=3.3(SD)/S, where SD= standard deviation of response (peak area of 40 ppm) and S= average of the slope of the calibration curve. Limit of Quantification (LOQ) was determined at a signal to noise ratio (S/N), which experimentally verified by diluting known concentrations of Tenofovir until the average responses were approximately 10 times the standard deviation of the responses for six replicate determinations, LOQ was calculated using following formula, LOQ=10(SD)/S.

2.3.3.6 Robustness:

Robustness is the measure of method capacity to retain unaffected by deliberate small changes in the chromatographic conditions like detection wavelength and flow rate. Detection wavelength was changed from 260 nm to 260 ± 2 nm and flow rate was changed from 1 ml/min to 1 ± 0.2ml/min. Effect of these changed parameters was studied by injecting the sample in to the system.

2.3.3.6 Assay of marketed formulation

Assay was performed by usingmarketed formulation TAVIN tablets which was procured from Emcure having 300 mg of Tenofovir disoproxil fumarate. It was carried out by making the drug concentration (100 µg/ml) with diluent(Methanol: Potassium di hydrogen ortho phosphate buffer) in a ratio of 70:30.20 µl of which was injected Triplicate.

2.4 Forced Degradation study of Tenofovir

Forced degradation studies of Tenofovir was carried out under conditions of acid-alkali hydrolysis and oxidation. Tenofovir was weighed (10 mg) and transferred into 10 ml volumetric flask and added 10 ml of methanol: buffer mixture to make 1000 ppm stock solution. Three such sets were prepared for acid, alkali and peroxide degradation.

2.4.1 Acid Degradation:

Forced degradation in acidic media was performed by adding 9 ml of 1 N HCl to 1 ml above stock solution (final volume was 100 ppm) and mixed it in one set of flasks and these mixtures were heated for up to 4h at 80°C. Samples were collected at 30 min, 1hr, 2hr and 4hr time interval respectively.

2.4.2 Base Degradation:

Forced degradation in basic media was performed by adding 9 ml of 1 N NaOH to 1 ml above stock solution (final volume was 100 ppm) and mixed it in one set of flasks and these mixtures were heated for upto 4h at 80°C. Samples were collected at 30 min, 1hr, 2hr and 4hr time interval respectively. Complete degradation observed initially so the concentration of NaOH reduced to 0.01 N NaOH.

2.4.3 Oxidative Degradation:

Forced degradation in oxidative media was performed by adding 9 ml of 3 % H_2O_2 to 1 ml above stock solution (final volume was 100 ppm) and mixed it in one set of flasks and these mixtures were heated for up to 4h at 80°C. Samples were collected at 30 min, 1hr, 2 hr and 4hr time interval respectively.

RESULTS AND DISCUSSION:

The optimized program of gradient elution is shown in Table 1. Based on the optimized chromatographic conditions the chromatogram is shown in figure no 3.

Table 1: **Gradient elution (solvent programming) runs**

Time (min)	Solution A (%)	Solution B (%)
0.5	30	70
5	90	10
8	30	70
4	30	70

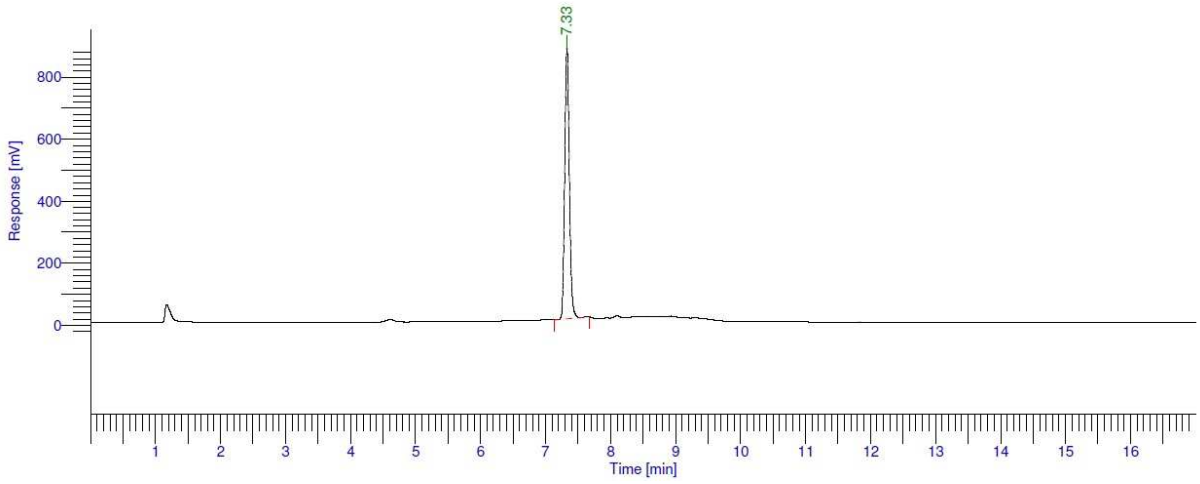

Figure: 3 Chromatogram of Optimised method.

3.1 Method Validation:

3.1.1 System Suitability:

The system suitability parameters were evaluated from standard chromatograms obtained by calculating the tailing factor and peak area from 3 replicate injections. The number of theoretical plates for Tenofovir peaks should not be less than 2000. The system suitability parameters of average of three replicate values are shown in Table 2

Table 2: System Suitability data

Retention time	7.32 min
Theoretical Plate	48244.76
Tailing Factor	1.14
Peak area	4398331

3.1.2 Linearity:

Linearity was done by using prepared standard solutions of five different concentrations levels ranging from 20 to 100 µg/ml that were injected in triplicate into the HPLC column, keeping the injection volume constant (20 µl) and Chromatograms was recorded at 260 nm. Calibration curve was plotted between the mean peak area vs. respective concentration (Table 3&Figure 4). The regression equation of calibration curves was obtained as y = 37363x + 80923 with a correlation coefficient of 0.9966. Where slope (m) is 37363 and intercept (c) is 80923.

Table 3: Linearity Data of Tenofovir

Concµg/ml	Area 1	Area 2	Area 3	Mean
0	0	0	0	0
20	892737.48	884775.36	883514.21	887009.01
40	1565144.92	1523543.54	1556435.84	1548374.76
60	2421329.91	2435973.24	2419732.86	2425678.67
80	3106174.26	3112642.25	3124782.91	3114533.14
100	3692311.07	3782680.95	3681546.25	3718846.09

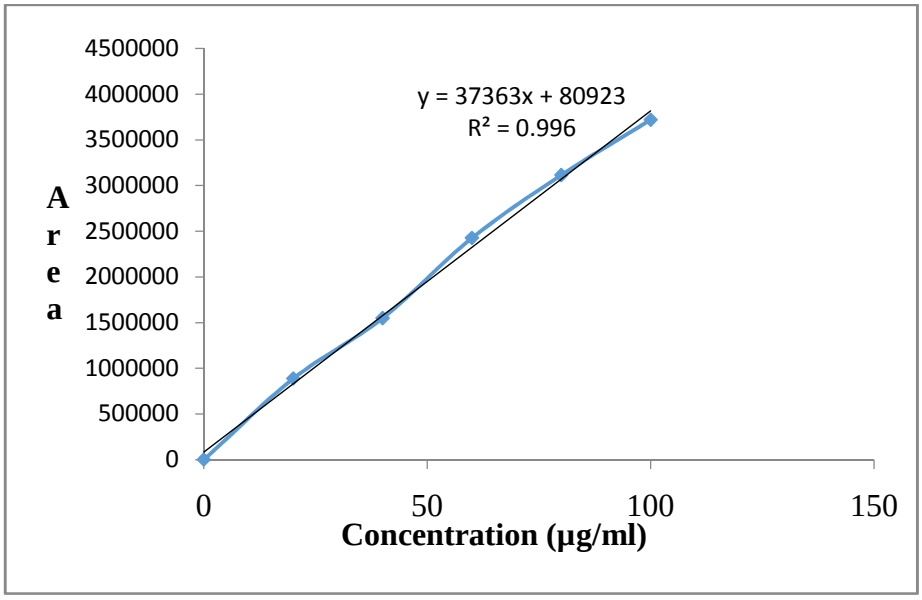

Figure 4: Figure:Linearity Plot of Tenofovir

3.1.3 Accuracy:

The accuracy of the method was carried out by adding known amounts of Tenofovir disproxil fumarate (API) corresponding to three concentration levels; 80%, 100%, and 120% to the drug solution of 50 **µg/ml (Formulation)**. The percentage recoveries at each level and each replicate were determined. The mean percentage recoveries found to be 99.13 to 100.21(Table 4). It was confirmed from results that the method is highly accurate.

Table 4: Accuracy data of Tenofovir

Sr. No.	Initial Conc. µg/ml (F)	Amt. added (µg/ml) (S)	Area	Amt. found µg/ml	Amt. Recover µg/ml	% Recovery	Mean %	%RSD
1	50	40	3303134.34	90.57	40.57	101.43	100.21	1.06
2			3275287.21	89.83	38.96	97.40		
3			3276243.99	89.85	39.85	99.63		
1	50	50	3659465.72	100.11	50.11	100.22	99.13	0.96
2			3626491.86	99.23	49.23	98.45		
3			3631525.62	99.36	49.36	98.72		
1	50	60	3994672.57	109.08	59.08	98.47	99.40	1.93
2			3986872.45	108.87	58.87	98.12		
3			4064920.24	110.96	60.96	101.60		

Where: F-Formulation, S-Standard API.

3.1.4 Precision:

The precision (intra-day repeatability) was established by analysing three replicates over three concentrations of Tenofovir shown in Table 5 and day to day precision (inter-day) was carried out by three concentrations with three

replicates shown in Table 6. Percentage relative standarddeviation (%RSD) is calculated, shown in Table 5 and Table 6 which are within the acceptable criteria of not more than 2.0%.

Table 5: Precision of Tenofovir (Intraday)

Sr.No	Conc. (µg/ml)	Area1	Area2	Area3	Mean	SD	RSD%
1	40	1541970.75	1556435.84	1536483.87	1544963.49	10307.16	0.67
2	60	2441080.59	2483642.58	2479732.86	2468152.01	23525.897	0.95
3	80	3194212.19	3183476.46	3124782.91	3167490.52	37373.372	1.18

Table 6: Precision of Tenofovir (Interday)

Sr. No	Conc. (µg/ml)	Area1	Area2	Area3	Mean	SD	RSD%
1	40	1534875.46	1522648.29	1523543.54	1527022.43	6815.64	0.45
2	60	2436583.20	2421329.91	2469493.59	2442468.9	24615.363	1.01
3	80	3106174.26	3126493.29	3112642.25	3115103.267	10380.665	0.33

3.1.5 LOD and LOQ:

The LOD was found to be 0.90µg/ml and the LOQ was found to be 2.71 µg/ml. Lower values of these parameter indicates more sensitivity of the method.

3.1.6 Robustness:

The results obtained from assay of the test solutions were not affected by varying the conditions and were in accordance with the results for original conditions. The value of assay determined for the same sample under original conditions and robustness conditions indicates that the developed method was robust for effect of changed wavelength (± 2 nm) and impact of flow rate (± 0.2), that evaluation were based on the system suitability parameters such as retention time, tailing factor and theoretical plates. Results were shown in Table 7.

Table 7: Robustness data for Tenofovir

Parameters	Variation	Rt (min)	Tailing factor	Plate Count
Wavelength(nm)	258	7.28	1.20	47213.81
	260	7.33	1.14	48244.76
	262	7.36	1.16	46121.12
Flow Rate(ml/min)	0.8	7.52	1.15	48213.13
	1	7.33	1.14	48244.76
	1.2	7.27	1.00	49124.67

3.1.7 Assay of marketed formulation

TENVIR tablet formulation was procured from Emcure, Which contains 300 mg of Tenofovir disoproxil fumarate. 100 µg/ml solution was prepared triplicate injection done for analysis and the percentage of drug found to be 99.53%-101.30% such as 298.60mg to 303.9mg. Chromatogram of TENVIR formulation given below Figure 5

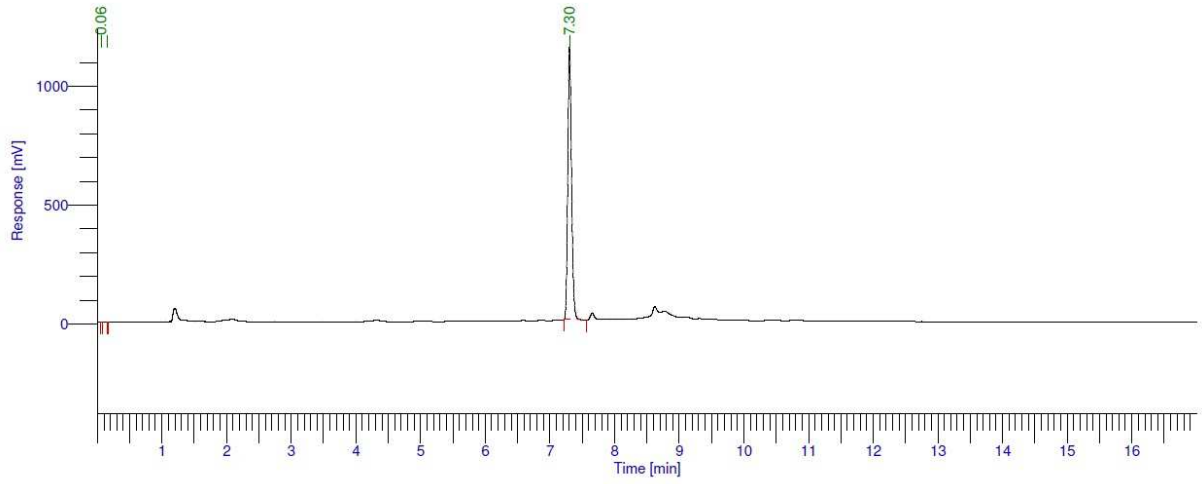

Figure 5: Chromatogram of Marketed formulation

3.2 Forced degradation studies

Forced degradation studies were conducted to conform whether the proposed method was able to detect the degraded product or not. Tenofovir standard is unstable at acid, alkali and oxidative conditions. A chromatogram of

Acid hydrolysis (1N HCl) performed at 80 ° C for ½ hr showed the degradation peaks at RT-1.71 min, 2.46 min and 4.52 min but major degradation occurs at 4.52 min .

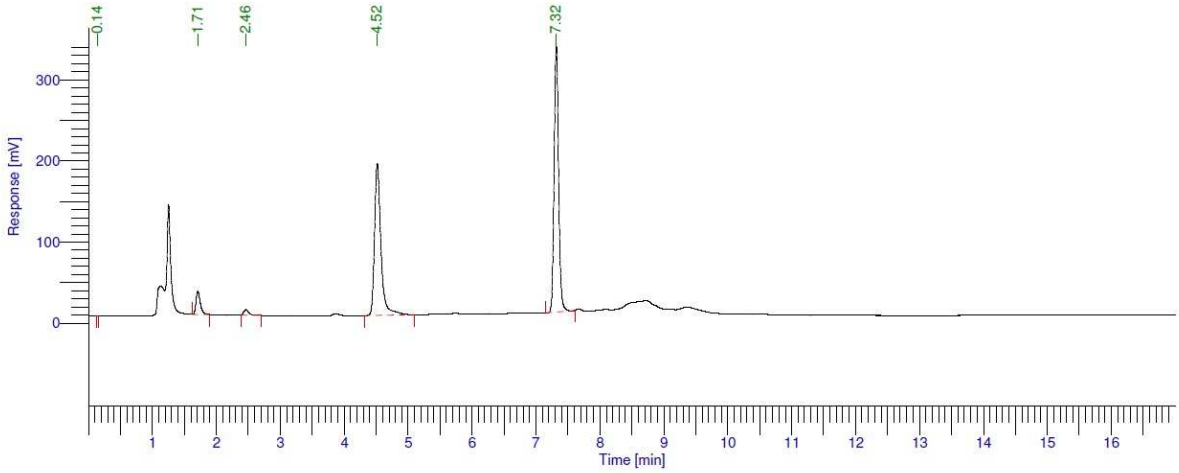

Figure 6: Chromatogram of 1 N HCl treated Tenofovir at 80 ° C for 30 min

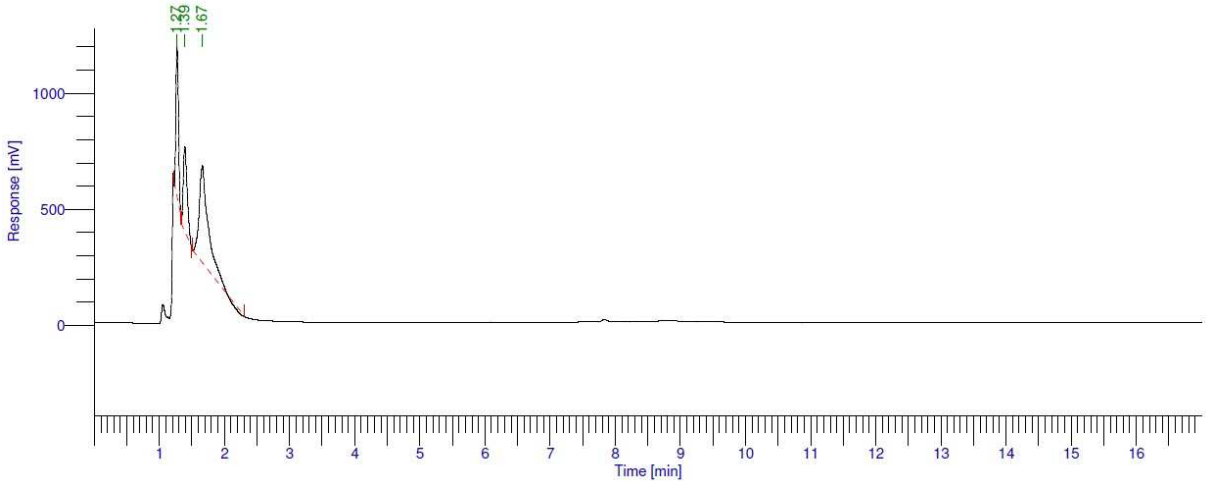

Figure 7: Chromatogram of 1 N NaOH treated Tenofovir at 80 ° C for 30 min

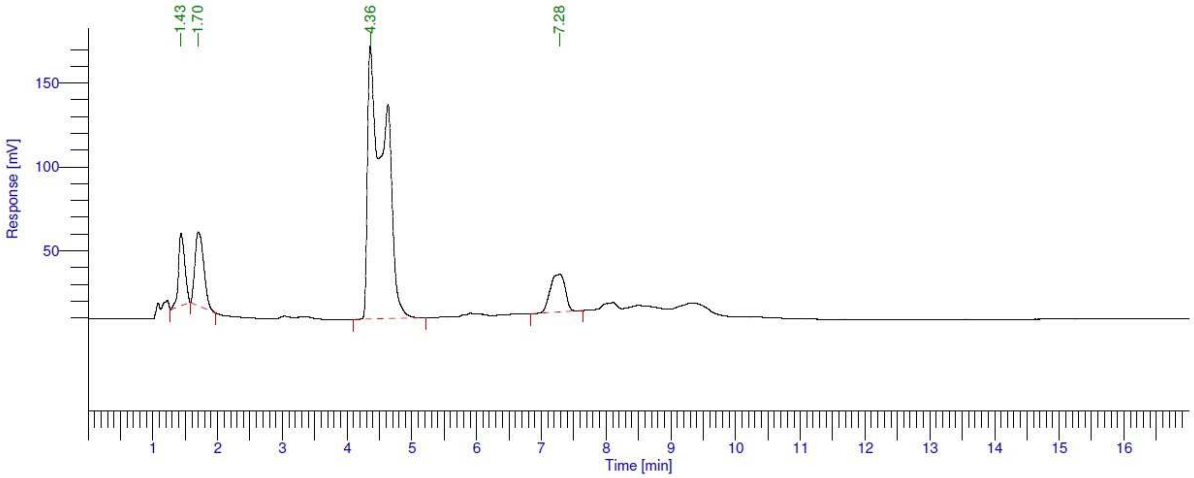

Figure 8: Chromatogram of 0.01 N NaOH treated Tenofovir at room temperature (0 min)

Total degradation found to be 48.04 % **(Figure 6)**A chromatogram of Basic hydrolysis(1 N NaOH) performed at 80 ° C for ½ hr showed the complete degradation at RT of 1.2 min, 1.3 min and 1.6 min respectively **(Figure 7)**.After observing this results degradation was again checked at 0min by using 1 N NaOH without applying any temperature, which showed the complete degradation at RT of 2 min**.**Finally the concentration was reduced to 0.01 N NaOH to check the degradation. The degradation peaks observed at 1.43 min, 1.7 min and 4.6 min respectively but major degradation observed at 4.36 min. Total degradation found to be 91.1%**(Figure 8).** A chromatogram of oxidized degradation performed at 80 °C for ½ hr showed the two major degradents observed at RT of 1.48 min and 4.71 min respectively. Total degradation found to be 47.44 %**(Figure 9).**The details of degradation was mentioned in table 8

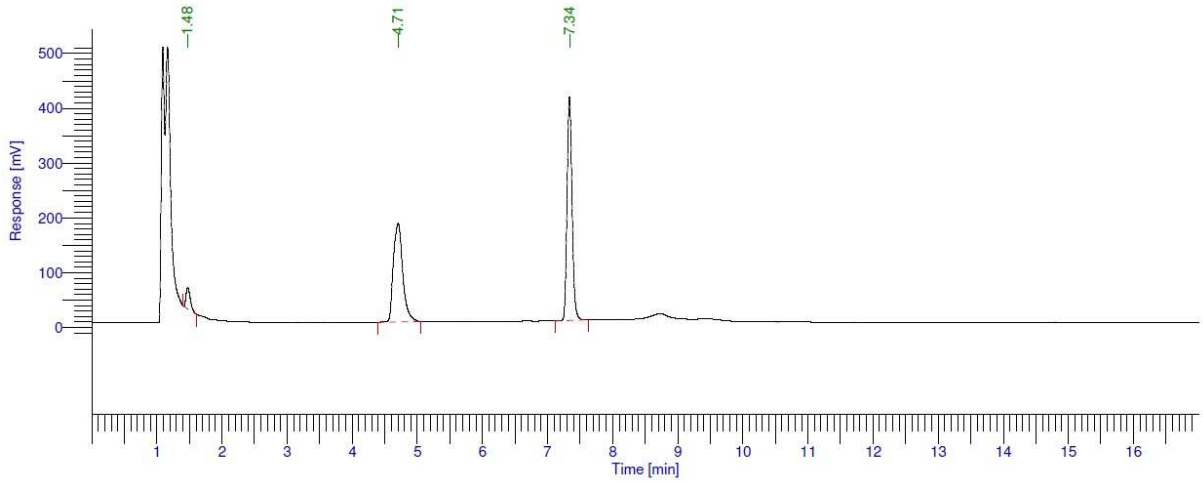

Figure 9: Chromatogram of 3 % H_2O_2 treated Tenofovir at 80 ° C for 30 min

Table 8: Forced degradation data of Tenofovir at different conditions

Degradation Condition	Time	% Degradation
Acid		
1 N HCl	30 min	48.04
Base		
1 N NaOH	30 min	100
0.01 N NaOH	0 min	91.1
Oxidative		
3 % H_2O_2	30 min	47.44

CONCLUSION

The present developed method is sensitive, rapid, precise, robust and accurate for the analysis of Tenofovir disoproxil fumarate.The stability indicating method can separate the API peak and degradation products separately without any interference under different conditions.After exposing to different conditions Tenofovir is susceptible to acid,alkali hydrolysis and Oxidation but more degradation was observed in Basic condition. Therefore the proposed method employed for monitoring the stability of Tenofovir drug substance in pharmaceutical formulation.

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