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STABILITY INDICATING LIQUID CHROMATOGRAPHIC METHOD FOR THE QUANTITATIVE DETERMINATION OF TENOFAVIR

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Abstract:

A selective, specific and sensitive stability-indicating high-performance liquid chromatographic method was developed and validated for the determination of tenofavir. Reversed-phase chromatography was performed on Hitachi Elite Lachrome, equipped with UVdetector using Develosil C18 column (150 mm × 4.6 mm, 5 µm). Isocratic elution was performed using methanol and water (55:45, v/v). The overall run time was 10 min. and the flow rate was 1.0 mL/min Detection wavelength was 240 nm and ambient temperature. Linearity was observed in the concentration range of 20-60 µg/mL with regression equation y = 47574x + 81945with correlation coefficient of 0.997. The LOQ and LOD were found to be 0.01 µg/mL and 0.03 µg/mL respectively. The percentage relative standard deviation in precision and accuracy studies was found to be less than 2%. Tenofavirwas subjected to stress conditions (acidic, alkaline, oxidation, UV and thermal degradation) and validated as per ICH guidelines.

Keywords: Tenofavir, Isocratic elution, RP-HPLC, Validation, Stability-indicating, LOD, LOQ.

Introduction:

Tenofaviris chemically known as ({[(2R)-1-(6-amino-9H-purin-9-yl)propan-2-yl]oxy}methyl)phosphonic acidwith molecular formula $C_9H_{14}N_5O_4P$ and molecular weight 287.213 g/mol as shown in Figure 1. Tenofovir, marketed by Gilead Sciences under the trade name Viread, belongs to a class of antiretroviral drugs known as nucleotide analogue reverse transcriptase inhibitors (nRTIs), which block reverse transcriptase, an enzyme crucial to viral production in HIV infected people. (1)

Tenofovir inhibits the activity of HIV reverse transcriptase by competing with the natural substrate deoxyadenosine triphosphate and, after incorporation into DNA, by DNA chain termination. Specifically, the drugs are analogues

ofthe naturally occurring deoxynucleotides needed to synthesize the viral DNA and they compete with the naturaldeoxynucleotides for incorporation into the growing viral DNA chain. However, unlike the natural deoxynucleotides substrates, NRTIs and NNRTIs (nucleoside/tide reverse transcriptase inhibitors) lack a 3'-hydroxyl group on the deoxyribose moiety. As a result, following incorporation of an NRTI or an NtRTI, the next incoming deoxynucleotide cannot form the next 5'-3' phosphodiester bond needed to extend the DNA chain. Thus, when an NRTI or NtRTI is incorporated, viral DNA synthesis is halted, a process known as chain termination. All NRTIs and NtRTIs are classified as competitive substrate inhibitors.(2)

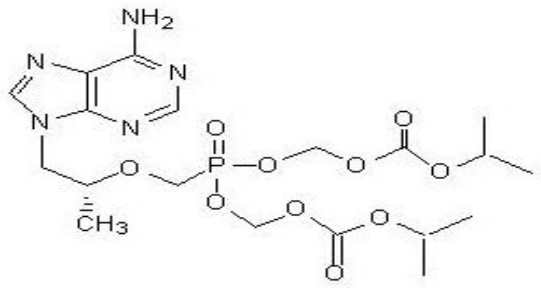

Fig 1: Chemical Structure of Tenofovir.

In the literature survey very few analytical methods have been reported for the determination of tenofavir which include spectroscopic techniques [4,5,6,7], LC method [3]. As per the literature available till date there was only one RP-HPLC method reported which is less sensitive. So, at present the authors have developed a stability indicating RP-HPLC method for the determination of tenofavirl in presence of its degradation products

Materials and Methods

Chemicals and reagents: Tenofavir standard was obtained from Reddy's lab (Visakhapatnam, India). Acetonitrile (HPLC grade), Sodium hydroxide (NaOH) and Hydrochloric acid (HCl), Potassium dihydrogen phosphate (KH2PO4), Potassium hydroxide (KOH) and Hydrogen peroxide (H2O2) were purchased from Merck (India). Tenofavir is available as solid pharmaceutical dosage form ,here tablets with brand name vired was used . All chemicals were of analytical grade and used as received.

Instrumentation and conditions: Chromatographic separation was achieved by using a hitachi elite lachromeHPLC system, equipped with uvdetector with develosil C18 (150 mm × 4.6 mm i.d., 5 µm particle size) column maintained at 25 ºC.

Preparation of stock solution:

Tenofavirstock solution (1000 µg/mL) was prepared by weighing accurately 25 mg of Tenofavirin a 25 mL volumetric flask with mobile phase. Working standard solutions were prepared on daily basis from the stock solution

with mobile phase and filtered through 0.45 μm membrane filter prior to injection. . Isocratic elution was performed using methanol and water (55:45, v/v). The overall run time was 10 min. and the flow rate was 1.0 mL/min. 20 μL of sample was injected into the HPLC system.

Preparation of Sample Solutions

Twenty tablets from each brand (vired) were procured, weighed and crushed to a fine powder. Powder equivalent to 25 mg tenofavir was accurately weighed into a 25 ml volumetric flask and made up to volume with mobile phase. The contents of the volumetric flask were sonicated for 30 min to enable complete dissolution of tenofavir. The solution was filtered and the filtrate was diluted with mobile phase. 20 μL of these solutions were injected into the system and the peak area was recorded from the respective chromatogram

Method Validation

The method was validated for system suitability, linearity, limit of quantitation (LOQ), limit of detection (LOD), precision, accuracy, selectivity and robustness,assay(8).

Linearity: Linearity test solutions for the assay method were prepared from a stock solution at different concentration levels (20–60 μg/mL) of the assay analyte concentration and 20 μL of each solution was injected in to the HPLC system and the peak area of the chromatogram obtained was noted. The calibration curve was plotted by taking the concentration on the x-axis and the corresponding peak area on the y-axis. The data was treated with linear regression analysis method. The limit of quantification and limit of detection were based on the standard deviation of the response and the slope of the constructed calibration curve (n=3), as described in ICH guidelines Q2 (R1).

Precision:

The intra-day precision of the assay method was evaluated by carrying out 9 independent assays of a test sample of Tenofavir at three concentration levels (30, 40 and 100 μg mL) (n=3) against a qualified reference standard. The %RSD of three obtained assay values at three different concentration levels was calculated. The inter-day precision study was performed on three different days i.e. day 1, day 2 and day 3 at three different concentration levels (20, 50 and 100 μg/mL) and each value is the average of three determinations (n=3). The % RSD of three obtained assay values on three different days was calculated.

Accuracy:

The accuracy of the assay method was evaluated in triplicate at three concentration levels (80, 100 and 120%), and the percentage recoveries were calculated. Standard addition and recovery experiments were conducted to determine

the accuracy of the method for the quantification of tenofavir in the drug product. The study was carried out in triplicate at 18, 20 and 22 µg/mL. The percentage recovery in each case was calculated.

Robustness: The robustness of the assay method was established by introducing small changes in the HPLC conditions which included wavelength (228 and 232 nm), percentage of acetonitrile in the mobile phase (68 and 72%) and flow rate (0.9 and 1.1 mL/min). Robustness of the method was studied using six replicates at a concentration level of 100 µg/mL of tenofavir.

Assay: The proposed method was applied to the determination of tenofavir tablets vired and the result of the assay yielded 99.98 with RSD < 2.0 %. The result of the assay (Table 5) indicates that the method is selective for the assay of tenofavir without interference from the excipients used in these tablets. The typical chromatograms for tenofavir obtained from the extracted marketed formulations were shown in Figure 4a and 4b.

Forced degradation studies

Forced degradation studies were performed to evaluate the stability indicating properties and specificity of the method (9).

Acid Hydrolysis: An accurately weighed 25 mg. of pure drug was transferred to a clean & dry 25 ml volumetric flask. To which 0.1 N Hydrochloric acid was added & make up to the mark & kept for 24 hrs. from that 4 ml was taken in to a 10 ml volumetric flask & make up to the mark with mobile phase, then injected into the HPLC system against a blank of HCl(after all optimized conditions)

Basic Hydrolysis: An accurately weighed 10 mg. of pure drug was transferred to a clean & dry 10 ml volumetric flask. To which 0.1 N Sodium hydroxide was added & make up to the mark & kept for 24 hrs. from that 4s ml was taken in to a 10 ml volumetric flask & make up to the mark with mobile phase, then injected into the HPLC system against a blank of .NaOH (after all optimized conditions).

Thermal Degradation: An accurately weighed 10 mg. of pure drug was transferred to a clean & dry 100 ml volumetric flask, make up to the mark with mobile phase & was maintained at 50 ^{0}C. for 24 hrs. then injected into the HPLC system against a blank of mobile phase (after all optimized conditions)

Photolytic Degradation: Approximately 10 mg. of pure drug was taken in a clean & dry Petri dish. It was kept in a UV cabinet at 254 nm wavelength for 24 hours without interruption. Accurately weighed 1 mg. of the UV exposed drug was transferred to a clean & dry 10 ml. volumetric flask. First the UV exposed drug was dissolved in methanol

& make up to the mark. Then injected into the HPLC system against a blank of mobile phase (after all optimized conditions).

Oxidation with (3%) H_2O_2:

Accurately weighed 10 mg. of pure drug was taken in a clean & dry 100 ml. volumetric flask. 30 ml. of 3% H2O2 and a little methanol was added to it to make it soluble & then kept as such in dark for 24 hours. Final volume was made up to 100 ml. using water to prepare 100 ppm solution. The above sample was injected into the HPLC system.

Results and Discussion

Initially the samples were analyzed using a mixture oftoacetonitrile :phosphate buffer with flow rate 1 mL/min and the drug sample was injected in to the loop where a sharp peak was eluted at 6.14 mins with tailing .. The mobile phase was changed methanol: water (80:20, v/v) with a flow rate of 0.5 mL/min in which the resolution and peak symmetry were not satisfactory. Finally the mobile phase composition was modified as 55:45, v/v and the drug peak eluted was sharp and symmetrical (UV detection at 240 nm) with retention time less than 8.47 mins (8.45 ± 0.05 min). Tenofavirshows linearity over a concentration range of 20-60µg/mL (Table 1) with % RSD 0.14 - 0.50. The linear regression equation was found to be y = 47574x + 81945 (r2 = 0.997)

Table 1: Optimized chromatographic conditions:

Mobile phase	Methanol: water 55:45
Flow rate	1 ml/min
Lambda max	240nm
Run time	13min
Column	Develosil c18
Injection volume	20µl
Linearity range	20-60µg/ml
Linearity equation	y = 47574x + 81945 (r2 = 0.997)

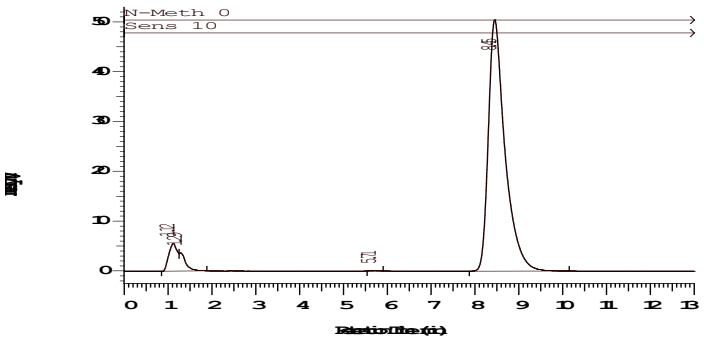

Fig 2: Chromatogram of tenofavir (optimized method).

Linearity:

Table-2: linearity data ConcvsAuc.

CONC.	AUC (n=6)
20	1082325
30	1400879
40	1938172
50	2473481
60	2959743

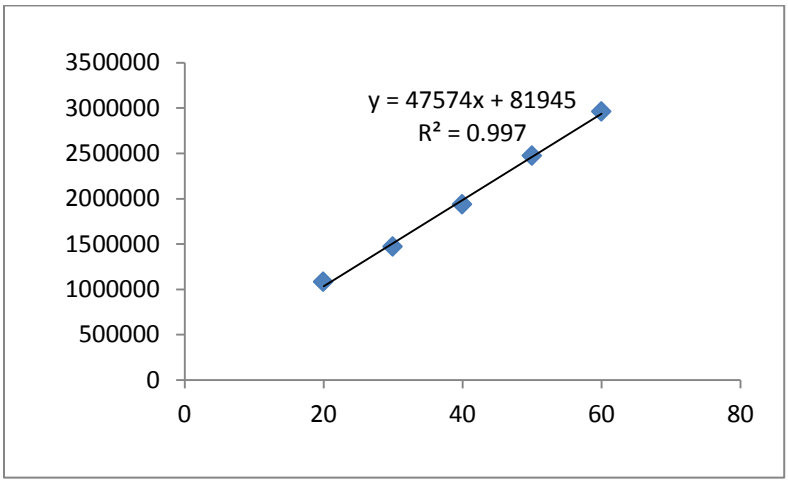

Fig-03: Calibration curve for Tenofovir.

The LOQ was found to be 0.03 µg/mL and the LOD was found to be 0.01 µg/mL. The % RSD in precision studies was found to be 0.28 – 1.00 (Intra-day) and 0.18 – 10.95 (Inter-day) (Table 2) and the percentage recovery was found to be 99.41 – 101 in accuracy studies with % RSD 0. 3 – 1.01 (<2.0 %) (Table 2)

Precision:

Table-3: Data showing the values of precision.

Conc. Of Tenofovir (API) (µg/ml)	Intra-Day precision		Inter-Day precision	
	Mean (n=6)	% RSD	Mean (n=6)	% RSD
30	30.24	0.28	30.75	0.82
40	40.76	0.73	40.86	0.74
100	99.61	0.95	99.73	0.18

Accuracy:

Table-4: Data showing the values of Accuracy.

Spiked concentration	Total concentration (µg/ml)	% Recovery	% RSD
80%	18	100.22%	1.062697277
100%	20	99.88666667%	0.636514851
120%	22	99.88666667%	0.326018736

The robustness of an analytical procedure refers to its ability to remain unaffected by small and deliberate variations in method parameters and provides an indication of its reliability for routine analysis [9]. The robustness of the method was evaluated by assaying the same sample under different analytical conditions deliberately changing from the original condition. The results obtained (Table 3) from assay of the test solutions were not affected by varying the conditions and were in accordance with the results for original conditions. The % RSD value of assay determined for the same sample under original conditions and robustness conditions was less than 2.0% (0.19-1.2) indicating that the developed method was robust

Robustness:

Table-5: Data showing the values of Robustness.

Change in parameter	**% RSD**
Flow (1.1 ml/min)	0.73
Flow (0.9 ml/min)	0.19
Temperature (27^0C)	0.53
Temperature (23^0C)	0.95
Wavelength of Detection (268 nm)	1.13
Wavelength of detection (264 nm)	1.02

Repeatability:

The precision of each method was ascertained separately from the peak areas & retention times obtained by actual determination of five replicates of a fixed amount of drug. Tenofovir (API). The percent relative standard deviation were calculated for Tenofovir are presented in the table below.

Table-6: Data showing the values of Repeatability.

HPLC Injection Replicates of Tenofovir	Retention Time	Area
Replicate – 1	8.47	1358039
Replicate – 2	8.47	1329282
Replicate – 3	8.45	1330602
Replicate – 4	8.46	1333599
Replicate – 5	8.47	1338172
Average	8.464	1337939
Standard Deviation	0.008944	11743.88
% RSD	0.105674	0.877759

System Suitability Parameter

System suitability testing is an integral part of many analytical procedures. The tests are based on the concept that the equipment, electronics, analytical operations and samples to be analyzed constitute an integral system that can be evaluated as such. Following system suitability test parameters were established. The data are shown in Table 39.

Table 7: Dataof System Suitability Parameter.

S.No.	Parameter	Limit	Result
1	Resolution	Rs > 2	4.15
2	Asymmetry	T ≤ 2	Tenofovir=0.14
3	Theoretical plate	N > 2000	Tenofovir=1439

Assay: The amount of drugs in vired tablet was found to be 122.98 (±0.498) mg/tab for Tenofovirand % assay was 99.98.

Table-8: Data showing the values of %assay

Brand name of tablets	Labelled amount of Drug (mg)	Mean (±SD) amount (mg) found by the proposed method (n=6)	% Assay
Vired	123	122.98 (±0.498)	99.98

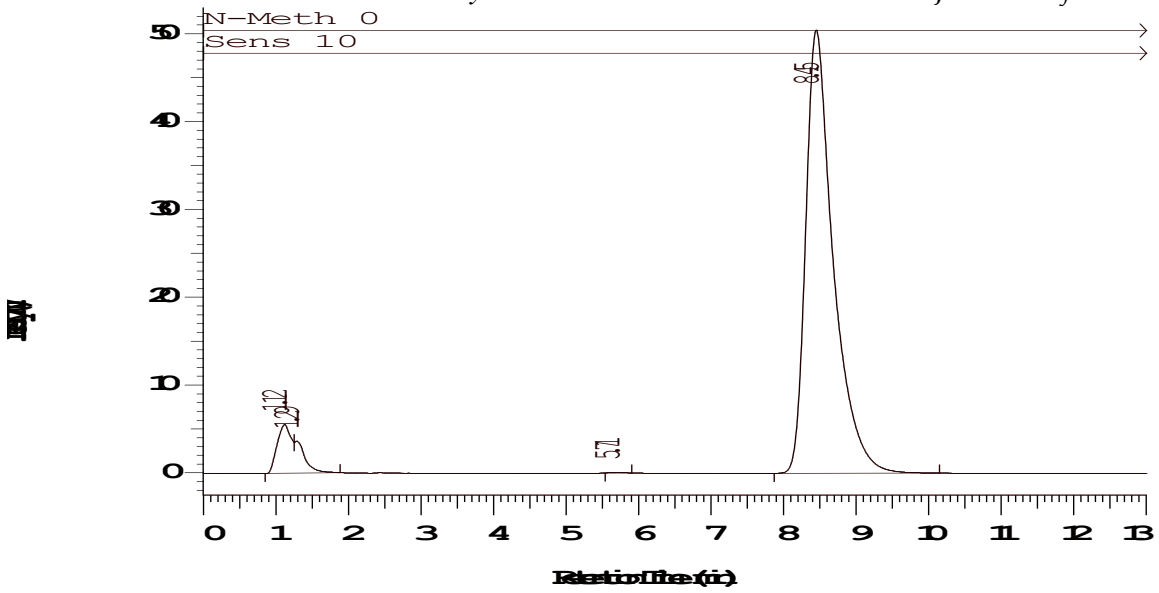

Fig 4: Chromatogram of tenofovirtablets(marketed formulation).

The results of the stress studies indicated the **specificity** of the method that has been developed. Tenofovir was degraded only in 3% H_2O_2& acidic stress conditions. The result of forced degradation studies are given in the following table.

Table-9:Results of force degradation studies of Tenofovir API.

Stress condition	Time	Assay of active substance	Assay of degraded products	Mass Balance (%)
Standard	24hrs	100	0	100
Acid Hydrolysis (0.1 M HCl)	24Hrs.	3.73	94.51	98.24
Basic Hydrolysis (0.I MNaOH)	24Hrs.	95.13	---------	95.13
Thermal Degradation (50 ^{0}C)	24Hrs.	98.52	----------	98.52
UV (254nm)	24Hrs.	97.13	----------	97.13
3 % Hydrogen peroxide	24Hrs.	91.37	8.27	99.64

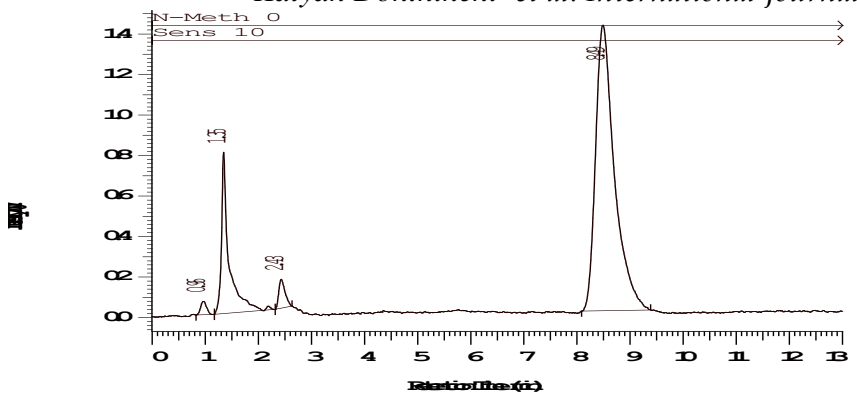

Fig 5: Chromatogram showing degradation for Tenofovir in 0.1 N HCl

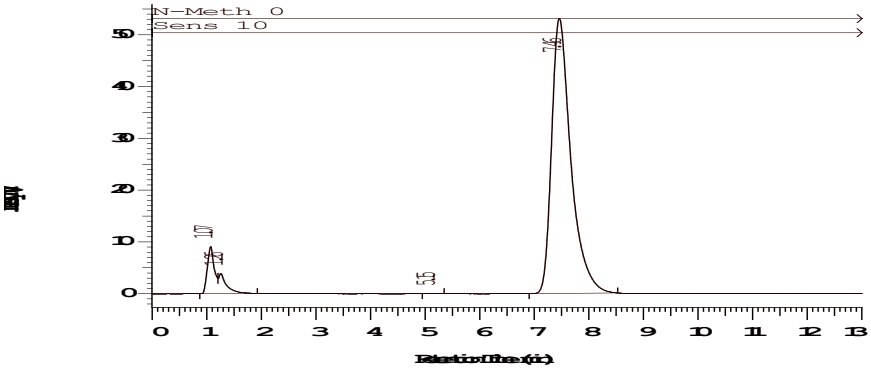

Fig 6: Chromatogram showing degradation related impurity in 0.1 N NaOH

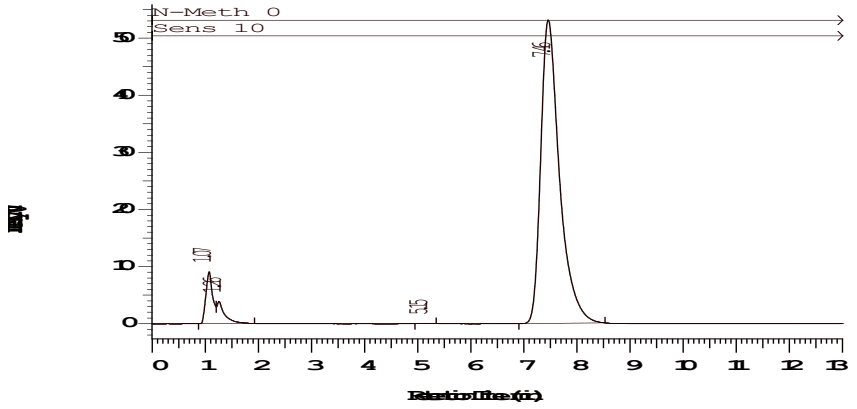

Fig 7: Chromatogram showing thermal degradation studies

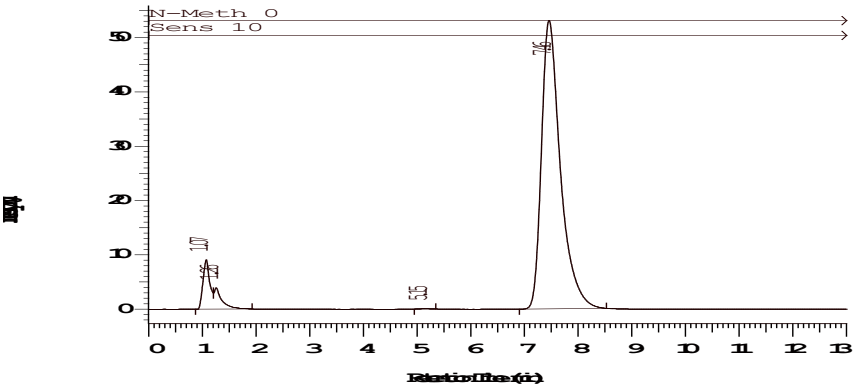

Fig 8: Chromatogram showing photolytic degradation.

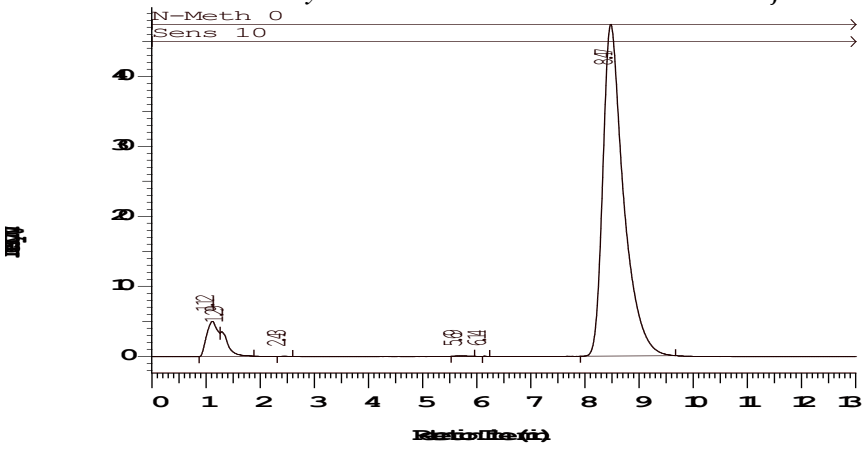

Fig 9: Chromatogram showing oxidative degradation.

Conclusions

The proposed stability-indicating HPLC method was validated as per ICH guidelines and applied for the determination of Tenofavir in bulk form and can be successfully applied to perform long-term and accelerated stability studies of tenofavirformulations. Suggestion

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