

METHOD DEVELOPMENT, VALIDATION AND FORCED DEGRADATION STUDIES OF TENOFOVIR ALAFENAMIDE FUMARATE IN BULK AND ITS PHARMACEUTICAL DOSAGE FORM USING RP-HPLC.

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ABSTRACT

A simple, Precised, Accurate method was developed for the estimation of Tenofovir Alafenamide by RP-HPLC technique. Chromatographic conditions used are stationary phase Kromasil C18 250mm x 4.6 mm, 5 μ , Mobile phase 0.1% Orthophosphoric acid Buffer: Acetonitrile in the ratio of 50:50. And flow rate was maintained at 1.0ml/min, detection wave length was 261 nm, column temperature was set to 30°C and diluent was mobile phase Conditions were finalized as optimized method. System suitability parameters were studied by injecting the standard six times and results were well under the acceptance criteria. Linearity study was carried out between 25% to 150 % levels, R² value was found to be as 0.999. Precision was found to be 0.5 for repeatability and 0.8 for intermediate precision. LOD and LOQ are 0.18 μ g/ml and 0.55 μ g/ml respectively. By using above method assay of marketed formulation was carried out 99.83% was present. Degradation studies of Tenofovir Alafenamide were done, in all conditions purity threshold was more than purity angle and within the acceptable range.

Key words: HPLC Tenofovir Alafenamide, Method development, ICH Guidelines

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1. INTRODUCTION

Tenofovir Alafenamide Fumarate (TAF), (*E*)-but-2-enedioic acid; propan-2-yl (2*S*)-2-[[[(2*R*)-1-(6-aminopurin-9-yl) propan-2-yl] oxymethyl-phenoxyphosphoryl] amino] propanoate is an antiretroviral drug which is used in treatment of chronic Hepatitis B and HIV/AIDS infection. It is nucleotide reverse transcriptase inhibitor. Tenofovir Alafenamide Fumarate (Fig. 1) is a Fumarate salt prepared from Tenofovir Alafenamide by reaction of one molecule of fumaric acid for every two molecules of Tenofovir Alafenamide, a prodrug for Tenofovir; it is used in combination therapy for the treatment of HIV-1 infection. It has a role as an antiviral drug, a HIV-1 reverse transcriptase inhibitor and a prodrug.

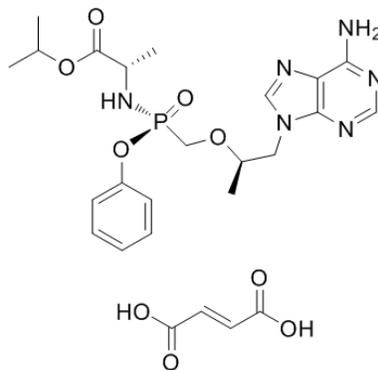


Fig. 1. Structure of TAF

A literature survey has revealed that only two articles on UV spectroscopic assay of TAF are reported. A few methods in literature review are found for determination of TAF in combined dosage forms and include High Pressure Liquid Chromatography (HPLC) (Sk. Mastanamma *et. al.*, 2018; N. MD. Akram *et. al.*, 2017; Benzil Dudekula *et. al.*, 2017; Bhushan P. Badgular⁵ *et. al.*, 2017), Ultra Performance Liquid Chromatography (UPLC) (K. Kranthi Kiran *et. al.*, 2017; S. Imam Pasha *et. al.*, 2017) Liquid Chromatography-Mass Spectrometry (LC-MS) (Mark A. Marzinkea *et. al.*, 2018). A method based on the measurement absorbance of the drug in water at 260nm has been reported (M. P. Shinde *et. al.*, 2018) and the method obeys Beer's law in the 2-10 µg/ml concentration range.

Simultaneous estimation of TAF and Emtricitabine by UV spectroscopic method is found in a literature. The method involving determination of TAF and Emtricitabine at 260nm and 280 nm over the concentration ranges of 5-30 µg/ml for both drugs has been described (Ashwini Shelke *et. al.*, 2018).

Most of the reported methods are often time consuming, expensive, use multi or expensive reagents, cumbersome and required expertise operational personnel. UV spectrophotometry, because of simplicity, reproducibility and speed and also it requires minimum solvent/reagent system and less analysis time, is widely used for the assay of the therapeutic compounds used as medications.

2. MATERIALS AND METHODS

Apparatus. The measurements were carried out using Waters Alliance HPLC system.

Materials. All chemicals used were of reagent grade. Distilled water was used to prepare solutions wherever required. Acetonitrile, hydrogen peroxide (H₂O₂), hydrochloric acid and sodium hydroxide were purchased from Merck (Mumbai, India). Tenofovir Alafenamide Fumarate sample (purity 99.5%) was kindly supplied by Mylan laboratories, Hyderabad, India. Commercial brand of tablets namely HepBest (Mylan Pharmaceuticals Ltd., Indore, India) were purchased from local commercial sources.

Reagents. Hydrochloric acid (1 M) was prepared by appropriate dilution of concentrated acid with water. A 5% solution of H₂O₂ was prepared by diluting suitable volume of the commercially available reagent to 100 ml with water in a volumetric flask. Sodium hydroxide solution (1 M) was prepared by dissolving required amount of the pellets in water.

Diluent. Acetonitrile: Distilled water (20:80) was chosen as the diluent for Tenofovir Alafenamide Fumarate depending on absorption at its analytical wavelength.

Mobile phase. 0.1% Orthophosphoric acid (pH 2.5): Acetonitrile (50:50) was used as mobile phase.

Standard drug solution. Preparation of stock solution (250 µg/ml): weigh about 25 mg of Tenofovir Alafenamide Fumarate and transfer to 25 ml volumetric flask, dissolve it in diluent and make up the final volume to 100 ml with diluent.

Chromatographic conditions. various combinations of mobile phases were screened and finally, the mobile phase consisting of 0.1% orthophosphoric acid: acetonitrile 50: 50 v/v was set with isocratic programming for 5mins optimized at a flow rate of 1ml/min, at 261nm wavelength, the injection volume of 25µL and 30 °C temperature was maintained during the entire process to obtain symmetric peaks of TAF shown in Fig. 2.

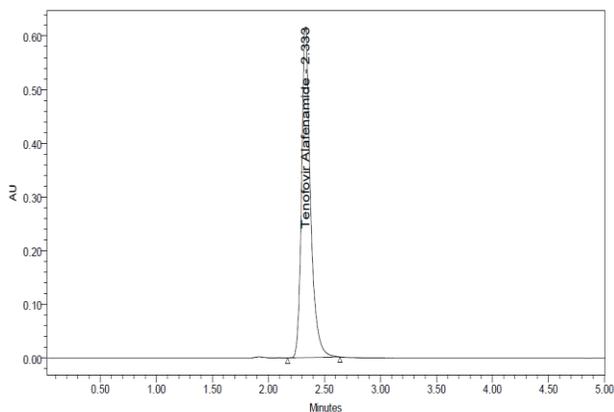


Fig. 2. Optimized chromatogram

METHOD VALIDATION

Specificity and selectivity. To determine the selectivity of the method, standard solution of TAF, commercial product solution and blank solutions were injected.

System suitability. Six replicate of sample containing TAF were injected to evaluate equipment, electronics, and analytical operations and sample suitability.

Linearity. Into a series of 10 ml calibration flasks, aliquots of standard drug solution (0.25–1.25 ml of 250µg/ml) equivalent to 6.25 -37.5 µg/ml TAF were accurately transferred and the volume was made up to the mark with the diluent. The absorbance of each solution was then measured at 261 nm against the respective diluent. Calibration curve was prepared by plotting the peak area versus concentration of drug.

Accuracy. The accuracy of the method was achieved at three concentration levels 50%, 100%, 150% for TAF known amount of standard drug solution was added to the sample and peak area was determined.

Precision. Precision is the degree of repeatability of an analytical method under normal operating conditions. It is of different types like system precision, method precision, intraday precision, interday precision.

LOD sample Preparation: 0.25ml of Standard stock solution was pipetted out and transferred to 10ml volumetric flasks and made up with diluents. From the above solution 0.1ml Tenofovir Alafenamide, were transferred to 10ml volumetric flasks and made up with the same diluents.

LOQ sample Preparation: 0.25ml of Standard stock solution was pipette out and transferred to 10ml volumetric flasks and made up with diluents. From the above solution 0.3ml Tenofovir Alafenamide, were transferred to 10ml volumetric flasks and made up with the same diluents.

Robustness. To evaluate the robustness of the method, the chromatographic conditions were deliberately altered and degree of reproducibility was evaluated. During this, each condition was varied separately, all other conditions being held constant at the optimized value.

ASSAY

Analysis of tablets. Twenty tablets from commercial brand (HepBest) were weighed and crushed into a fine powder using a Pestle and Mortar. An amount of tablet powder equivalent to 10 mg of TAF was transferred into a 100 ml volumetric flask. The content was shaken well with about 50 ml of the respective diluent for 20 min. The mixture was diluted to the mark with the same diluent. It was filtered using Whatmann No 42 filter paper. First 10 ml portion of the filtrate was discarded and a subsequent portion was diluted to get a working concentration and subjected to analysis.

FORCED DEGRADATION STUDIES

A 1 ml aliquot of the standard 25 μ g/ml TAF was taken (in triplicate) in a 10 ml volumetric flask and mixed with 5 ml of 1 M HCl (acid hydrolysis) or 1 M NaOH (alkaline hydrolysis) and boiled for 2 h at 40 °C on a hot water bath or 3% H₂O₂ (oxidative degradation) at room temperature for 2h. The solution was cooled to room temperature, neutralized and diluted to the mark with diluent. In thermal degradation, solid drug was kept in Petri dish in an oven at 40 °C for 2h. After cooling to room temperature, 10 mg of TAF was weighed and transferred to a 100 ml volumetric flask, dissolved in and diluted up to the mark with the respective diluent. For UV degradation study, suitable aliquot of the stock solution (250 μ g/ml) was exposed to UV radiation for 4h in a UV chamber. Finally, the absorbance of all the resulting solutions (25 μ g/ml) obtained from acid and alkaline hydrolysis, oxidative degradation, thermal and UV degradation of TAF was measured at 261nm against the respective solvent as blank in each case.

3. RESULTS AND DISCUSSION

Optimization of chromatographic conditions. The goal of this work is to develop a sensitive and precise method for quantification of TAF. Initial method development was started with mobile phase water: methanol (50:50) at flow rate 1ml/min using Ascentis C18 column (150mm x 4.6 mm, 2.7microns). This resulted to a bad peak shape.

Paying attention to the issues in the earlier trail, various modifications were opted like change in mobile phase, change of the column, changing the injection volume, maintaining the column temperature, etc. All the optimized conditions are given in table 1.

Table 1. Optimized chromatographic conditions

Column	Kromasil C18 250 x 4.6 mm, 5 μ .
Temperature	30 °C
Mobile phase	0.1% OPA (PH-2.5): Acetonitrile (50:50)
Flow rate	1.0 ml/min
Injection Volume	25.0 μ L
Detector	PDA 261nm
Pump mode	Isocratic
Retention time	2.3mins

METHOD VALIDATION

Specificity and selectivity

The results of the tests proved that the components other than the drug did not produce any detectable signal at the retention time of TAF as shown in figure 3.

System suitability

Parameters calculated for system suitability were given in table 2. Various parameters like theoretical plates, tailing factor were calculated.

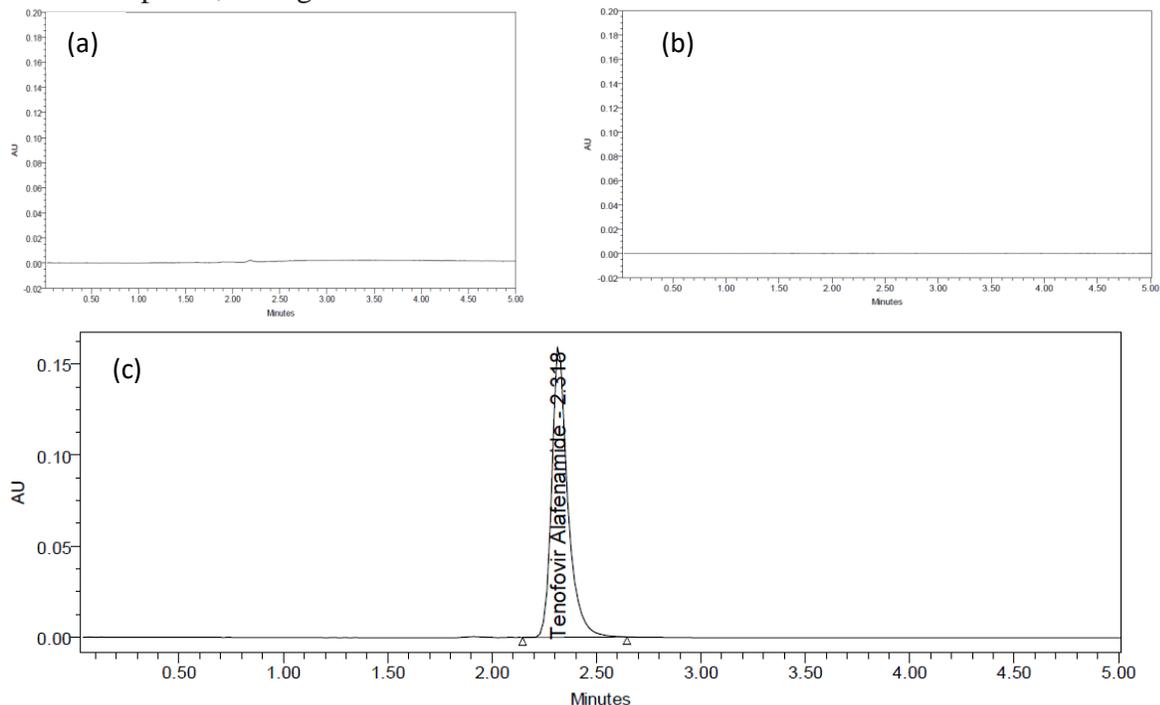


Fig. 3. Chromatogram of blank (a), placebo (b) and TAF (c)

Table 2. System suitability parameters

Parameter	Value
Retention time	2.3 mins
Peak area	912386
USP plate count	4771
Tailing factor	1.36

Linearity. Linearity was established in the range of 6.25 $\mu\text{g/ml}$ -37.5 $\mu\text{g/ml}$. Plot a graph to concentration versus peak area. Slope obtained was 36736 Y-Intercept was 7994 and Correlation Co-efficient was found to be 0.999 and calibration curve was shown in Fig 4.

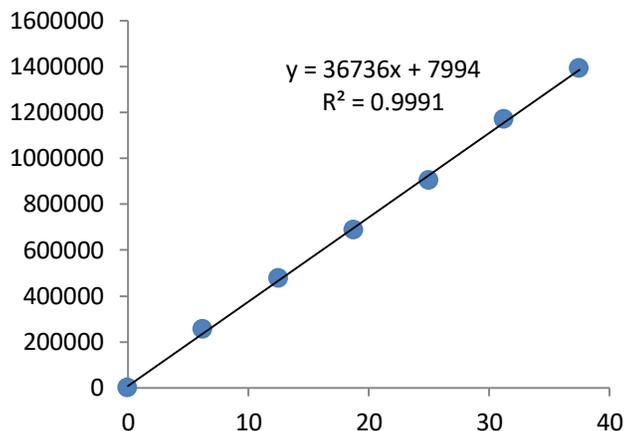


Fig. 4. Calibration curve of TAF

Accuracy. Accuracy as recovery was evaluated by spiking previously analyzed test solution with additional placebo at three different concentration levels. The results are shown in table 3.

Precision. Percentage RSD of peak area of six injections were calculated for system precision, method precision, interday and intraday precision (table 4).

LOD and LOQ. Detection limit of the Tenofovir Alafenamide in this method was found to be 0.19 µg/ml. Quantification limit of the Tenofovir Alafenamide in this method was found to be 0.57 µg/ml. The chromatograms are shown as figure 5.

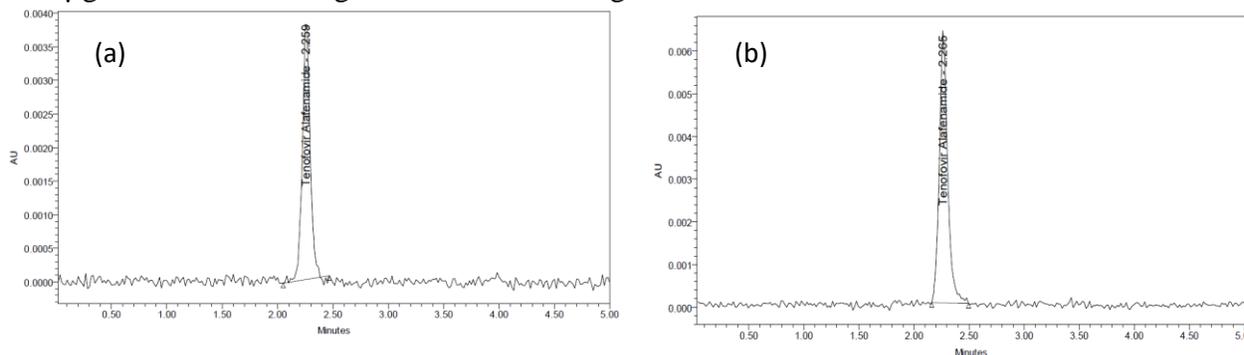


Fig. 5. Chromatogram of LOD (a) and LOQ (b).

Robustness. Small Deliberate changes in the method are made like Flow minus, flow plus, Mobile phase minus, Mobile phase plus, Temperature minus, Temperature Plus. %RSD of the above conditions are calculated and shown in table 3.

ASSAY

Six injections of TAF sample solutions were injected and the average peak area of these injections was compared to the average peak area of the standard solution of TAF. The % assay of marketed formulation was calculated and was found to be 99.83%.

Table 3. Accuracy and recovery studies of TAF

Concentration (%)	Preanalyzed concentration (µg/ml)	Spiked (µg/ml)	Founded (µg/ml)	Recovery (%)	%RSD
50%	25	12.5	12.60	100.78	0.57
	25	12.5	12.46	99.64	

	25	12.5	12.56	100.50	
100%	25	25	24.83	99.31	0.54
	25	25	25.08	100.34	
	25	25	24.86	99.43	
150%	25	37.5	37.60	100.26	0.27
	25	37.5	37.44	99.85	
	25	37.5	37.51	100.02	

Table 4. Precision and robustness data

Precision parameters	RSD (%)	Robustness parameters	RSD (%)
System precision	0.39%	Flow Minus	0.4
Method precision	0.52%	Flow Plus	0.2
Intraday precision	0.44%	Mobile phase Minus	0.2
Interday precision	0.50%	Mobile phase Plus	0.1
		Temperature minus	0.1
		Temperature plus	0.3

Forced degradation studies

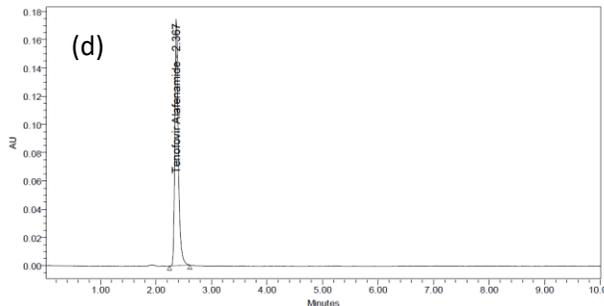
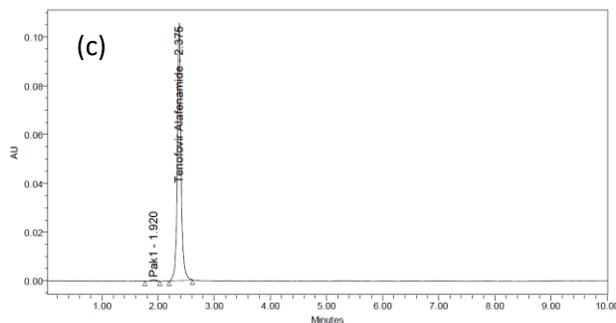
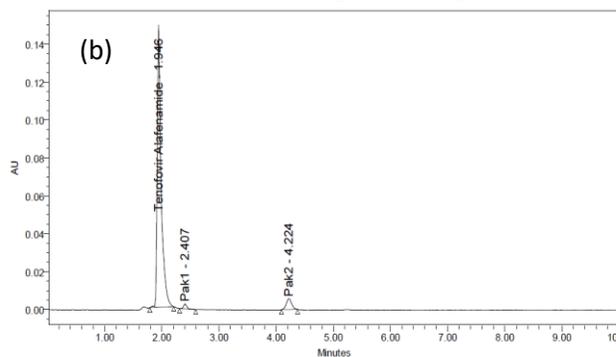
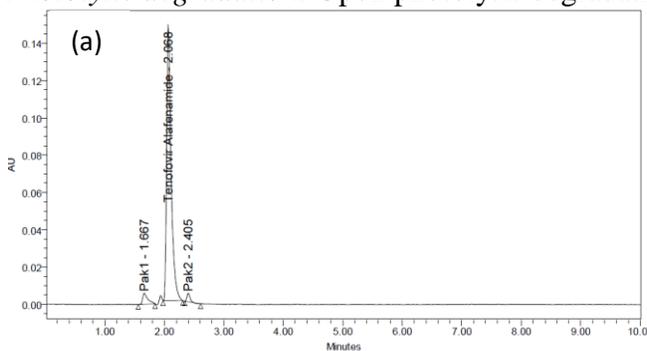
Acid hydrolysis. Upon acid degradation, 6.85 % of TAF was degraded (figure 6a).

Base hydrolysis. Upon base degradation, 5.16 % of TAF was degraded (figure 6b).

Oxidation. Upon peroxide degradation, 10.81 % of TAF was degraded (figure 6c).

Thermal degradation. Upon thermal degradation, 3.82 % of TAF was degraded (figure 6d).

Photolytic degradation. Upon photolytic degradation, 1.89 % of TAF was degraded (figure 6e).



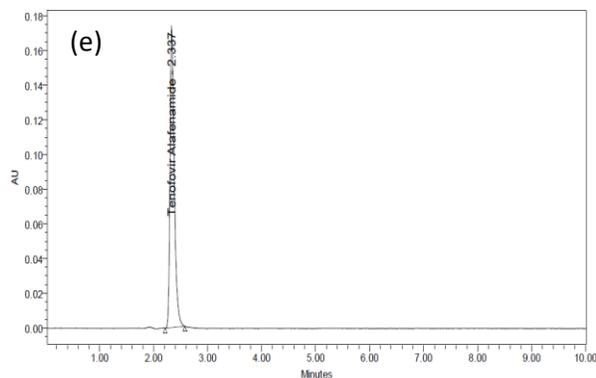


Fig. 6. Chromatograms of acid (a), base (b), peroxide (c), thermal (d) and photolytic (e) TAF degradations

CONCLUSION

In this study, the degradation behavior of TAF was studied by subjecting the drug to various stress conditions recommended by ICH. The additional findings in this study show that the drug undergoes an extensive degradation under oxidative (peroxide) condition. The method was validated for parameters like linearity, precision, accuracy, ruggedness and robustness. Application of this method for the analysis of TAF tablet dosage forms showed that there was no interference of excipients in the determination. The method is advantageous over most of the reported methods in-terms of sensitivity, simplicity, cost-effectiveness and experimental conditions. The method does not involve any tedious procedural steps; do not require any extra reagents or longer analysis time and a very simple instrument is required. The method can be used to determine the purity of the drug available from various sources.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest in this research article.

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