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A validated stability indicating RP-HPLC method for the determination of tenofovir in bulk and tablet dosage forms

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Abstract

A rapid, precise, accurate, specific and simple RP-HPLC method was developed for the estimation of Tenofovir in its tablet form. A High performance liquid chromatograph 10AT SHIMADZU- SPD10A, using Phenomenex - Luna RP-18(2),250X4.6mm, 5 µm column, with mobile phase composition of Acetonitrile: water [78:22 %(v/v)] was used. The flow rate of 1.0 mL min^{-1} and effluent was detected at 260 nm. The retention time of Tenofovir was 5.541 minutes. Linearity was observed over concentration range of 500-4000 ng mL^{-1}. The Limit of detection was found to be 74.80 ng mL^{-1} while quantification limit was 226.68 ng mL^{-1}. The accuracy of the proposed method was determined by recovery studies and found to be 98.465 to 100.003%. Commercial tablet formulation was successfully analyzed using the developed method and the proposed method is applicable to stability studies and routine analysis of Tenofovir in bulk and pharmaceutical formulations. The proposed method was validated for various ICH parameters like linearity, limit of detection, limits of quantification, accuracy, precision, range and specificity.

Keywords:

Tenofovir, RP-HPLC, Stability studies, Validation, ICH guidelines

1. Introduction

Tenofovir is chemically [(2R)-1-(6-aminopurin-9-yl) propan-2-yl] oxymethyl phosphonic acid (Fig. 1). It is a white crystalline powder used as antiretroviral agents, for the treatment of HIV infection. It has an empirical formula of $C_9H_{14}N_5O_4P$ and molecular weight of 287.2123. Tenofovir belongs to a class of antiretroviral drugs known as nucleotide analogue reverse transcriptase inhibitors (NtRTIs), which block reverse transcriptase, an enzyme crucial to viral production in HIV-infected people [1]. Food and Drug Administration granted approval to market Viread (TENOFOVIR) for the treatment of chronic hepatitis B [2]. Literature survey reveals that very few analytical methods has been established for the estimation of Tenofovir and Emtricitabine in bulk and in tablet Dosage form by Spectrophotometric method [3], Simultaneous determination of Emtricitabine and Tenofovir by area under curve and dual wavelength spectrophotometric method [4], Relevance of a combined UV and single mass spectrometry detection for the determination of tenofovir in human plasma by HPLC in therapeutic drug monitoring [5], Segmented polyurethane intravaginal rings for the sustained combined delivery of antiretroviral agents dapivirine and tenofovir [6]], Simultaneous quantification of a non-nucleoside reverse transcriptase inhibitor efavirenz, a nucleoside reverse transcriptase inhibitor emtricitabine and a nucleotide reverse

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transcriptase inhibitor tenofovir in plasma by liquid chromatography positive ion electrospray tandem mass spectrometry [7], RP-HPLC Method for the Determination of Tenofovir in Pharmaceutical Formulations and Spiked Human Plasma [8] were reported.

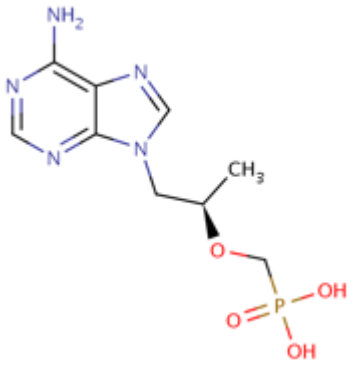

Fig. 1. Chemical structure of Tenofovir

The stability of a drug substance or drug product is defined as its capacity to remain within established specifications, i.e. to maintain its identity, strength, quality, and purity until the retest or expiry date [9]. Stability testing of an active substance or finished product provides evidence of how the quality of a drug substance or drug product varies with time under a variety of environmental conditions, for example temperature, humidity, and light. Knowledge from stability studies is used in the development of manufacturing processes, selection of proper packaging and storage conditions, and determination of product shelf-life [10, 11]. There was no reported stability-indicating analytical method for analysis of tenofovir in the presence of its degradation products in bulk and pharmaceutical dosage forms. The objective of this work was to develop a new, simple, economic, rapid, precise, and accurate stability-indicating HPLC method for quantitative analysis of tenofovir, and to validate the method in accordance with ICH guidelines[12] with shorter retention time, runtime, and economic mobile phase.

2. Experimental

2.1. Materials and methods

Pure standard of Tenofovir (Assigned purity 99.98%) was obtained as a gift sample from Ranbaxy labs Pvt. Ltd, Gurgaon, India. The gift samples were used as standard without further purification. HPLC grade water, Acetonitrile and methanol (Qualigens), Hydrochloric acid, Sodium hydroxide, Hydrogen peroxide (S.D. fine chemicals, Mumbai, India), were used throughout the experiment. Commercial pharmaceutical preparation (Viread) which was claimed to contain 300mg of Tenofovir is used in analysis. The chemical structure and purity of the sample obtained was confirmed by TLC, IR, Melting point studies.

2.2. Instrumentation and chromatographic conditions

High performance liquid chromatograph, Shimadzu pumpLC-10AT VP equipped with universal injector (Hamilton 25 µL) SPD10A, UV-VIS detector SPD10A-10A VP (Shimadzu) was used. Isocratic elution of mobile phase comprising of Acetonitrile and water in the ratio of 78:22% (v/v) with flow rate of 1.0 mL min^{-1} was performed on C18 column (250x4.6 mm, 5µm). The effluent was detected at 260 nm. The retention time of Tenofovir was 5.541 minutes. The column temperature was maintained at ambient and the volume of injection was 20 µL. Prior to injection of analyte, the column was equilibrated for 30- 40 min with mobile phase.

Different kinds of equipments viz Analytical weighing balance (Shimadzu AX 200), Sonicator (model SONICA 2200MH), Water purification system, Vacuum pump (model XI 5522050 of Millipore), Millipore filtration kit for solvents and sample filtration were used throughout the experiment. The Spinchrom CFR software was used for acquisition, evaluation and storage of chromatographic data.

2.3. Preparation of mobile phase

The HPLC grade solvents of Acetonitrile and water were used for the preparation of mobile phase in the ratio of 78:22% (v/v). The contents of the mobile phase were filtered before use through a 0.45 µm membrane filter, sonicated and pumped from the solvent reservoir to the column at a flow rate of 1 mL min^{-1}.

2.4. Preparation of standard solution

A stock solution of drug was prepared by dissolving 100 mg of Pure Tenofovir in a 100 mL volumetric flasks containing sufficient amount of methanol (HPLC grade) to dissolve the drug, sonicated for about 15 min and then made up to volume with mobile phase. Daily working standard solutions of Tenofovir was prepared by suitable dilution of the stock solution with the mobile phase. Six sets of the drug solution were prepared in the mobile phase containing Tenofovir at a concentration of 1400-3400 ng mL^{-1}. Each of these drug solutions (20µl) was injected six times into the column, the peak area and retention times were recorded.

2.5. Procedure for Sample solution (from Formulation)

Twenty tablets were weighed accurately and powdered. An amount of the powder equivalent to 300 mg of Tenofovir (content of one tablet) was dissolved in 50 mL of mobile phase. The solution was stirred for 10 min using a magnetic stirrer and filtered into a 100 mL volumetric flask through 0.45 µm membrane filter. The residue was washed 3 times with 10 mL of mobile phase, and then the volume was completed to 100 mL with the same solvent. Further add mobile phase to obtain a stock solution of 10 µg mL^{-1}. An aliquot of this solution (1 mL) was transferred to a 10 mL volumetric flask and made up sufficient volume with the mobile phase to give an expected concentration of 1 µg mL^{-1}. All determinations were conducted in triplicate.

2.6. Stability Studies

2.6.1. Thermal degradation at different temperature and different time interval

Expose about 2 to 3 gm of sample at different time intervals viz. 0, 90, and 180 days and at different temperatures viz. -20^0C, 25^0C, and 40^0C. After that sample solution (Tenofovir) in mobile phase was demonstrated by injected the sample solution in HPLC, no degradants were observed in the chromatogram. However, after 180 days and 40^0C the chromatographic peak area of tenofovir decreased insignificantly. Hence, the sample was stable at least for 180 days at 40^0C.

2.6.2. Photochemical degradation

The photochemical stability of the tenofovir was studied by exposing the methanolic stock solution to direct sunlight for 8 h (from 9 AM to 5 PM, at 20^oC).

2.6.3. Thermal Stress (test sample exposed to sunlight)

Transfer about 2 to 3 gm of sample into a clean dry watch glass and spread evenly. Expose to sunlight for 10 hours. After the sample got exposed to prescribed time, weigh accurately 25 mg of sample into a clean dry 50 mL volumetric flask, dissolve and dilute to the

mark with mobile phase, finally make a concentration of 2200 ng mL^{-1} with mobile phase and inject 20 µL of this sample into HPLC, observe the degradation.

2.6.4. Forced degradation of Tenofovir and tablets of Tenofovir

In order to establish whether the analytical method and the assay were stability indicating, the tablets and pure active pharmaceutical ingredient of tenofovir were stressed under various conditions to promote degradation. As this drug was freely soluble and stable in methanol and methanol was used as solvent in all forced degradation studies. All solutions were prepared to use in forced degradation studies were prepared by dissolving Tenofovir or drug product in small volume methanol and later diluted with 3% hydrogen peroxide, 0.1N hydrochloric acid and 0.1 N sodium hydroxide to achieve concentration of 100 µg mL^{-1}. After the degradation, these solutions were diluted with mobile phase to get starting concentration of 10 µg mL^{-1} with the objective of evaluating stability of tenofovir. The degradants were observed in the chromatogram and showing good resolution with the tenofovir.

2.6.5. Hydrolysis (Acid and alkaline)

Initially for hydrolytic degradation the tenofovir was dissolved in known amount of methanol and diluted with 0.1 N HCl or 0.1 N NaOH to obtain a concentration of 100 µg mL^{-1}. After completion of degradation process, both the solutions were neutralized with acid or base, as necessary and diluted with the mobile phase to achieve a concentration of 10 µg mL^{-1}. The solutions for hydrolysis were prepared in methanol and 0.1 N HCl and 0.1N NaOH (60:40 v/v). The prepared solutions in acid were injected to the chromatographic system at 0 h (immediately after preparing the solution) and after reflux at 60 ºC about 2h and the solutions prepared in alkali were injected at 0 h and after reflux at 60 ºC about 2h. The respective chromatograms were recorded for the study of extent of degradation.

2.6.6. Peroxide degradation

The solutions for peroxide degradation were prepared in methanol and 3% hydrogen peroxide (60:40 v/v). The prepared solution was refluxed at 60^0c about 2h and injected into chromatographic system after 2 h. The respective chromatogram was recorded for the study of extent of degradation.

3. Results and Discussions

3.1. Stability Studies

3.1.1. Thermal degradation at different temperature and different time intervals

Expose about 2 to 3 gm of sample at different time intervals viz. 0, 90, and 180 days and at different temperatures viz. -20 ºC, 25 ºC, and 40 ºC. After that sample solution (Tenofovir) in mobile phase was demonstrated by injected the sample solution in HPLC, no degradants were observed in the chromatogram. However, after 180 days and 40 ºC the chromatographic peak area of tenofovir decreased insignificantly. Hence, the sample was stable at least for 180 days at 40^0C (Fig. 2a-g)

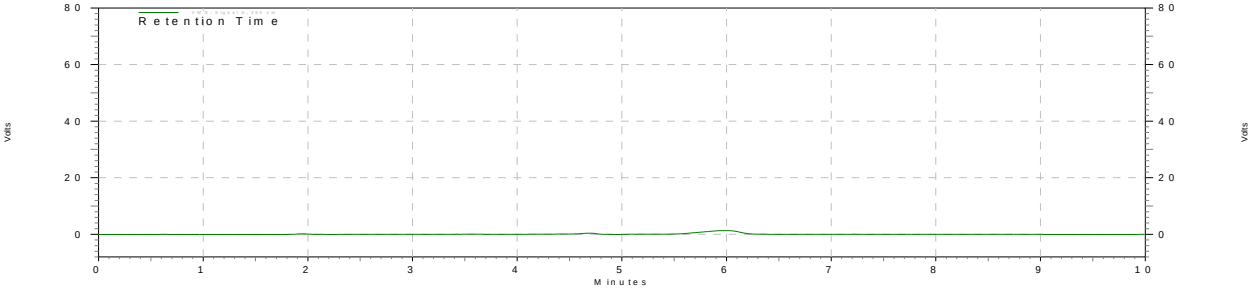

Fig.2a. Stability Chromatogram No.1 (blank)

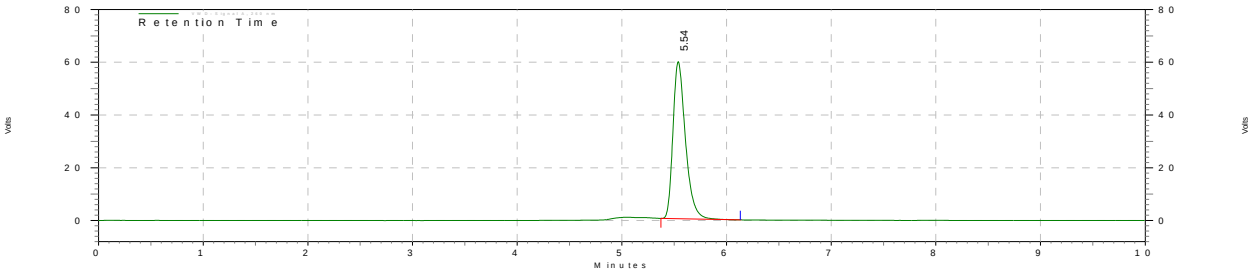

Fig.2b. Stability Chromatogram No.2 (initial)

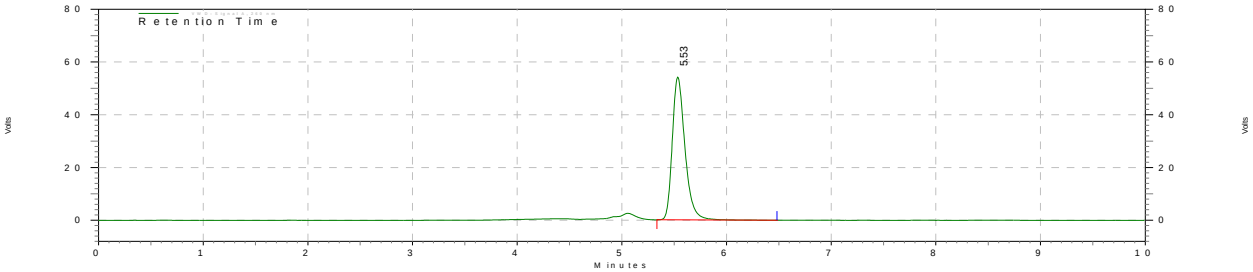

Fig.2c. Stability Chromatogram No. 3 (25^0C, 90 Days)

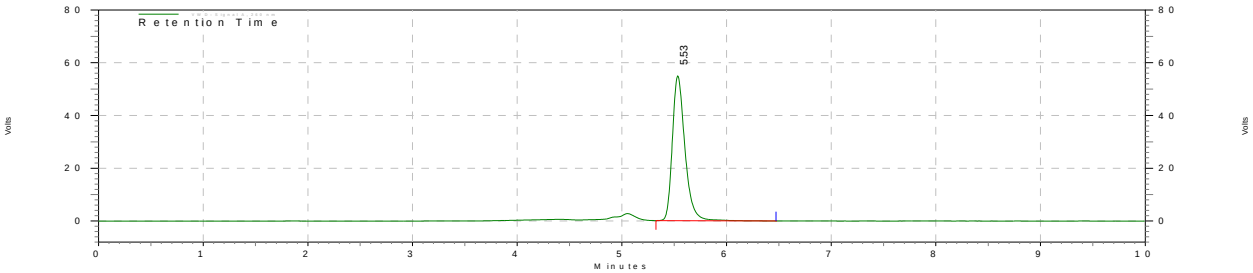

Fig.2d. Stability Chromatogram No. 4 (25^0C, 180 Days)

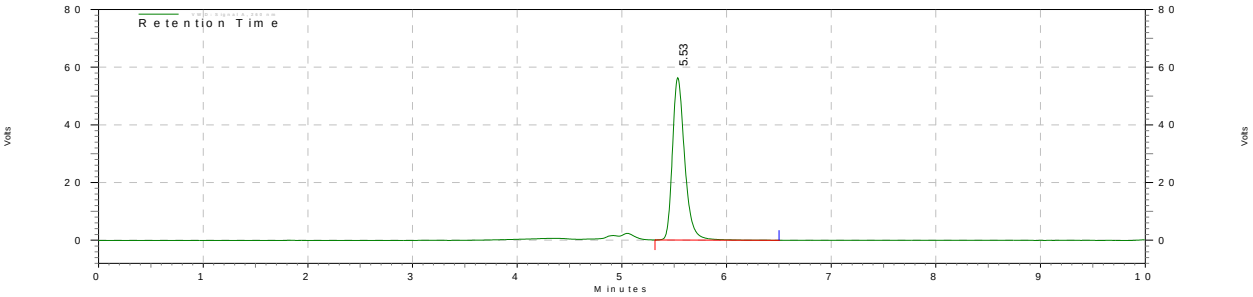

Fig.2d. Stability Chromatogram No. 5 (-20^0C, 90 Days)

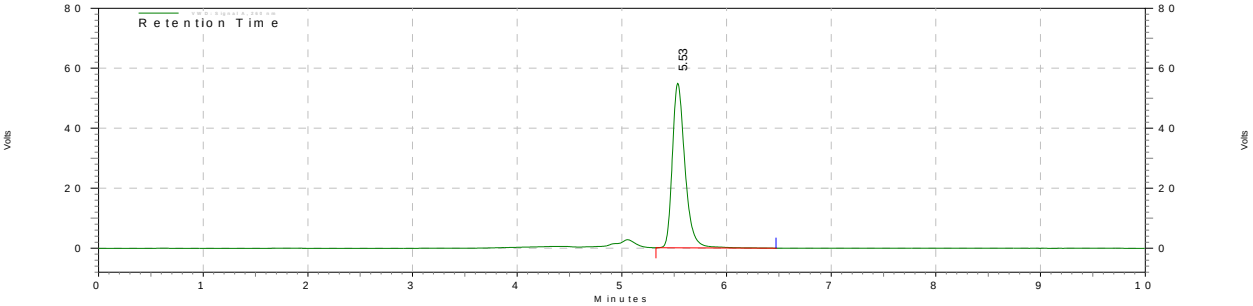

Fig.2e. Stability Chromatogram No. 6 (-20^0C, 180 Days)

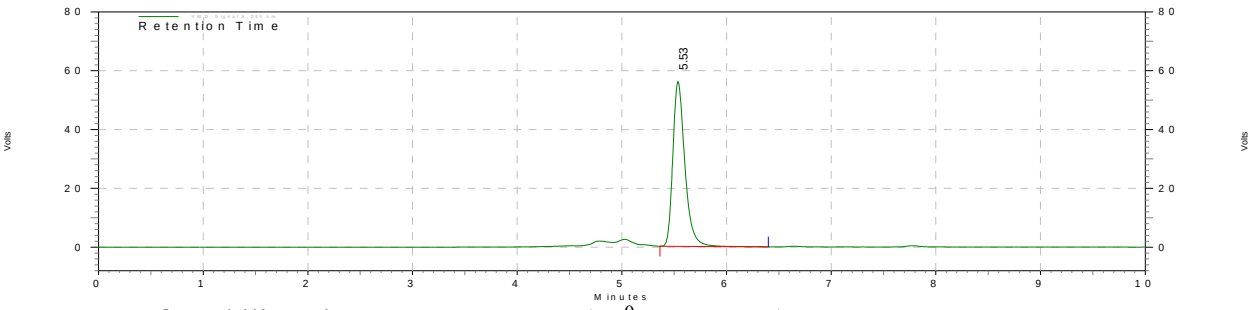

Fig.2f. Stability Chromatogram No. 7 (40^0C, 90 Days)

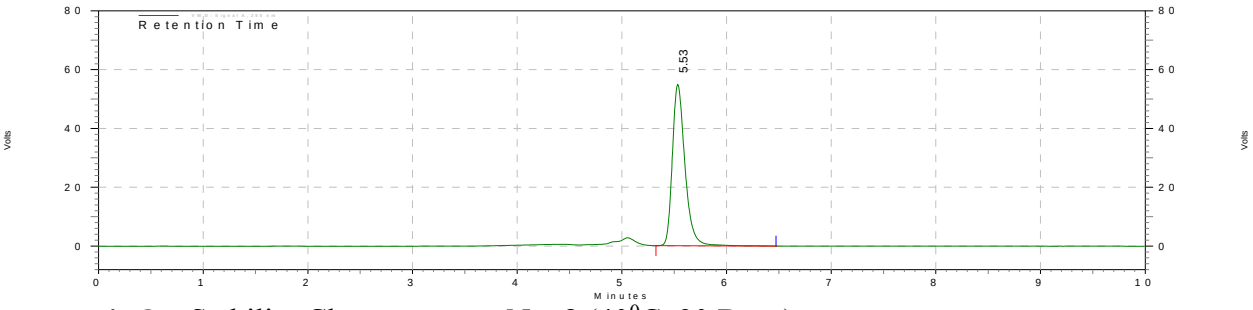

Fig.2g. Stability Chromatogram No. 8 (40^0C, 90 Days)

The result obtained by thermal degradation at different temperature and different time interval viz. Initial, 25 ºC at 90 days, 25 ºC at 180 days, -20 ºC at 90 days, -20 ºC at 180 days, 40 ºC at 90 days, 40 ºC at 180days was found to be 99.43%, 99.25%, 99.13%, 99.29%, 99.17%, 98.36%, 97.43% respectively. The further study was carried out by employing the following tests: hydrolysis (neutral, acidic and basic), photolysis and thermolysis. No decomposition was observed when the tenofovir was exposed to sunlight, temperature, UV; whereas significant change i.e., decrease of assay about 20 to 25% observed when sample was treated with 0.1N NaOH and 0.1 N HCl. The sample treated with 3% H_2O_2 was almost completely degraded.

3.1.2. Validation of Analytical Method

Validation of an analytical method is process to establish by laboratory studies that the performance characteristics of the method meet the requirements for the intended analytical application. Performance characteristics are expressed in terms of analytical parameters.

Typical analytical parameters used in validation area:

- Linearity
- Accuracy
- Precision
- Specificity
- Limit of detection
- Limit of quantification
- Range
- Ruggedness
- Robustness
- System suitability
- Solution stability

3.2. Linearity

A stock solution of drug was prepared by dissolving 100 mg of Pure Tenofovir in a 100 mL volumetric flasks containing sufficient amount of methanol (HPLC grade) to dissolve the drug, sonicated for about 15 min and then made up to volume with mobile phase. Daily working standard solutions of Tenofovir was prepared by suitable dilution of the stock solution with the mobile phase. Six sets of the drug solution were prepared in the mobile phase containing tenofovir at a concentration of 1400-3400 ng mL^{-1}. Each of these drug solutions (20 µL) was injected in six concentrations in three replicates times into the column, the peak area and retention times were recorded. (Table 1 and Fig.3). The coefficient of correlation (r^2) should be greater than 0.998.

Table 1. Peak areas of Tenofovir for several dilutions.

Replicate*	Dilution I	Dilution II	Dilution III	Dilution IV	Dilution V	Dilution VI
1	6768459	7476074	8525623	9379921	10224371	11225410
2	6752369	7462105	8512632	9441257	10326598	11201254
3	6742851	7461025	8501255	9221463	10212852	11092541
Average	6754560	7466401	8513170	9347547	10254607	11173068
SD	12943.789	8394.162	12192.905	113416.963	62611.500	70776.879
RSD%	0.19	0.11	0.14	1.21	0.61	0.63

*Average of five readings

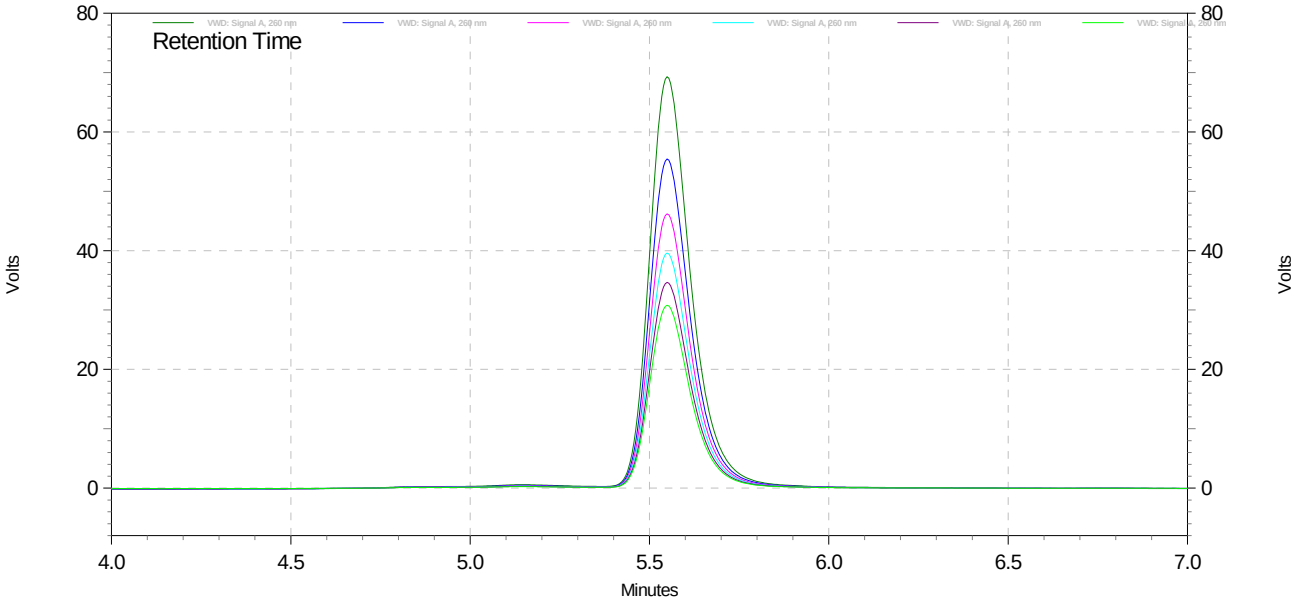

Fig. 3. Chromatogram No. 9 (Overlay spectra for Linearity)

The correlation coefficient (r^2) for tenofovir was found to be 0.9992, indicating the linearity and the method is linear between the concentrations of 500-4000 ng mL^{-1}.

3.3. Accuracy

The accuracy is the closeness of the measured value to the true value for the sample. Accuracy was found out by recovery study from prepared solution (three replicates) with standard solution, of the label claim. Aliquots of 0.4 mL, 1.2 mL and 2.0 mL of sample drug

(Tenofovir) solution of 10 µg mL^{-1} were pipetted into each of three volumetric flasks. To this 1.0 mL of standard drug (Tenofovir) solution of 10 µg mL^{-1} was added to each volumetric flask respectively. The volume was made up to 10 mL with mobile phase. 20 µL of each solution was injected and chromatograms were recorded. The range was found between 98.465 to 100.003 % respectively

The values of recovery justify the accuracy of the method. The % recovery values were obtained within the standard limit which confirms that the method is accurate and free from any positive or negative interference of the excipients. The recovery data was generated for Tenofovir are presented in the Table 2.

Table 2. Result of recovery studies of drug

Conc. taken in ng mL^{-1} (A)	Std addition in ng mL^{-1} (B)	Total drug conc. in ng mL^{-1} (A+B)	Peak Area*	Recovery, %
400	1000	1400	6650903	98.465
1200	1000	2200	8497081	99.811
2000	1000	3000	10254984	100.003

*****Average of three readings

The percentage recovery by the proposed method was ranging from 98.465 to 100.003% indicating no interference of the tablet excipients with drug under analysis.

3.4. Precision

Precision is measure of repeatability or reproducibility and it was determined by injecting 5 times the expected operating range concentration. The chromatograms were recorded to determine mean standard deviation and relative standard deviation. The acceptance criteria for the precision is RSD<2.0% for peak area and retention time.

Table 3. The precision of Tenofovir analysis

S.No.	Area Response
1	8335874
2	8335214
3	8346327
4	8356251
5	8328369
Average	8340407
S.D	10937.407
R.S.D	0.13

From Table 3, it is observed that RSD for the assay is 0.13 which indicates that the method is precise and reproducible.

3.5. Specificity

Specificity is the ability to assess the analyte in the presence of components that may be expected to be present in the sample matrix (USP 2004). For demonstrating the specificity

of the method for drug formulation the drug was spiked and the representative chromatogram (Fig. 4).

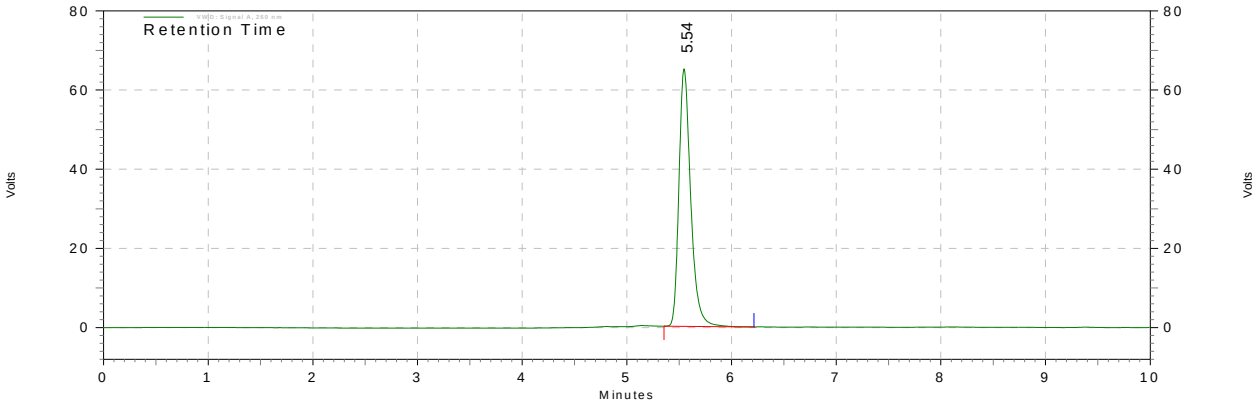

Fig. 4. The specificity chromatogram of Tenofovir

The excipients used in different formulation products did not interfere with the drug peak and thus, the method is specific for tenofovir.

3.6. Solution Stability

The solution stability of the standard and sample prepared in mobile phase was studied for 5 days at bench top. The solution under study was compared with freshly prepared standard solution, the samples were found to be stable for period of more than 72 hours.

3.7. Range

The specific range derived from the linearity studies. The range was calculated from the linearity graph. From the lower to higher concentration between which the response is linear, accurate and precise. The acceptance criteria for the range is RSD < 2.0. It was found to be 500-4000 ng mL^{-1}.

3.8. Limits of Detection and Quantification

The detection limit (LOD) is the lowest amount of an analyte in a sample that can be detected, but not necessarily quantitated, under the stated experimental conditions. It may be expressed as a concentration that gives a signal-to-noise ratio of 2:1 or 3:1. The lower limit of detection for tenofovir is 74.80 ng mL^{-1} in reference material and formulation. Limit of Quantification (LOQ) is the lowest amount analyte in a sample that can be determined with acceptable precision and accuracy under the stated experimental conditions. A signal-to-noise ratio of 10:1 can be taken as LOQ of the method. The LOQ values were found to be 226.68 ng mL^{-1} for raw material and formulations.

3.9. System Suitability

A solution of 2200 ng mL^{-1} (Approx.) of tenofovir (in five replicates) was prepared and same was injected, then the system suitability parameters were calculated from the following chromatogram. (Fig. 5).

3.10. Theoretical plates per column

Theoretical plates column were calculated from the data obtained from the peak.

$$n = (5.54Vr^2)/W_h^2$$

Where, 'n' is number of theoretical plates per meter, 'Vr' is the distance along the base line between the point of injection and a perpendicular dropped from the maximum of the peak of interest and 'W_h' is the width of the peak of interest at half peak height.

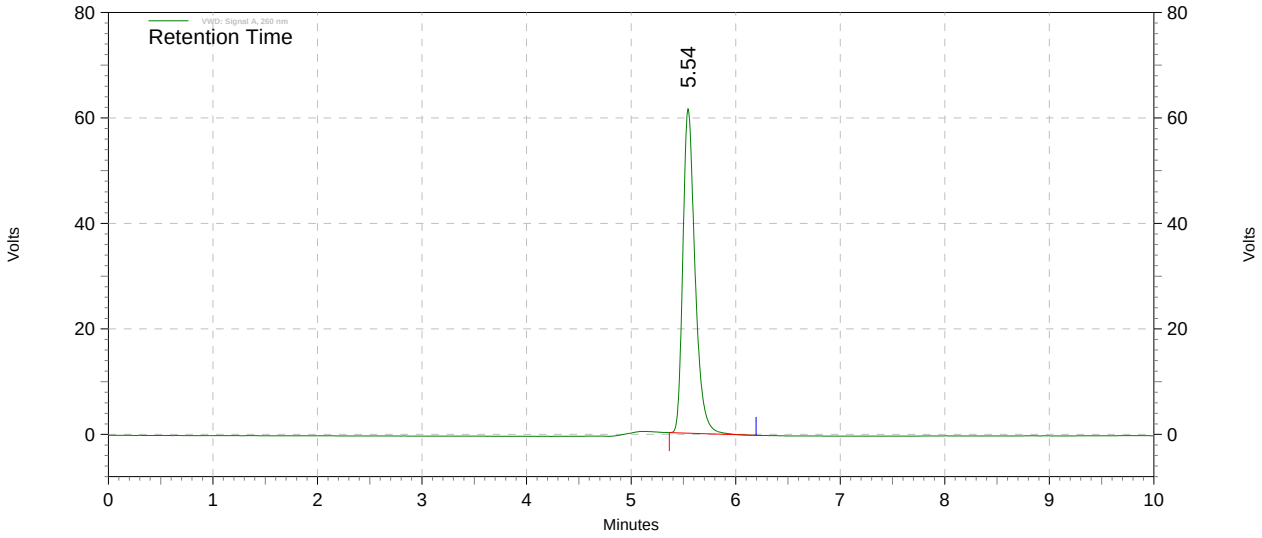

Fig.5. Chromatogram of system suitability

3.11. Tailing Factor (USP Method)

A measure of the symmetry of a peak, given by the following equation where $W_{0.05}$ is the peak width at 5% height and f is the distance from peak front to apex point at 5% height. Ideally, peaks should be Gaussian in shape or totally symmetrical.

$$T = W_{0.05} / 2f$$

The accuracy of quantitation decreases with increase in peak tailing because of the difficulties encountered by the integrator in determining where/when the peak ends and hence the calculation of the area under the peak. Integrator variables are preset by the analyst for optimum calculation of the area for the peak of interest.

Recommendations:

T of ≤ 2

$T= (a+b) / 2a$

Where:

T = tailing factor (measured at 5% of peak height)

b = distance from the point at peak midpoint to the trailing edge

a = distance from the leading edge of the peak to the midpoint

Table 4. Results of system suitability parameters of Tenofovir

Parameters	Data obtained
Theoretical plates per column	5390
Symmetry factor/Tailing factor	1.36

Acknowledgement

The authors thank Ranbaxy labs Pvt. Ltd, Gurgaon, India, for providing a sample of Tenofovir as a gift.

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