

DEVELOPMENT AND VALIDATION OF STABILITY INDICATING RP-HPLC METHOD FOR TENOFOVIR NANOPARTICLE FORMULATION

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ABSTRACT

The objective of the study was to develop a novel stability-indicating high performance liquid chromatographic (HPLC) method for Tenofovir Disproxil fumarate (TEN) with photodiode array (PDA) detection and validated as per International Conference on Harmonisation (ICH) guidelines. The developed method was successfully applied for assay of Tenofovir Disproxil fumarate to nanoparticle formulation. A Lichrocart (C₁₈) (250mm × 4.6mm, 5 μm particle size) column and a mobile phase composed of acetonitrile and 0.025M potassium di hydrogen phosphate buffer (pH 3.0 adjusted by using 10% v/v Orthophosphoric acid) in the ratio 35:65 (v/v) was used, and the detection wavelength of 260 nm. The method was validated with the parameters like specificity, linearity, precision, accuracy, limits of detection and quantification as per ICH guidelines. Forced degradation studies under different stress conditions like Acid, Base and Oxidation was successfully achieved, TEN was found to degrade significantly in alkaline and acidic conditions, PDA peak purity test confirmed the specificity of the developed method. The method was found to be precise and accurate with a linearity range of 0.1 μg mL⁻¹ to 50 μg mL⁻¹ ($r^2 > 0.999$).

Keywords: Tenofovir, HPLC, Stability indicating, Nanoparticle.

INTRODUCTION

Tenofovir Disproxil fumarate is a nucleotide reverse transcriptase inhibitor which is used as a part of HAART (highly active anti-retroviral therapy) for HIV patients and it's administered in combination with other anti-retroviral or as a single dosage form. Tenofovir Disproxil fumarate is highly water soluble drug [1] this property results in limited drug entrapment in polymer as nanoparticle formulation. Dima Alukda et al [2] (2011) have worked on solid lipid nanoparticle formulation of Tenofovir gel 1 mg where in vitro release is calculated by using UV spectrophotometer with a sensitivity of 2 μg mL⁻¹ to 100 μg mL⁻¹. Rohan LC et al [3] (2010) have worked Tenofovir gel formulation and used HPLC method for in vitro release but no specific chromatography conditions were discussed. Meng, J et al [4] (2011) have worked on Tenofovir chitosan nanoparticles where encapsulation efficiency was calculated by using UV spectrophotometer. Tao Zhang a et al [5] (2011) have worked on pH responsive nanoparticles and drug entrapment efficiency was calculated by UV spectrophotometer with 2 μg mL⁻¹ to 100 μg mL⁻¹. Few analytical methods for conventional dosage forms were reported by N.Appala raju [6] et al (2008) reported simultaneous method for Tenofovir, Emtricitabine, Efavirenz tablet formulations and which involved high amount of organic ratio in mobile phase and a gradient run which sometimes results in pump problems. P.B Kandgal [7] et al (2008) reported a method for tenofovir disproxil fumarate in tablet dosage form but the linearity range is less sensitive for lower concentrations. The need of the study was to have a standard method to analyze Tenofovir in nanoparticle formulation as it is a precursor to bioavailability studies. A few bioanalytical methods were reported like S. Sentenaca [8] et al. (2003) have reported the method for human plasma by HPLC which included solid phase extraction due to complex plasma matrix. Vincent Jullien [9] et al. (2003) reported spectrofluorometric method of Tenofovir which has a long sample preparation procedure. Nazer L. Rezk et al. [10] (2005) reported simultaneous estimation of Tenofovir, Emtricitabine in human plasma by HPLC using solid phase extraction. Tom Delahunty et al. [11] (2006) have reported estimation of Tenofovir by LC/MS/MS and Noel Gomes et al. [12] (2008) have reported simultaneous estimation of Tenofovir, Emtricitabine in human plasma by LC-MS/MS. The above methods are for Human plasma which involves expensive cartridges and mass spectrophotometer, as newer drug delivery systems like Tenofovir nanoparticle formulations are evolving no standard sensitive analytical method is published. As a result there was a need to develop a simple method with high

sensitivity, stability and which can be applied to Tenofovir Nanoparticle formulation and with minimal optimization can also be applied to tablet formulations.

MATERIALS AND METHODS

Chemicals and Reagents

Tenofovir disproxil fumarate was obtained as gift sample from Aurobindo pharmaceuticals, Hyderabad, India. HPLC grade Acetonitrile, methanol and analytical grade potassium dihydrogen phosphate from Merck Laboratories Pvt. Ltd., Mumbai, India. Milli Q water, Orthophosphoric acid analytical grade from Ranbaxy Fine chemicals New Delhi, India were used.

Instrumentation

The LC systems of Shimadzu LC 2010 Japan with SIL- 10ADVP auto-injector and column oven of CTO-10ASVP model, SPD M-10AVP photo diode array detector, SCL-10 AVP System controller were used for the study.

Chromatographic conditions

Detection wavelength of 260 nm was set with mobile phase comprising of 25mM phosphate buffer pH adjusted to 3.0 using Orthophosphoric acid (Control dynamics pH meter Bangalore) and acetonitrile in the ratio of 65:35 with 1ml min⁻¹ flow rate. Lichrocart C₁₈ 250 x 4.6mm, 5μ column was used as stationary phase. Mobile phase was filtered through 0.2μ nylon filter (Millipore Pvt Ltd Bangalore) and degassed in ultrasonic bath for 5 minutes. The sample injection volume was kept at 50μL and column oven temperature maintained to 25°C.

Standard preparation

Amount equivalent to 10 mg of TEN is weighed in 10 ml volumetric flask and dissolved in methanol (1mg/ml). Subsequent dilutions for 0.1 μg mL⁻¹, 0.25 μg mL⁻¹, 0.5 μg mL⁻¹, 1.0 μg mL⁻¹, 2 μg mL⁻¹, 5 μg mL⁻¹, 10 μg mL⁻¹, 25 μg mL⁻¹, and 50 μg mL⁻¹ were prepared by using mobile phase as diluent. Mobile phase is used as blank solution.

Nanoparticle sample preparation

Definite amount of Nanoparticle formulation equivalent to 10 mg of TEN was weighed in a 10 ml volumetric flask. 2ml of organic solvent comprising of 1:1 ratio of acetonitrile and methanol was added to dissolve polymer of formulation, sonicated for 10minutes. 5ml of mobile phase was added and vortexed the mixture for 5 minutes and

then volume was made up to with mobile phase. The sample was centrifuged at 10,000 RPM for 15 minutes and the supernatant was injected into the HPLC system.

Analytical Method Validation

Specificity study

Mobile phase along with placebo (excipients present in nanoparticle formulation other than the drug) were injected to check the interference at the retention time of TEN in the above mentioned chromatographic condition and no interference were observed at the designated retention Time which was established by peak purity of the chromatogram by PDA detection.

Forced degradation studies

Acid, Alkaline and Oxidative degradation studies were planned and TEN was subjected to these conditions. 0.1N HCL, 0.1N NaOH, 30% Hydrogen peroxide were used for forced degradation studies. Placebo and mobile phase were also subjected to same treatment as sample to check for interferences.

Linearity

Nine point linearity plot was done in order to accommodate wide range from 0.1 $\mu\text{g mL}^{-1}$ to 50 $\mu\text{g mL}^{-1}$, so that nanoparticle analysis can be carried out easily with $r^2 = 0.999$ for the above plot.

Precision

ICH describes precision as closeness of individual measure of analytes when the procedure is applied repeatedly to multiple times Interday and intraday precision has been established in the method. Six independent assay samples were prepared and injected for intraday precision, there %RSD was calculated. For Interday precision under the same experimental conditions the

assay samples were prepared and injected by another analyst, %RSD calculated.

Accuracy

It's was evaluated on three levels of 80%,100% and 120% by adding known amount of drug to placebo and extracting the sample. Three sets were prepared and sample was analyzed.

Solution stability

Tenofovir API and its Nano formulation stability was carried out for a period of 24 hours at sample cooler temperature at 25°C. (Table 2)

Robustness

Varying conditions of flow rate, buffer pH, column temperature, and wavelength were carried out as per ICH guidelines to estimate the effects on the method.

RESULTS AND DISCUSSIONS

Method development and optimization

The method development was carried out to achieve a simple, reliable method, various phosphate buffers with different concentrations were tried out, and 25mM buffer strength was selected as its compatibility with C_{18} column was ideal. Change in pH 3.0 to pH 7.0 was carried out but lower pH resultant in better peak shape and symmetry. Theoretical plates were good. Phosphate buffer was more compatible than other volatile buffers like TEA and acetate buffer. Mobile phase composition organic phase ratio was kept under fifty percent to prevent pump problems. Various compositions were tried out and the retention time of around 7.5 minutes was found to be ideal with no interference at 65:35 ratios. The final standard and Nano formulation chromatogram was shown as figure 1 & 2.

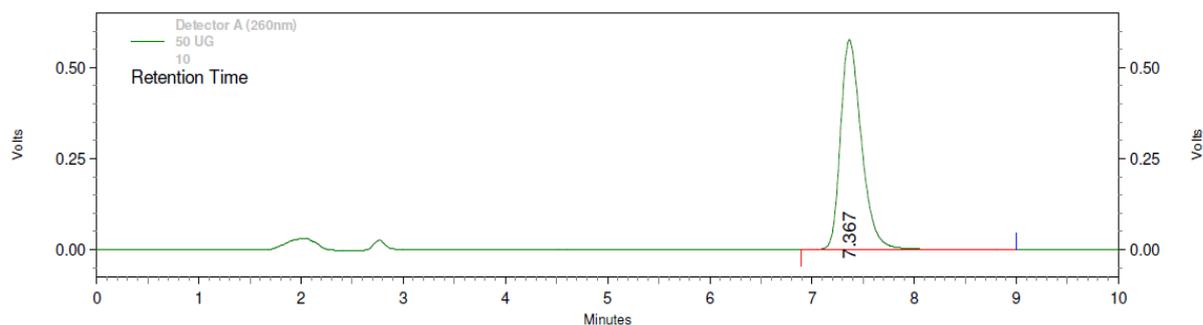


Fig. 1: It shows standard chromatogram of Tenofovir

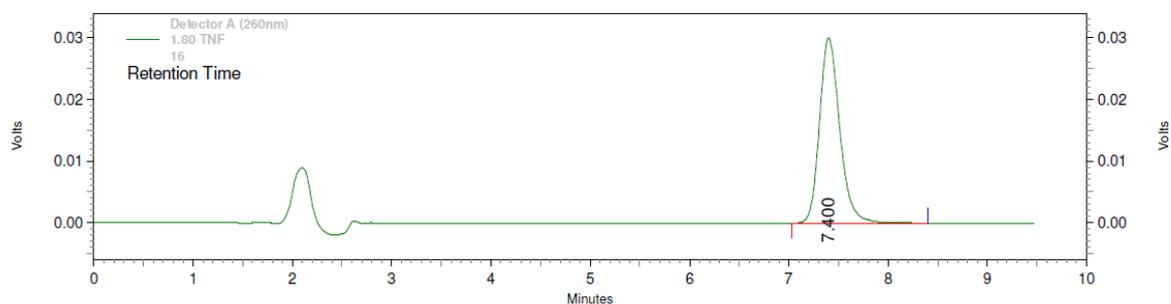


Fig. 2: It shows chromatogram of Tenofovir nano formulation

Table 1: It shows System suitability parameters

Parameter	Tenofovir	Acceptance Criteria
Tailing factor	1.15	not more than 2.0
Theoretical plates	7571.88	not less than 2000
% RSD of 6 injections (area)	0.88	not more than 1.0
% RSD of 6 injections (retention time)	0.10	not more than 1.0

System suitability

System suitability parameters like theoretical plates per meter, tailing factor, percentage relative standard deviation of area and retention time of six injections were carried out and the values are well within the limits.

Linearity

A linear calibration plot of Tenofovir was carried out at nine point concentration levels $0.1 \mu\text{g mL}^{-1}$ to $50 \mu\text{g mL}^{-1}$ in duplicate. Peak area of Tenofovir was plotted against respective concentrations and linear regression analysis was performed. Correlation coefficient was found to be more than 0.999 (Table 2).

Precision

The precision of the assay method was evaluated for Intraday and Interday precision, the percentage relative standard deviation of Tenofovir was found to be 0.22% and 0.06% respectively. These values were well within the generally acceptable limit as per ICH guidelines.

Table 2: It shows Precision, Solution stability and Linearity (n=6)

Parameter	% RSD	Limit
Precision Repeatability	0.22	2.0%
Intermediate	0.06	2.0%
Solution Stability for 24 hours	0.98	3.0%
Linearity (r^2)	0.9995	0.999

LOD and LOQ

Limit of detection and limit of quantification were calculated by using slope method ($3.3 \times \text{SD/S}$ and $10 \times \text{SD/S}$ respectively) and found to be $0.025 \mu\text{g mL}^{-1}$ and $0.10 \mu\text{g mL}^{-1}$ respectively.

Accuracy

The known amount of standard was spiked in 80%, 100%, 120% concentration in triplicate to test solution and recovery of drug was calculated. The accuracy of method was established at three concentration levels at 8, 10 and $12 \mu\text{g mL}^{-1}$ of Tenofovir standard. The recoveries at three different concentrations were found to be within the range of 98 to 102 % as per ICH guidelines. Mean % recovery (mean \pm SD) was found to be 100.51 ± 1.01 . The results indicated that the recovery of Tenofovir in three different concentrations (Table 3)

Table 3: Accuracy

Amount added	% Recovery	% RSD
$0.8 \mu\text{g/ml}$	99.20	0.97
$1.0 \mu\text{g/ml}$	101.1	0.32
$1.2 \mu\text{g/ml}$	98.21	0.99

Robustness

The robustness of assay method was studied by incorporating small but deliberate changes in the analytical method (e.g. variations in flow rate, column temperature, mobile phase composition, pH of buffer, etc.) and also by observing the stability of the drugs for 24 hours at room temperature in the dilution solvent. In all the varied chromatographic conditions, there was no significant change in chromatographic parameters.

Table 4: Method Robustness

Parameter	Acceptance criteria: (% RSD \leq 2.0 %)	
Flow rate	0.9 ml/min	0.10
	1.1 ml/min	0.06
pH	2.8	0.54
	3.2	1.21
Wavelength	+2nm	0.23
	-2nm	0.08
Temperature	+2°C	0.21
LOD	$3.3 \times \text{SD/S}$	$0.025 \mu\text{g/ml}$
LOQ	$10 \times \text{SD/S}$	$0.1 \mu\text{g/ml}$

Main peak areas relative standard deviation represents mean of three determinations.

Alkali hydrolysis

Initially 1.0M and 0.1M concentration of Sodium hydroxide were tried which resulted in complete degradation of TEN so 0.01 M Sodium hydroxide concentration 10% v/v for 5 minutes was used for the degradation of Tenofovir and it was found that the drug degradation was 20.0% with area normalisation method. The TEN peak retention time was found to be around 7 minutes. Peak purity index (0.9999) was greater than peak purity threshold (0.9991) which indicates that there is no merging of impurity peaks and peak is pure.

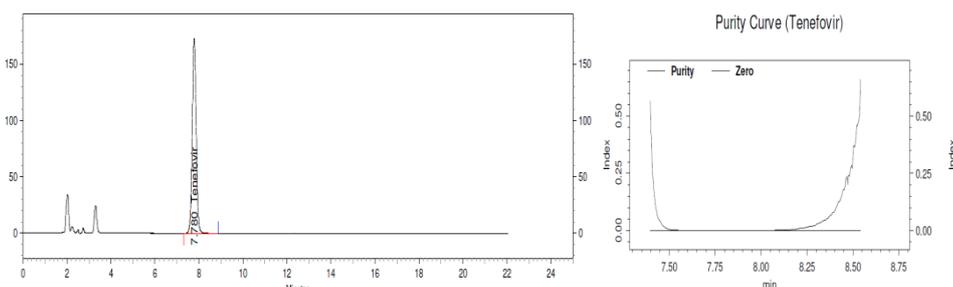


Fig. 3: It shows alkaline degradation chromatogram along with peak purity curve

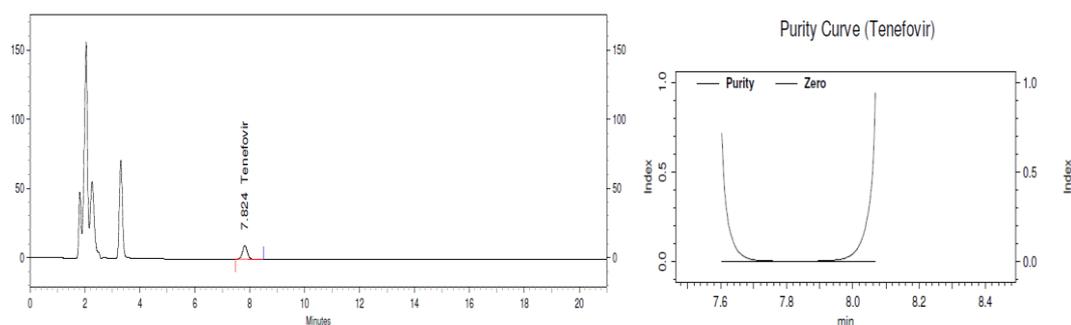


Fig. 4: It shows acid degradation chromatogram with its peak purity curve

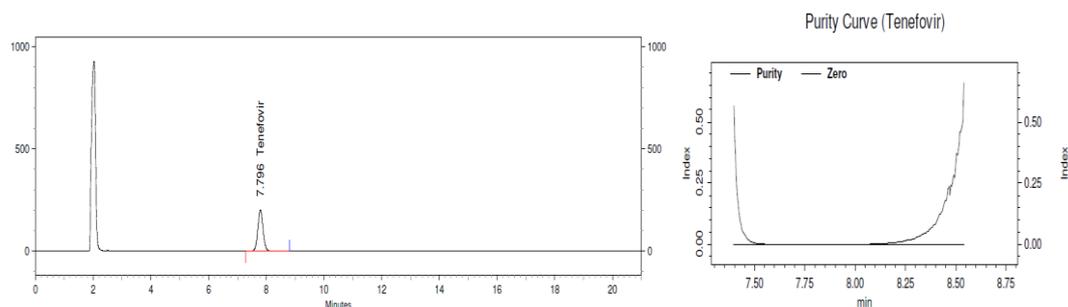


Fig. 5: It shows oxidative degradation chromatogram and its peak purity curve

Acid Hydrolysis

Initially 1.0M and 0.1M concentration of Hydrochloric acid were tried which resulted in complete degradation of TEN so 0.01 M Hydrochloric acid concentration 10% v/v for 5 minutes was used for the degradation of Tenofovir and it was found that the drug degradation at was 14.0% with area normalisation method. The TEN peak retention time was found to be around 7 minutes. Peak purity index (0.9999) was greater than peak purity threshold (0.9991) which indicated that there is no merging of impurity peaks and peak is pure.

Oxidative degradation

30% hydrogen peroxide 2.5% v/v for 5 minutes was used for the degradation of Tenofovir, it was found that the drug degradation was 9.0% with area normalisation method. The TEN peak retention time was found to be around 7 minutes. Peak purity index (0.9999) was greater than peak purity threshold (0.9991) which indicated that there is no merging of impurity peaks and peak is pure.

CONCLUSION

The developed method can be successfully applied to API, Nano formulation and on availability of placebo we can apply to tablet formulation also once the specificity is established. The above method was found to be stability indicating under different stress conditions and selective for Tenofovir disoproxil fumarate.

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