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NEW HPLC METHOD DEVELOPMENT AND VALIDATION FOR THE ESTIMATION OF TENOFOVIR IN MARKETED PREPARATIONS

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ABSTRACT: The RP-HPLC method developed and validated allows simple and fast quantitative determination of Tenofovir disoproxil fumarate from bulk and formulation. A mobile phase composed of only methanol with a short run time (< 5 min) and isocratic elution used are advantageous and made the routine analysis easy. Maximum absorbance (λ_{max}) obtained at 260.0 nm. Different solvents and solvent mixtures were tried for mobile phase and 100% methanol was used as mobile phase for further method development. Retention time was observed at 4.733min. Flow rate employed for analysis was 1.2 ml/min. Calibration curve was prepared between conc. of 10, 20, 40, 60, 80, and 100 ppm and area under the curve (AUC). Linearity equation was y = 7259 x - 7619 and regression coefficient (R^2) = 0.999. All data prove that method is linear. Mean % Recovery studies was found to be 99.84% of tenofovir. Recovery studies data summarized in Table. Recovery greater than 98 % with low standard deviation justifies the accuracy of the method. For Intraday precision, Repeatability of injection and sample in term of % RSD were found to be 0.968 and 0.5035 % respectively, % RSD was found to be less than 2% (which is recommended by ICH guideline) for within a day and day to day variation, which proves that method is precise. LOD and LOQ value was found to be 3.41µg/ml and 10.34µg/ml. Assay value was found to be 98.60% for tenofovir. The proposed validated method was successfully applied for estimation of tenofovir. Stability indicating RP-HPLC studies reveals that Acid and alkaline degradation of tenofovir was performed 0.1N HCl and 0.1N NaOH. Tenofovir was highly susceptible to attack by HCl and NaOH. Tenofovir under neutral hydrolysis did not give rise to the presence of degradants as the peak area remained constant which indicated drug stability under the conditions investigated. The drug was found to be unstable to oxidative degradation. Drug was stable against thermal stress. The developed HPLC method proved to be simple, accurate, precise and specific.

KEYWORDS: RP-HPLC, Method Developed, Validated, Tenofovir, Stability indicating

INTRODUCTION

Analytical method development and validation plays an important role in the discovery, development and manufacture of pharmaceuticals[1]. These methods used to ensure the identity, purity, potency and performance of drug products. There are many factors to consider when developing methods[2]. The initially collect the information about the analyte's physiochemical properties (pKa, logP, solubility) and determining which mode of detection would be suitable for analysis[3]. The majority of the analytical development effort goes in to validating a stability indicating HPLC method[4]. The goal of the HPLC method is to try and separate quantify the main active drug, any reaction impurities, all available synthetic intermediates and degradants[5]. High-performance liquid chromatography is a separation technique based on a solid stationary phase and a liquid mobile phase. Separations are achieved by partition, adsorption, or ion exchange processes, depending upon the type of stationary phase used[6].

Stability is defined as the capacity of a drug substance or drug product to remain within established specifications to sustain its identity, strength, quality and purity all over the retest or expiration dating periods[7]. Physical, chemical, and microbiological data are generated as a function of time and storage conditions (e.g., temperature and relative humidity [RH]). Stability testing provides confirmation that the quality of a drug substance or drug product under the influence of various environmental factors changes with time [8]. Stability plays an important role in the drug development process. It explains several factors that influence the expiration dating of drug products, including the chemical and physical stability during the pre-clinical formulation stages, process development, packaging development, and post-marketing life[9]. Stability testing allows the establishment of suggested storage conditions, retest periods, and eventually product shelf-life and expiry dating. In pharmaceutical field stability studies finds an application in the following areas of drug development program[10].

The aim and objective of this work was stability indicating method development and validation for the estimation of Tenofovir in bulk drug or in marketed formulation, in dosage forms or in body fluids and validation of developed Analytical method according to ICH guideline.

MATERIALS AND METHOD

Tenofovir disoproxil fumarate was obtained from Mylan, Indore (India) as gift. Methanol, Water (HPLC grade), Ethanol (HPLC grade) were purchased from Himedia lab(Mumbai) India.

Method Development for Assay of Tenofovir by HPLC

(A) Selection of Wavelength:

(a) Preparation of Standard Stock Solution:10 mg of Tenofovir was weighed accurately and transferred to a 10ml volumetric flask and the volume was adjusted up to the mark with the mobile phase methanol, to give a stock solution of 1000ppm.

(b) Preparation of Working Standard Solution: From stock solutions (1000ppm) of Tenofovir 1ml solution was taken and diluted up to 10 ml results 100ppm solution, from this 100ppm solution, 1ml was taken and diluted up to 10 ml with mobile phase methanol, to give a stock solution of 10 ppm and again 2ml, 4ml, 6ml, 8ml, 10ml, was pipette out and volume make up to 10 ml so 2, 4, 6, 8 & 10 ppm dilution was prepared.

(c) Determination of λ_{max} of Drugs: Standard solution of conc. 10µg/ml of pure Tenofovir was prepared. The pure drug solutions were scanned on UV spectrophotometer from 200–400 nm, which showed maximum absorbance at 364.4nm Tenofovir. The UV spectrogram was recorded.

(B) Selection of mobile phase: The criteria employed for assessing the suitability of a particular solvent system for the analysis was cost, time required for analysis, sensitivity of the assay and solvent noise. The mobile phase was selected in terms of its components and proportions.

Taking into consideration the system suitability parameter like RT, Tailing factor, No. of theoretical plates and HETP, the mobile phase found to be most suitable for analysis was Methanol in the ratio of 100. The mobile phase was filtered through Whattman filter paper to remove particulate matter and then degassed by sonication.

(C) Assay of marketed Tablet Formulation: Powdered Tenofovir tablet (Ricovir) equivalent to 10 mg was weighed and transferred to a 10 ml volumetric flask and volume was made up to 10 ml with methanol to obtain concentration of 1000µg/ml. Resultant solution was filtered through Whatmann filter paper 1 ml of filtrate was taken in 10 ml volumetric flask and volume was made up to 10 ml with methanol to obtain concentration of 100µg/ml.

Stability Indicating RP-HPLC Studies (Forced Degradation Study)

Forced degradation is the process, in which pure drug and drug products are subjected to chemical and environmental stress conditions to know the degradation pathway of drug and degradation products which can be used to determine the stability of the drug. For acid and alkali stress conditions, 0.1 N HCl and 0.1 N NaOH were added, respectively, and kept at room temperature for 6h, for oxidative degradation, 30% H_2O_2 was added and kept at 60°C for 1 h, and water added and kept at 60°C for 1 h for water hydrolysis degradation. Thermal degradation was performed by keeping the sample in a Petri dish and then placed them in an oven at 60°C for 1 h.

RESULTS AND DISCUSSION

Identification of drug

White color fine solid tenofovir crystals melts on 279-281 ^{O}C, with bitter taste, was soluble in ethanol, methanol water etc. Different aliquots were prepared with methanol by serial dilution. Solution of 10µg/ml was scanned between 200 to 400 nm on UV spectrophotometer using methanol as blank. Maximum absorbance (λ_{max}) obtained at 260.0 nm.

Method Development for Assay of Tenofovir by RP-HPLC Method

(a) Mobile Phase Selection:

Table No. 1: Selection of Mobile phase

Mobile phase	Ratio	Flow rate	Conclusion
Water: Methanol	80:20	1.0ml/min	No peak found
Water :Methanol	70:30	1.2ml/min	Peak Broadening in Drug
Acetonitrile:Water	40:60	1.2ml/min	No Peak found
Acetonitrile: methanol(Ph adjust 4.0 with GAA)	70:30	1.2ml/min	More Tailing in peakes
Methanol	100	1.2ml/min	Most suitable peak

Discussion: Taking into consideration the system suitability parameter like RT, Tailing factor, No. of theoretical plates and HETP, the mobile phase found to be most suitable for analysis was Methanol in the ratio of 100. The mobile phase was filtered through Whatman filter paper to remove particulate matter and then degassed by sonication. Flow rate employed for analysis was 1.2 ml/min.

(b) Method Optimization:

Chromatographic conditions were as Phenomenex C_{18} HPLC column Octadecylsilane (C_{18}), flow rate 1.2 ml/min, isocratic mode, Injection volume (20 µl), Wavelength used (260.0 nm), 100% Methanol (HPLC grade) was used Mobile phase, pH was 7.4, Retention Time 4.732 ± 0.2 min, total Run time was 8.0 min.

Effect of mobile phase: After confirming the mobile phase, change in the ratio of mobile phase was done for the optimization of the peak. The ratio of water::methanol 80:20 observed no peak, Water: Methanol 70: 30 broad peak, acetonitrile:Water 40:60, acetonitrile::methanol tailing in peak, were tried. In that case of 100% methanol shows good retention time and resolution.

Effect of flow rate: After confirming the ratio of mobile phase, flow rate of the mobile phase was changed, at 0.8ml/min it shows increased retention time the flow rate of 1.2ml/min resulted in fronting of the peak. The flow rate of 1ml/min has given a good result.

Selection of column: The literature review showed the usage of C-18 column for the determination of Tenofovir Mostly C-18 column is used for analytical purpose and the column selected was C_{18} Phenomenex column. The columns with different dimensions were available but that showing shifting of retention time. Phenomenex C-18 (25×0.46cm, 5µ) column shows good results.

Selection of detector wavelength: The sensitivity of the HPLC method that uses UV detector depends upon the proper selection of wavelength. An ideal wavelength selected by spectra of Tenofovir gives maximum absorbance and good response for the drugs to be detected. UV spectrum of Tenofovir was recorded at λ max at 260.0 nm.

Prerparation of Solvent mixture: 100% Methanol (HPLC grade) was taken separately filtered through membrane nylon filters of size 4.5 µ, to the filtered solution 1.5 ml of Ammonium Hydroxide Solution was added and the mixed solution was sonicated for 15 minutes and filtered through membrane nylon filters of size 4.5 µ.

Determination of Retention Time: The working standard solution (10µg/ml) of Tenofovir was injected into the chromatograph separately and their retention time recorded at 4.735min, at detection wavelength of 260.0 nm. The result is presented below.

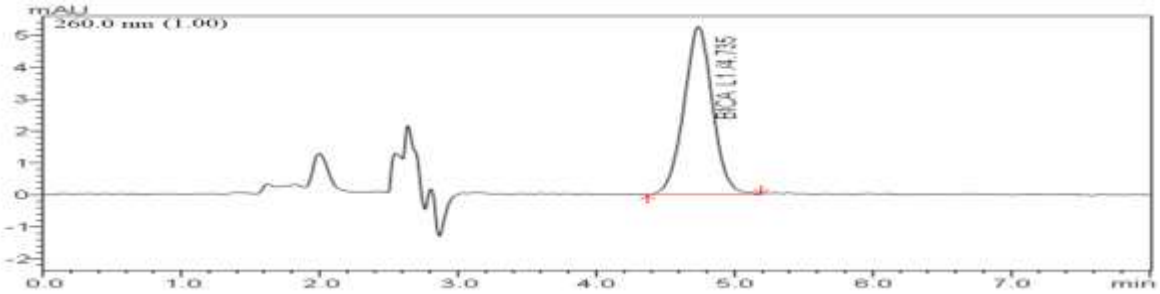

Figure 1: HPLC Chromatogram of Tenofovir Standard

Validation of Developed Method

Linearity: 10mg of the drug sample was weighed and transferred in 100ml volumetric flask and was dissolved in methanol and volume was made up to 100ml with methanol. Out of the above solution 1ml, 2ml, 4ml, 6ml, 8ml was taken and diluted to 10ml with methanol to make the conc. of 10, 20, 40, 60, 80, and 100 ppm. Result found that the given method is linear.

Table No. 2: Linearity of Standards for Tenofovir

Concentration (ppm)	0	10	20	40	60	80	100
Area Under Curve (AUC)	0	65805	136953	274463	423232	568089	728553

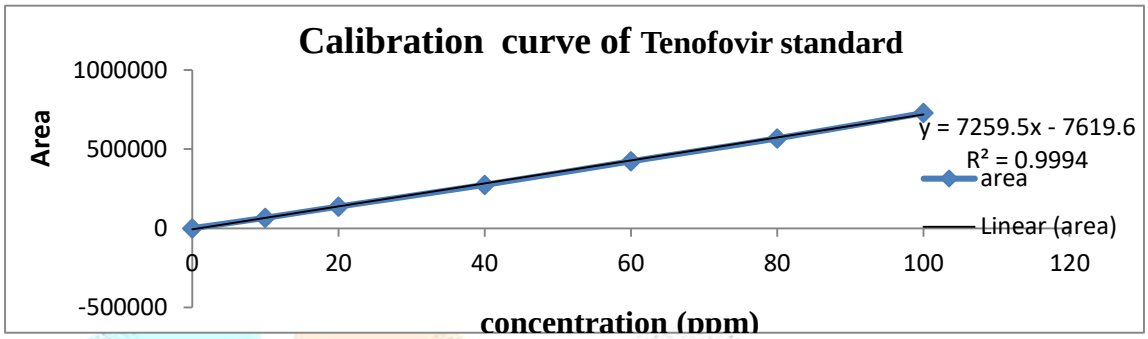

Figure 2: Calibration curve for Tenofovir

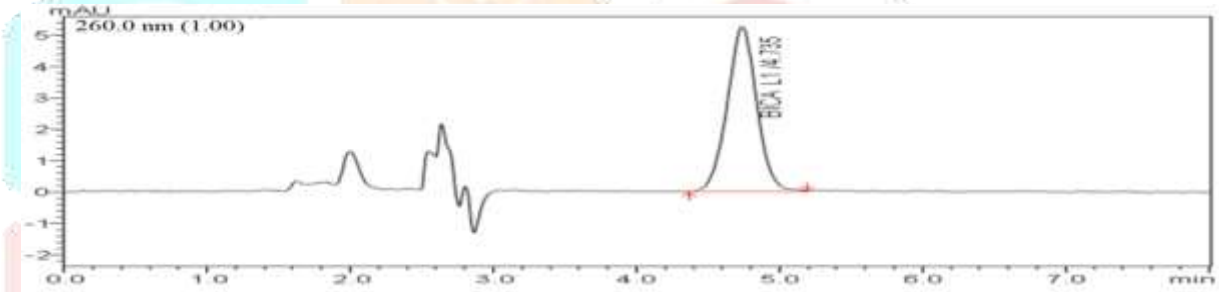

Figure 3: Chromatogram of Tenofovir at 10µg/ml

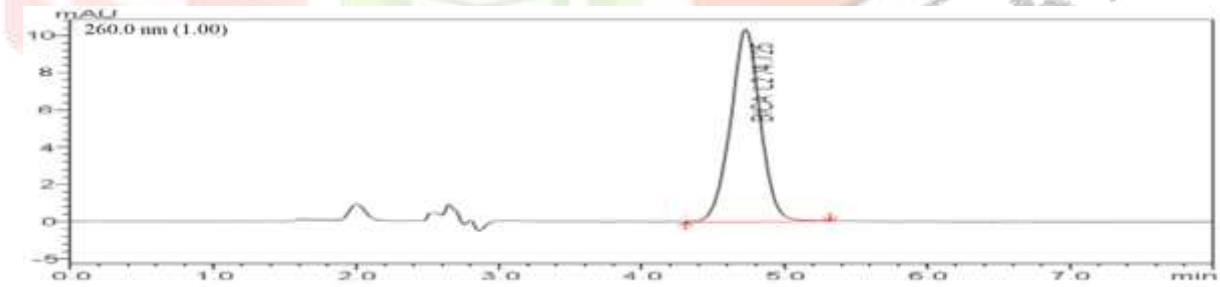

Figure 4: Chromatogram of Tenofovir at 20µg/ml

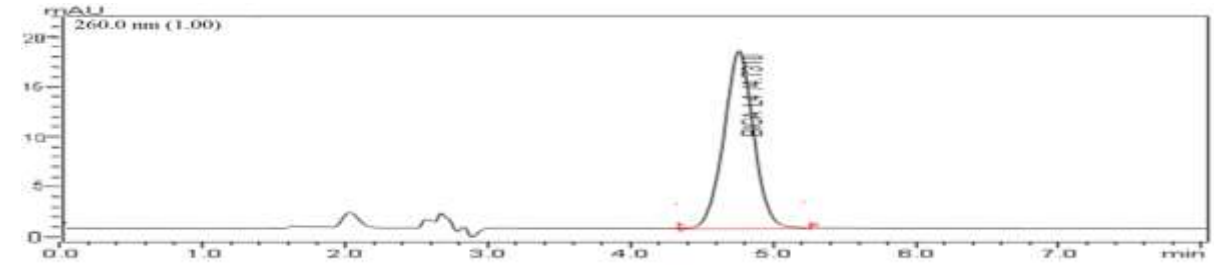

Figure 5: Chromatogram of Tenofovir at 40µg/ml

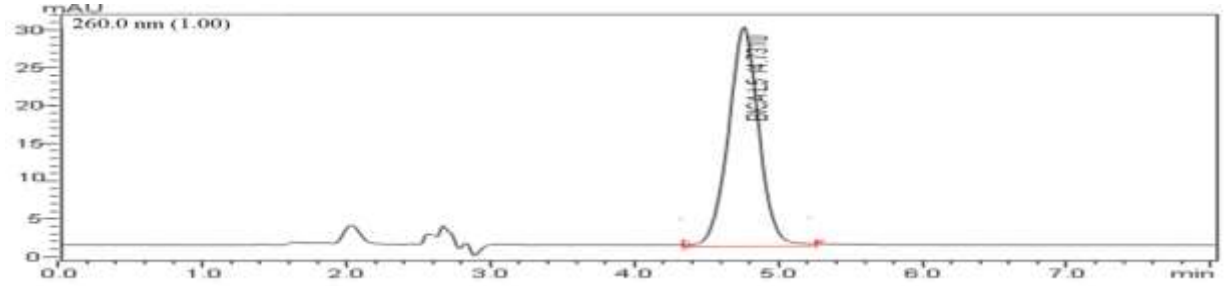

Figure 6: Chromatogram of Tenofovir at 60µg/ml

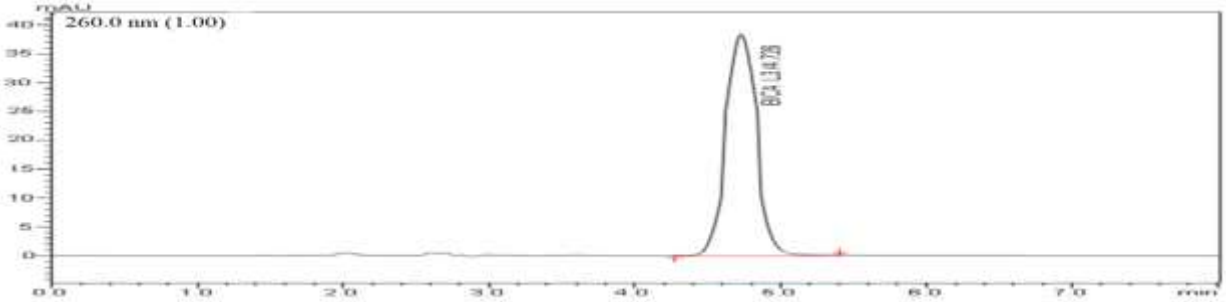

Figure 7: Chromatogram of Tenofovir at 80µg/ml

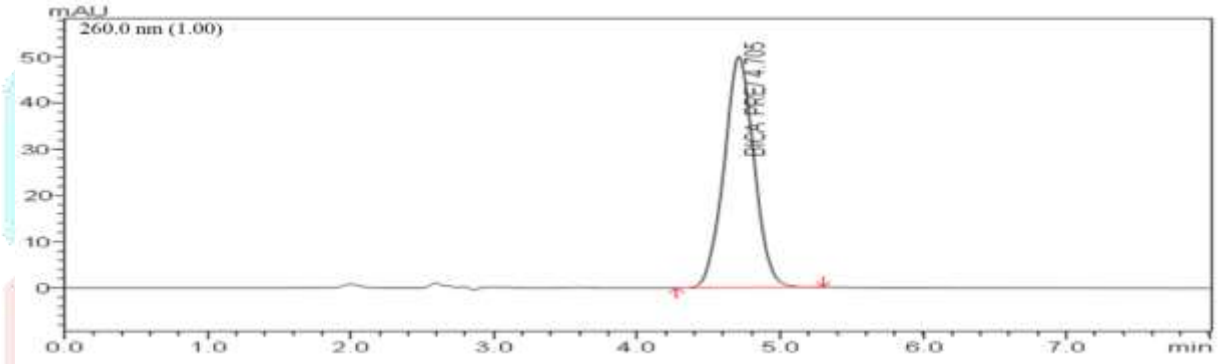

Figure 8: Chromatogram of Tenofovir at 100µg/ml

Accuracy: The accuracy of the method was done by addition of standard drug solution to pre-analyzed sample solution at three different levels 80, 100 and 120 %. Mean percentage recovery was determined.

Table No. 3: Recovery Studies and Statistical Validation for Accuracy of Formulation

Statistical Validation Recovery (%)	80%	100%	120%
Amount Present (mg/ml)	10	10	10
	10	10	10
	10	10	10
Amount of Std. Added (mg/ml)	8	10	12
	8	10	12
	8	10	12
% Recovery	99.90	99.74	99.74
	99.90	99.56	99.88
	99.96	99.96	99.94
Mean Recovery	99.92	99.75	99.85
SD	0.0346	0.2003	0.1026
%RSD	0.0346	0.2008	0.1028

Precision: The given method is repeatable the acceptable limit of RSD was not more than 2.0%

(a) Repeatability of Injection:

Table No. 4: Repeatability of injection

Concentration (ppm)	Area	RSD in %	Acceptance Criteria
20	136974 137220 136664	0.968	**NMT 2**

(b) Repeatability of Sample:

Table No. 5: Repeatability of Sample

Concentration (ppm)	Area	RSD in %	Avg % RSD
10	65787 65894 95733	0.375	0.5035%
20	136974 137220 136664	0.968	
40	278431 279246 276712	0.607	
60	423232 424712 425993	0.148	
80	566654 566581 571032	0.118	
100	727881 729193 728586	0.805	

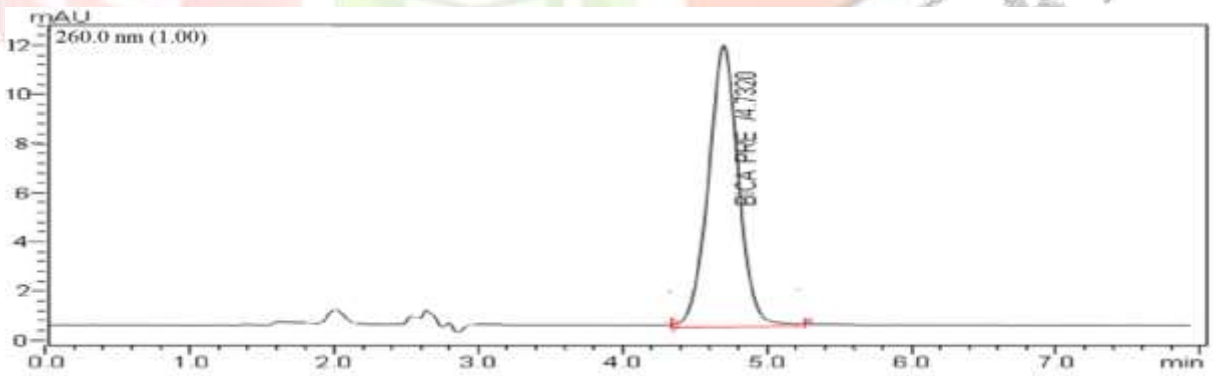

Figure 9: Chromatogram of Tenofovir at 20µg/ml for Repeatability

Limit of Detection (LOD): The limit of detection (LOD) and The Limit of Quantification (LOQ) was calculated as:

Table No. 6: LOD for Tenofovir

Sample Name	LOD	LOQ
Tenofovir	3.41 µg/ml	10. 34 µ g/ml

Specificity of Sample:

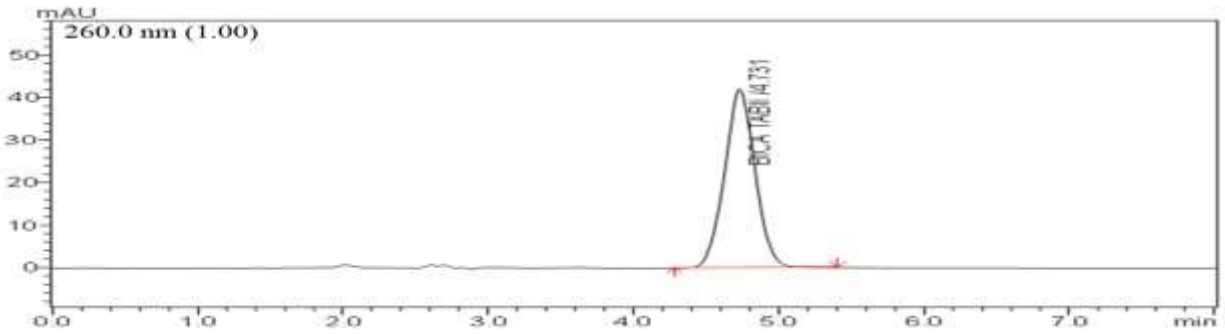

Figure 10: HPLC chromatogram of standard Tenofovir at 100µg/ml

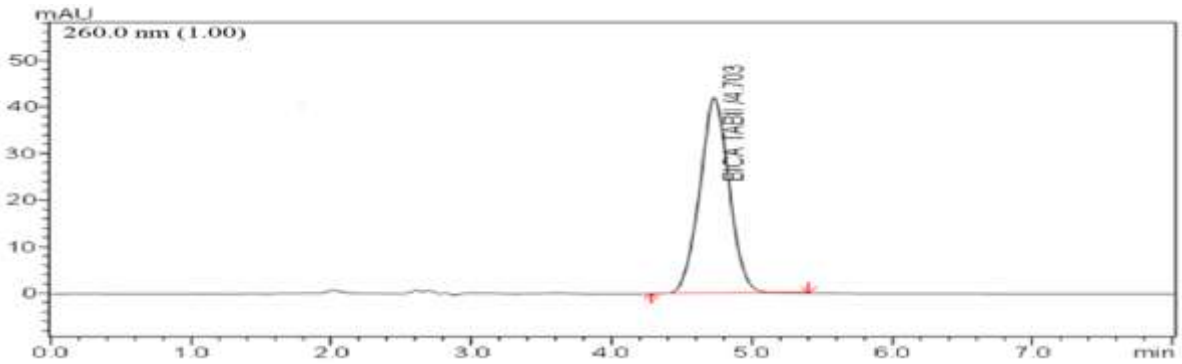

Figure 11: HPLC chromatogram of Tenofovir (Ricovir) Formulation at 100µg/ml

Assay of marketed Tenofovir (Ricovir) Tablet Formulation

For Tenofovir: Potency of Tenofovir working Standard: 99.95%

Table No. 7: Assay of Tenofovir Tablet (Ricovir)

Product Name	Retention Time	Amount of drug taken	Area Under the Curve	Label Claim	% Assay
Tenofovir standard	4.731	50 mg	728553	--	--
Tenofovir (Ricovir) formulation	4.703	49.3 mg	718356	50mg	98.60%

Stability indicating RP-HPLC studies (Forced degradation study)

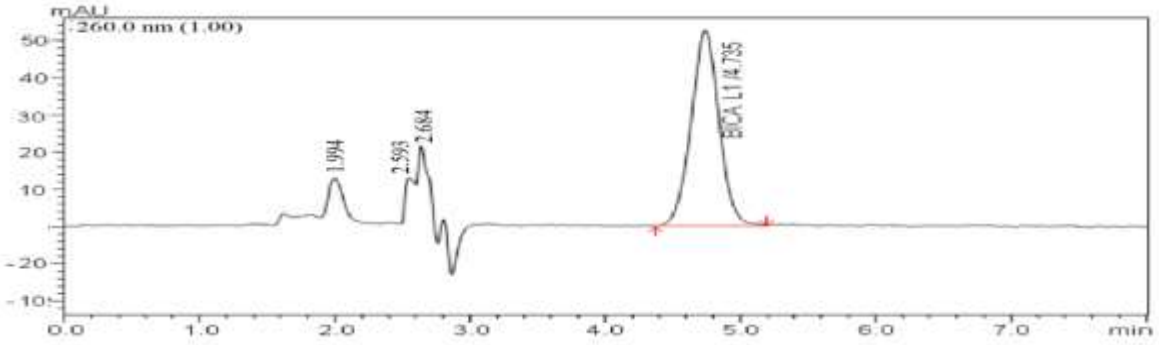

Figure 12: Chromatogram of degradation of tenofovir (100 µg/ml); peak 1 (degraded) (Rt: 1.994 min), peak 2 (degraded)(Rt: 2.593 min), peak 3 (degraded)(Rt: 2.684 min), peak 4 (tenofovir disoproxil fumarate) (Rt:4.735min)

Table No. 8: Stability indicating study by developed RP-HPLC Assay Method

S. No.	Exposure condition	Exposure time (Hour)	Temperature	Degradant Peak	Rt (min)
1	0.1 N HCl	6 hour	Room temperature	3	1.994, 2.593 & 2.684
2	0.1 N NaOH	6 hour	Room temperature	3	1.994, 2.593, & 2.684
3	Hydrolytic Degradation	1 hour	60°C	Nil	-
4	Oxidation Degradation	6 hour	Room temperature	3	1.994, 2.593, & 2.684
5	Thermal Degradation	1 hour	60°C	Nil	-
6	Photo Degradation	1hour	Room temp. UV light	3	1.994, 2.593, & 2.684

DISCUSSION

White color fine solid tenofovir crystals melts on 279-281 ^{O}C, with bitter taste, was soluble in ethanol, methanol water etc. Different aliquots were prepared with methanol by serial dilution. Solution of 10µg/ml was scanned between 200 to 400 nm on UV spectrophotometer using methanol as blank. Maximum absorbance (λ_{max}) obtained at 260.0 nm.

Different solvents and solvent mixtures were tried for mobile phase and 100% methanol was used as mobile phase for further method development. Retention time was observed at 4.733min. Flow rate employed for analysis was 1.2 ml/min. Calibration curve was prepared between conc. of 10, 20, 40, 60, 80, and 100 ppm and area under the curve (AUC). Linearity equation was y = 7259 x - 7619 and regression coefficient (R^2) = 0.999. Which are within specified criteria of ICH guideline, all data prove that method is linear. Mean % Recovery studies was found to be 99.84% of tenofovir. Recovery studies data summarized in Table. Recovery greater than 98 % with low standard deviation justifies the accuracy of the method. For Intraday precision, Repeatability of injection and sample in term of % RSD were found to be 0.968 and 0.5035 % respectively, % RSD was found to be less than 2% (which is recommended by ICH guideline) for within a day and day to day variation, which proves that method is precise. LOD and LOQ value was found to be 3.41µg/ml and 10.34µg/ml. Assay value was found to be 98.60% for tenofovir. The proposed validated method was successfully applied for estimation of tenofovir.

Stability indicating RP-HPLC studies reveals that Acid and alkaline degradation of tenofovir was performed 0.1N HCl and 0.1N NaOH. Tenofovir was highly susceptible to attack by HCl and NaOH. Complete degradation occurred immediately after addition of HCl and NaOH at room temperature. Tenofovir under neutral hydrolysis did not give rise to the presence of degradants as the peak area remained constant which indicated drug stability under the conditions investigated. The drug was found to be unstable to oxidative degradation. In 0.3% H_2O_2 complete degradation occurs immediately at room temperature. There was no significant degradation of solid tenofovir on exposure to heat at 60°C 1 hour, which indicated that drug was stable against thermal stress. Tenofovir was degraded in photochemical degradation after exposing drug to ultra violet energy for 1 hrs forming three degradation products. Over all observation was that with on peak of tenofovir disoproxil fumarate (Rt: 4.735min), three major degradation peaks were observed peak-1 (Rt: 1.994 min), peak-2 (Rt: 2.593 min), peak-3 (Rt: 2.684 min) in chromatogram of degradation of tenofovir (100 µg/ml). All type of degradation chromatograms were shows similar pattern and retention time of all three major degrdant.

CONCLUSION

The RP-HPLC method developed and validated allows simple and fast quantitative determination of Tenofovir disoproxil fumarate from bulk and formulation. A mobile phase composed of only methanol with a short run time (< 5 min) and isocratic elution used are advantageous and made the routine analysis easy. Among the significant advantages of this method are simplicity, selectivity, accuracy and precision ensuring that it is suitable for determining the content of Tenofovir disoproxil fumarate in tablet formulation. Thus, the proposed method can be used for routine analysis of Tenofovir disoproxil fumarate alone and also in combination; likewise the same can be applied to other formulations. We have also got the similar results from the method that was developed by UV Visible spectroscopy. This assures us to our work of analysis. Further, evaluation of degradation & stability indicating method was employed. The degradation & stability indicating study showed that tenofovir disoproxil fumarate was found to be unstable under acidic, alkaline and oxidative conditions as it degraded completely, but it was found that it is labile to photolysis and that complete separation of degradants was carried out using an isocratic stability-indicating HPLC method. Tenofovir disoproxil fumarate was observed to be stable when exposed to neutral hydrolytic condition, and dry heat. The developed HPLC method proved to be simple, accurate, precise and specific. Hence, it is recommended for industrial analysis of drug and degradation products obtained from stability procedures

CONFLICTS OF INTEREST

There are no conflicts of interest.

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