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Stability Indicating Assay Method Development and Validation for Tenofovir Alafenamide Fumarate by RP-HPLC

Aggarwal NN, Bhat KI* and Jacob JT

Department of Pharmaceutical Chemistry, NGSM Institute of Pharmaceutical Sciences of Nitte-Deemed to be University, Paneer, Deralakatte, Mangalore-575 018, Karnataka, India

Abstract

Aim: Development and validation of a stability indicating assay method for Tenofovir Alafenamide Fumarate tablets (25 mg strength) by RP-HPLC.

Methodology: An efficient experimental design based on systematic scouting of all key components of the RP-HPLC method and stress studies were performed. The separations were carried out on a C-18 reversed phase column (Inertsil ODS, 100 x 4.6 mm, 5 µ) using a mobile phase consisting of pH 6.0 ammonium acetate buffer and a solvent mixture (30:70) of ACN and THF in the ratio of 990:10 (Mobile phase A) and 500:500 (Mobile phase B) in a gradient elution mode at a flow rate of 1.50 mL/min and column oven temperature of 45°C. The wavelength of detection was 260 nm. Analytical validation parameters such as selectivity, linearity, accuracy, precision and robustness were evaluated as per ICH Q2 (R1) guidelines.

Results: USP plate count and the USP tailing factor for the pure drug peak was found to be 9082 and 0.98 respectively which are well within the acceptance criteria. Forced degradation studies performed revealed that none of the degradants generated interfered with the pure drug peak.

Conclusion: The proposed method can hence be used for routine analysis of Tenofovir Alafenamide Fumarate.

Keywords: RP-HPLC; Tenofovir alafenamide fumarate; Validation

Introduction

Tenofovir Alafenamide Fumarate (TAF) belongs to the class of nucleotide reverse transcriptase inhibitor (NRTI). It is a novel ester prodrug of the antiretroviral Tenofovir. It is chemically called as (2E)-but-2-enedioic acid; bis(propan-2-yl (2S)-2-{[(S)-({[(2R)-1-(6-amino-9H-purin-9-yl)propan-2yl]oxy}methyl)(phenoxy)phosphoryl]amino} propanoate). It has a molecular formula of $C_{23}H_{31}N_6O_7P$ and a molecular weight- 476.47 g/mol. It has the following structure (Figure 1) [1].

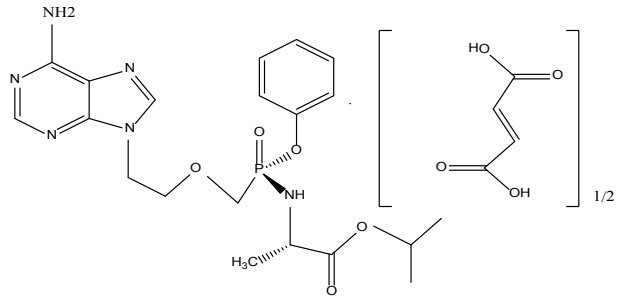

Figure 1: Structure of tenofovir alafenamide fumarate.

Experimental

Chemicals and reagents

The reference standards as well as the test samples and placebos were provided by Mylan laboratories. Ammonium acetate, orthophosphoric acid, Hydrochloric acid, (AR grade); Water, methanol, Tetrahydrofuran (THF) and Acetonitrile (ACN) of HPLC grade were used. The 0.45 µm pump nylon, PVDF filter were obtained from advanced micro devices (Ambala Cantt, India).

Instrumentation

The development and validation of the method was carried out in Agilent Technology HPLC with PDA detector, using Inertsil ODS column (100 x 4.6 mm, 5 µm). Recording of the data was done using Empower 2 and Empower 3 software.

Chromatographic conditions

After numerous trials using different combinations of solvents, the mobile phase was optimized to be

Mobile Phase A: Ammonium acetate buffer (pH 6.0): 70%THF+30%ACN (990:10) and

Mobile Phase B: Ammonium acetate buffer (pH 6.0): 70%THF+30%ACN (500:500).

The chromatographic separation was carried out using Inertsil ODS column (100 x 4.6 mm, 5 µm) at a flow rate of 1.50 mL/min and column oven temperature of 45°C. The sample temperature was set at 8°C. The wavelength of detection was 260 nm.

100% methnol was optimized as diluent-1 and 50% aqueous methanol was optimized as diluent-2.

***Corresponding author:** Natasha Naval Aggarwal, Department of Pharmaceutical Chemistry, NGSM Institute of Pharmaceutical Sciences of Nitte-Deemed to be University, Paneer, Deralakatte, Mangalore-575 018, Karnataka, India, Tel No: +91 7795578072; E-mail: aggarwalnatasha01@gmail.com

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Column	Inertsil ODS-3V column (4.6 mm x 100 mm) with 5 µm
Mobile phase	**Mobile Phase A**: Ammonium acetate buffer (pH 6.0) : 70%THF+30%ACN (990:10) **Mobile Phase B**: Ammonium acetate buffer (pH 6.0) : 70%THF+30%ACN (500:500)
Injection Volume	10 µL
Flow rate	1.5 mL/min
Column temperature	45˚C
Sample temperature	8˚C
Detection wavelength	260 nm
Run Time	15 minutes

Table 1: Optimized chromatographic parameters.

For analysis of forced degradation samples, the PDA detector was used in scan mode with a scan range of 200-400 nm. The peak homogeneity was expressed in terms of peak purity and was obtained spectral analysis report using previously mentioned software (Table 1).

Tenofovir alafenamide fumarate standard stock solution preparation

Accurately weighed 57 mg of Tenofovir Alafenamide Fumarate was taken in a 100 mL dry volumetric flask and was sonicated to dissolve after adding about 80 mL of diluent-1. It was then made up to mark with diluent-1.

Tenofovir alafenamide fumarate standard solution preparation

From the above prepared stock solution, 5 mL was pipetted into a 25 mL volumetric flask and made up to mark with diluent-2 to get a final concentration of 100 ppm .

Analysis of formulation

Firstly, the average weight of twenty tablets was taken. Ten tablets were then taken randomly in a 250 mL volumetric flask. To this, about 30 mL of water was added to disintegrate the coating of the tablet. Thereafter, about 170 mL of diluent-1 was added and the sample was sonicated for 30 minutes. It was then made up to mark with diluent-1 and centrifuged. From the supernatant, 5 mL was taken in a 50 mL volumetric flask and then made up to mark with diluent-2.The solution was then filtered through 0.45 µm PVDF filter. (Sample concentration: 100 ppm) (Figure 2).

Calculations

$$\% \text{ Assay of TAF} = \frac{AT \times WT \times V1 \times 25 \times V4 \times 476.5 \times P \times AWs}{ST \times 100 \times V2 \times WS \times V3 \times 534.5 \times 100 \times LC}$$

Where,

AT= Average peak area of Tenofovir Alafenamide Fumarate from test preparation

ST= Average peak area of Tenofovir Alafenamide Fumarate from standard preparation

WT= Weight of Tenofovir Alafenamide Fumarate standard (mg), for standard preparation

LC= Label claim of Tenofovir Alafenamide Fumarate (mg), per unit dose (25 mg)

P = Potency of Tenofovir Alafenamide Fumarate standard in % w/w, on as is basis

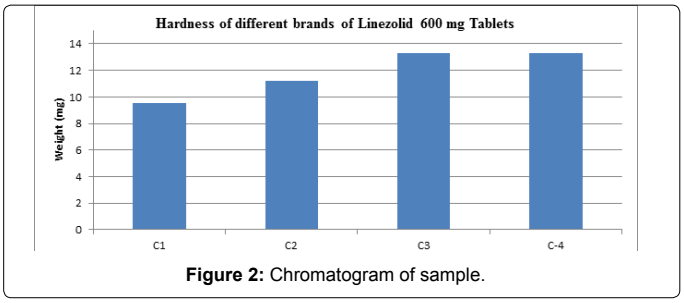

Figure 2: Chromatogram of sample.

WS= Weight of sample taken in 'mg' for test preparation

AWs= Average weight of Tenofovir Alafenamide Fumarate tablets (mg)

476.5= Molecular weight of Tenofovir Alafenamide

534.5= Molecular weight of Tenofovir Alafenamide Fumarate

V1, V2= Standard dilutions

V3, V4= Sample dilutions

The chromatogram obtained from the optimized method is shown below

Forced degradation studies

The International Conference on Harmonization (ICH) guideline entitled stability testing of new drug substances and products needs that stress testing is carried out to demonstrate the inherent stability characteristics of the active substance [2]. To evaluate the interference from degradation products, forced degradation study has been conducted by stressing simultaneously placebo, standard and drug product under the following stress conditions (Figure 3 and Table 2) [3].

TAF has 10 known impurities.

Standard solution preparation: Standard solution of 100 ppm of Tenofovir alafenamide fumerate was prepared and injected into the HPLC system.

Acid degradation: 156 mg of sample was accurately weighed and transferred into 25 mL volumetric flask. Added to it 2.5 mL of 0.1 N HCL and kept on bench top for 2 minutes. Added 2.5 mL of 0.1 N NaOH and shaken for the neutralization step to take place. Thereafter, 10 mL diluent-1 was added and sonicated for 30 minutes. It was then made up to mark with diluent-1 and centrifuged. 5 mL from the supernatant was pipetted into a 50 mL volumetric flask and made up to mark with diluent-2. Placebo and API equivalent to the amount present in one tablet (131 mg and 25 mg respectively) was treated in a similar manner and analyzed as per the method. The resulted degradation was 9.6% (Figure 4).

Base degradation: Weighed accurately about 156 mg of sample and transferred into 25 mL volumetric flask. Added to it 0.5 mL of 0.05 N NaOH and kept on bench top for 2 minutes. Added 0.5 mL of 0.1 N HCL and shaken for the neutralization step to take place. Thereafter, 10 ml diluent-1 was added and sonicated for 30 minutes. It was then made up to mark with diluent-1 and centrifuged. 5 mL from the supernatant was pipetted into a 50 mL volumetric flask and made up to mark with diluent-2. Placebo and API equivalent to the amount present in one tablet (131 mg and 25 mg respectively) was treated in a similar manner

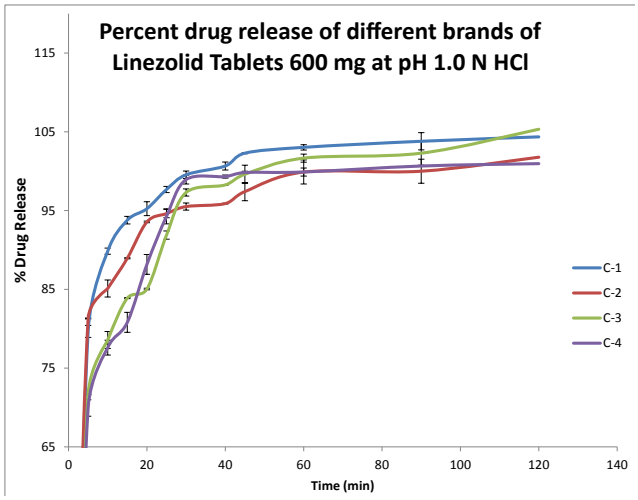

Figure 3: Linearity of TAF.

Sr. No.	Name of impurities	RT of Impurities from Known Impurity Injection (min)
	PMPA Anhydro Impurity	0.671
	TAF impurity	0.713
	Mono Phenyl PMPA Impurity	1.233
	PMPA monoamidate impurity	1.241
	Methyl Impurity	2.901
	Ethyl impurity	5.198
	PMPA bisamidate Impurity	5.424
	Diastereomer- 3 impurity	6.171
	Diastereomer- 2 impurity	6.173
	n-Propyl Impurity	8.534

Table 2: Results of impurities interference.

System Suitability Parameters	Observed Value	Acceptance Criteria
USP Plate count	**9082**	¨2000
USP Tailing factor	**0.98**	¨¨2.0
% Relative standard deviation*	**0.05**	¨¨2.0
*Six replicate injections ¨Not less than ¨¨Not more than		

Table 3: Results of system suitability.

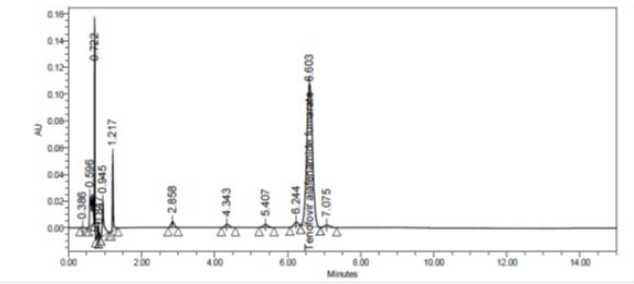

Figure 4: Typical chromatogram of standard preparation spiked with known impurities (1% spiking).

and analyzed as per the method.

TAF degradation was reported as 8.7% (Figure 5).

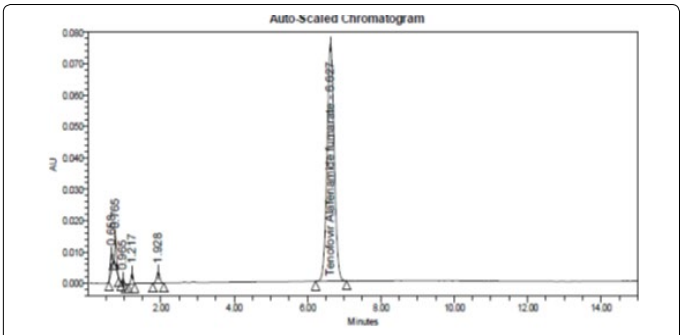

Figure 5: Chromatogram of acid degradation sample.

Oxidative degradation: Weighed accurately about 156 mg of sample and transferred into 25 mL volumetric flask. Added to it 2.5 mL of 30% H_2O_2 and kept on bench top for 3.5 hours. Thereafter, 10 mL diluent-1 was added and sonicated for 30 minutes. It was then made up to mark with diluent-1 and centrifuged. 5 ml from the supernatant was pipetted into a 50 mL volumetric flask and made up to mark with diluent-2. Placebo and API equivalent to the amount present in one tablet (131 mg and 25 mg respectively) was treated in a similar manner and analyzed as per the method.

Oxidative degradation of 8.4% was recorded (Figure 6).

Hydrolysis degradation: Weighed accurately about 156 mg of sample and transferred into 25 mL volumetric flask. Added to it 2.5 mL of H_2O and kept at 80°C for 3.5 hours. Thereafter, 10 mL diluent-1 was added and sonicated for 30 minutes. It was then made up to mark with diluent-1 and centrifuged. 5 mL from the supernatant was pipetted into a 50 mL volumetric flask and made up to mark with diluent-2. Placebo and API equivalent to the amount present in one tablet (131 mg and 25 mg respectively) was treated in a similar manner and analyzed as per the method.

Hydrolysis degradation of 18.6% was achieved (Figure 7).

Humidity degradation: Weighed accurately 156 mg of sample exposed to 40°C/5% RH for 7 days and transferred into 25 mL volumetric flask. Thereafter, 10 mL diluent-1 was added and sonicated for 30 minutes. It was then made up to mark with diluent-1 and centrifuged. 5 mL from the supernatant was pipetted into a 50 mL volumetric flask and made up to mark with diluent-2. Placebo and API equivalent to the amount present in one tablet (131 mg and 25 mg respectively) was treated in a similar manner and analyzed as per the method.

7.4% of the drug degraded (Figure 8).

Thermal degradation: Weighed accurately 156 mg of sample exposed to 105°C for 3 hours and transferred into 25 mL volumetric flask. Thereafter, 10 mL diluent-1 was added and sonicated for 30 minutes. It was then made up to mark with diluent-1 and centrifuged. 5 mL from the supernatant was pipetted into a 50 mL volumetric flask and made up to mark with diluent-2. Placebo and API equivalent to the amount present in one tablet (131 mg and 25 mg respectively) was treated in a similar manner and analyzed as per the method.

This resulted in 6.9% degradation (Figure 9).

Method Validation

System suitability

System suitability testing is an important segment of any analytical procedure [4]. The rationale of the test is the fact that electronics analytical operations, equipments and sample to be analyzed form

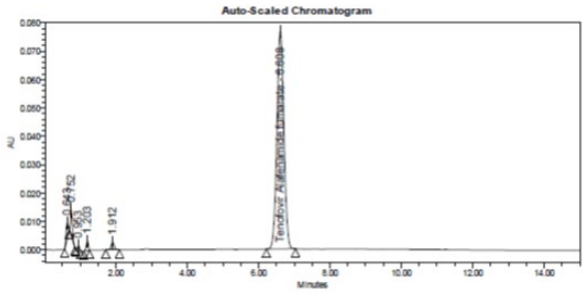

Figure 6: Chromatogram of base stressed sample.

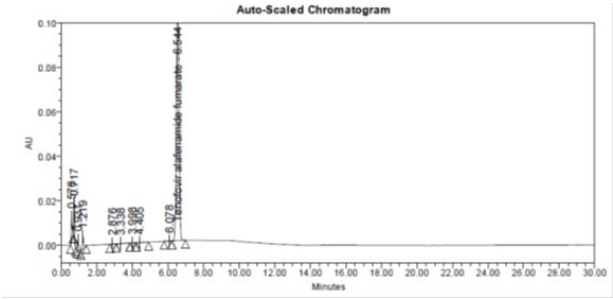

Figure 7: Chromatogram of peroxide stressed sample.

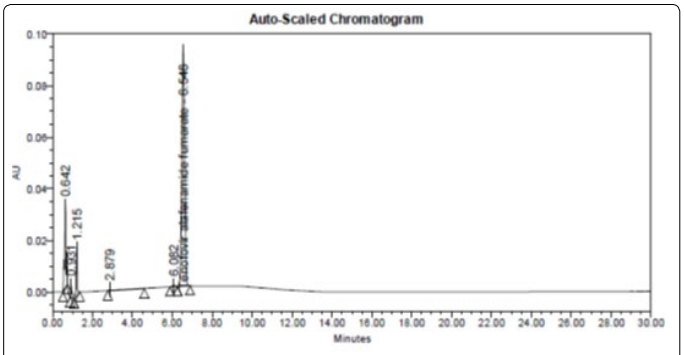

Figure 8: Chromatogram of hydrolysis stressed sample.

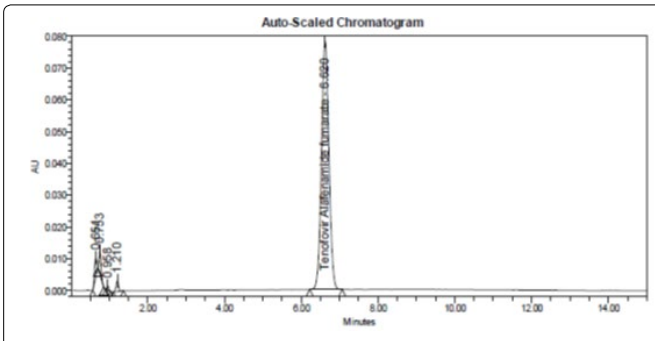

Figure 9: Chromatogram of humidity stressed sample.

a vital system that can be evaluated as such [5]. System Suitability was determined by preparing and injecting standard solution in six replicates (Table 3).

Linearity

Linearity of the method has been studied across 10% to 150% of working concentration, using standard preparation [6]. Linearity graph has been plotted between response and concentration (Table 4 and Figure 10).

Solution	Concentrated (µg/mL)	Area	Area (Linear Fit)
1	10.132	119331	118736.2113
2	50.662	597860	596486.7824
3	75.994	893509	895089.7301
4	101.325	1192185	1193680.89
5	121.59	1431648	1432556.176
6	151.987	1792880	1790863.21

Table 4: Linearity Data of Tenofovir.

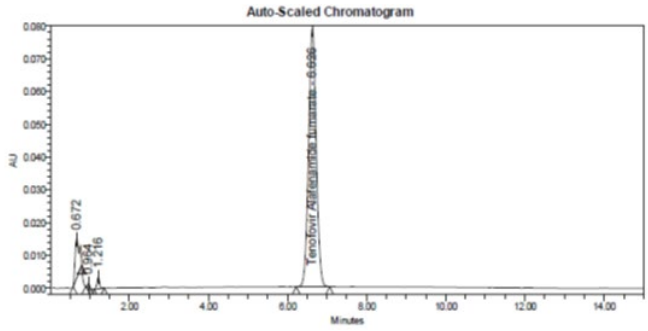

Figure 10: Chromatogram of thermal stressed sample.

Precision

System precision: System precision has been established by injecting six replicates of standard preparation. % RSD shall be calculated for area responses (Table 5).

Method precision (Repeatability): Method precision has been determined by injecting six test preparations, representing a single batch to determine the Assay. %RSD shall be calculated for observed results (Table 6).

Accuracy

A study of recovery was conducted for TAF intact tablet from about 50% to 150% of the initial assay concentration. Sample solutions were prepared in triplicate for each level and analyzed as per test method [7]. The individual % recovery, %average recovery and % RSD for recovery at each level were calculated (Table 7).

Range

The range of an analytical procedure is the interval between the upper and lower concentration (amounts) of analyte in the sample (including these concentrations) for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity [7]. The range was confirmed as 10% to 150% of the test concentration for TAF.

Robustness

Method robustness has been determined by making changes in the chromatographic parameters and sample preparation parameters [8]. The assay value determined for the same sample under original and robustness conditions depicts that the developed method was robust for effect of changed column temperature (± 5°C), pH of the mobile phase(± 0.2), organic & aqueous variation in the mobile phase and impact of flow rate (± 0.2 ml/min). The evaluation was based on the system suitability parameters such as tailing factor, retention time, and theoretical plates.

Injection No	System precision (Peak area)
	TAF
1	1126672
2	1126954
3	1126811
4	1127849
5	1130718
6	1126872
Mean	1127801
%RSD*	0.150235
*n=6	

Table 5: System precision results.

Sample No	Area Count	(% Assay)
	TAF	
1	1135522	101.9
2	1132359	101.8
3	1133270	101.8
4	1130741	101.8
5	1132992	101.9
6	1134736	101.9
Mean		101.85
%RSD*		0.05
*n=6		

Table 6: Method precision results.

Results and Discussion

Optimization of chromatographic conditions

In recent years an ART (antiretroviral) therapy has been developed, which includes single or combination therapy by antiviral drug [9]. The present investigation has been reported with an intention to develop a new validated method for the estimation of Tenofovir Alafenamide Fumarate tablets of strength 25 mg by RP-HPLC method using a photo diode array detector. Literature reveals that there are no analytical methods reported for the estimation Tenofovir Alafenamide Fumarate individually by RP-HPLC method. To develop a stability-indicating method different makes of C18, C8 column (Xterra, Hypersil and Thermosil) and different mobile phases containing buffers (pH 2-6): methanol as well as acetonitrile in different ratios were tried but failed to get optimum peak shape and freedom from impurity interference. This challenge was met by using 0.03 M Ammonium acetate buffer (adjusted to pH 6 by glacial acetic acid): organic modifier (99:1%v/v) as mobile phase A and pH 6, 0.03 M ammonium acetate buffer: organic modifier (50:50%v/v) as mobile phase B where optimum resolution and good symmetric peaks were observed by using Inertsil C18 (4.6 mm × 100 mm, 5 µm) analytical column in gradient mode at a flow rate of 1.5 mL/min and column temperature at 45°C. Under the above optimized conditions, the retention time reported for formulation was 6.5 min. The linearity of an analytical procedure was demonstrated by preparing and analyzing the standard preparation at five different concentrations of (10-150 ppm). The calibration curve constructed for Tenofovir by plotting the peak area versus concentration yielded coefficient of regression R_2=0.99996. The mean recovery value was 99.9. The robustness study and percentage of assay of the formulation were found within limit as per ICH Guidelines .The theoretical plates were more than 2500 and tailing factor was less than 1.5. Specificity of the method was proved since the chromatograms of blank, placebo solution do not show any interference at the retention time of Tenofovir. Therefore, the developed method was free from the inference of diluents as well as the excipients used in the formulation.

% Level Spiked	Sample No.	% Recovery	% Recovery Mean	%RSD
50	**1**	100.1	101.03	**1.35**
	2	102		
	3	100.4		
100	**1**	101.9	101.83	**0.05**
	2	101.8		
	3	101.8		
150	**1**	100.8	100.93	**0.15**
	2	100.9		
	3	101.1		

Table 7: Results of accuracy.

Forced degradation study

Specificity of the developed method in the presence of the degradants is shown by the forced degradation studies [4]. Degradation was carried out on standard drug, drug formulation as well as on the placebo. Out of the ten known impurities of Tenofovir Alafenamide Fumerate, none of them were reported at the retention time of Tenofovir (Table 8).

The developed method was validated for accuracy, precision, reproducibility, specificity, robustness in accordance with ICH guidelines [10].

Maximum absorbance was shown at 260 nm and hence this was the wavelength selected.

For the purpose of method development, many columns were employed. A satisfactory symmetric peak as well as separation between the main peak and the impurities peak was obtained using C-18 Inertsil ODS column (100 × 4.6 mm, 5 µ) since Tenofovir alafenamide fumarate is a polar drug.

With regard to the drug pKa, Ammonium Acetate buffer of pH 6 was selected. In order for the complete separation of all the 10 impurities of Tenofovir Alafenamide Fumarate, a solvent mixture (ACN:THF) in 30:70 ratio was added to the buffer in different ratios. The most optimum ratio was found to be when mobile phase A contained 1% solvent mixture and mobile phase B contained 50% solvent mixture, the rest being buffer.

A retention time of 6.5 minutes was observed wherein the column oven temperature was set at 45°C and the sample temperature was 8°C. However, in order for the elution of all the impurities, a run time of 15 minutes was opted.

Once all the method parameters were optimized, stability of the method was determined by performing forced degradation studies. Degradation by acid, base, peroxide, water, humidity and heat was done. Degradation of drug substances between 5% and 20% has been accepted as reasonable for validation of chromatographic assays and the observed values of degradation were within this limit suggesting that the developed method was stable. After the establishment of the stability of the method, validation parameters such as accuracy, linearity, precision, system suitability and robustness were evaluated. With the flow rate of 1.5 mL/min, the method was found to be accurate, precise and linear within a range of 10 ppm-150 ppm. Robustness studies as well as filter validation studies were performed indicating that the developed method is rugged.

Hence, the aim of developing a stable method along with its validation was achieved for Tenofovir alafenamide fumarate.

Conclusion

A simple, precise and specific RP-HPLC assay method was

Stress	Conditions	% Degradation	TAF		Purity Flag	Acceptance Criteria
			Purity Angle	Purity Threshold		
Initial		-	0.106	0.295	No	The Purityangle should be less than Purity Threshold and no purity flag for Tenofovir Alafenamide Fumarate peak.
Acid Stress 0.1 N HCl	2 min, 2.5 mL, BT	9.6	0.134	0.302	No	
Peroxide Stress 30% H_2O_2	2.5 mL,3.5 hours	8.4	0.126	0.213	No	
Base Stress 0.05 N NaOH	2 Min,0.5 mL	8.7	0.129	0.314	No	
Humidity stress	40°C/ 75%RH7 days	7.4	0.106	0.3	No	
Water stress	80°C, 3.5 hours	18.6	0.129	0.228	No	
Thermal stress	105°C, 1 hour	6.9	0.14	0.313	No	

Table 8: Forced degradation study.

developed for estimation of TAF and validated for determination in commercial tablet dosage form of 25 mg. The procedure was validated for all compendial and non compendial parameters in accordance with ICH guidelines. The study showed that the reverse phased liquid chromatography is sensitive and selective for detecting TAF and its impurities. The method was validated by using various validation parameters like system suitability, specificity, linearity, precision, accuracy, solution stability, filter interference and robustness. All the validation parameters were found to be within the acceptance criteria. Stress studies were performed under acid, base, thermal, hydrolysis and humidity conditions. The method developed could detect the main peak without any interference from the degradant peaks formed under these conditions. The HPLC method was found to be accurate, precise and reproducible. The method can be applied for routine estimation of TAF in pharmaceutical formulations.

Acknowledgements

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References

1. https://www.drugbank.ca/
2. Ettre LS (1993) Nomenclature for chromatography (IUPAC Recommendations 1993). Pure Appl Chem 65: 819-872.
3. Bakshi M, Singh S (2002) Development of validated stability-indicating assay methods-critical review. J Pharm Biomed Anal 28: 1011-1040.
4. Sanjay B, Dinesh S , Neha S (2012) Stability testing of pharmaceutical products. J Appl Pharm Sci 02: 129-138.
5. Badgujar BP, Mahajan MP, Sawant SD (2017) Development and validation of RP-HPLC method for the simultaneous estimation of tenofovir alafenamide and emtricitabine in bulk and tablet dosage form. Intl J of Chem Tech Res 10: 731-739.
6. Varanasi MB, Mohammed I (2017) Stability indicating RP-UPLC-PDA method development, validation of multi drug combination of emtricitabine, tenofovir alafenamide and rilpivirine in bulk drug and its tablet formulation. Oriental J Chem 33: 925-929.
7. Akram NMD, Umamahesh MA (2017) New validated RP-HPLC method for the determination of emtricitabine and tenofovir af in its bulk and pharmaceutical dosage forms. J Chem Pharm Sci 10: 54-59.
8. Gandla KS, Rajkumar M, Pranay K, Sudheer KD (2017) New stability indicating RP-HPLC method for the simultaneous estimation of tenofovir alafenamide and emtricitabine in bulk and combined tablet dosage forms. Asian J Pharm Anal Med Chem 5: 142-149.
9. Szepesi G, Gazdag M, Mihalyfi K (1991) Selection of high-performance liquid chromatographic methods in pharmaceutical analysis: III. Method validation. J Chrom A 464: 265-278.
10. Bhavana N, Likhitha P, Hanumanth, Suresh P (2017) Design and development of stability indicating assay methods as per ICH guidelines-a review. Int J Pharm Chem Res 3: 252-258.