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Original Article

VALIDATED GRADIENT STABILITY INDICATING RP-HPLC METHOD FOR THE SIMULTANEOUS QUANTIFICATION OF 11 RELATED SUBSTANCES IN THE COMBINED DOSAGE FORMS OF LAMIVUDINE AND TENOFOVIR DISOPEOXIL FUMARATE

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ABSTRACT

Objective: Development of a stability-indicating reverse phase liquid chromatographic (RP-HPLC) method for the simultaneous quantification of 11 impurities in the combined dosage forms of lamivudine and tenofovir disoproxil fumarate drug substances.

Methods: Efficient chromatographic separation of all analytes was achieved on a Waters X-terra RP18 column (150 x 4.6 mm, 3.5 µm) using mobile phase A (ammonium acetate buffer, pH adjusted to 5.0±0.05 with dilute orthophosphoric acid) and mobile phase B (mixture of methanol and ammonium acetate buffer in the ratio of 20:80) with the flow rate of 1.0 ml/min in gradient elution mode at 260 nm.

Results: The method was validated in terms of the limit of detection, limit of quantification, linearity, accuracy, precision and robustness according to the international conference on harmonisation (ICH Q2R1). Regression analysis showed that the correlation coefficient (r^2) is greater than 0.997 for individual active drug substances as well as their related substances. The method has proven very accurate (94.6 % to 108.2 % with % RSD not more than 4.9), highly precise (% RSD of the Intra-day and the inter-day study was not more than 8.9) and robust enough to deliver accurate results, when the chromatographic conditions were altered intentionally. Forced degradation studies were conducted in acidic, basic, thermal, photolytic, humid and peroxide stress conditions, where all the degradation peaks were monitored. Highest degradation of lamivudine was observed under oxidative stress condition and tenofovir was more susceptible to degradation under acidic and alkaline conditions.

Conclusion: The present method is able to separate all the related compounds with each other and with the main drug substances with the resolution more than 2.0. The test solution was found to be stable in diluent up to 24 h. The mass balance of forced degradation of formulations, close to 99 %, made this method as a stability indicating method.

Keywords: Related Substances, Lamivudine and Tenofovir disopeoxil fumarate, HPLC method development, Validation

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INTRODUCTION

Tenofovir disoproxil fumarate {9-[(*R*)-2-[[bis [[isopropoxycarbonyl] oxy] methoxy] phosphonyl] methoxy] popyl] adenine fumarate} is a nucleotide analog reverse transcriptase inhibitor (NRTI) and is used for treating HIV infection in adults in combination with other antiretroviral agents. Lamivudine {4-amino-1-[(2*R*, 5*S*)-2-(hydroxyl methyl)-1,3-oxathiolan-5-yl]-1,2-dihydro pyrimidin-2-one} is an another NRTI used in the treatment of HIV infection and chronic hepatitis B virus (HBV) [1-2]. The combination of these two drugs is available in the market with brand names of Forstavir 30s tablet, Tavin 30s tablet, Ricovir 30s tablet, Tenolam 30s tablet, Envir 30s tablet with 300 mg strength of each rugs substance. Total eleven specified impurities are present in both lamivudine and tenofovir out of which, four impurities are related to lamivudine and seven impurities (including one dimer) are related to tenofovir. As per the ICH guidelines, all these related substances must be controlled below 0.2 % of the drug concentration except monoester and isopropyl ethers and limits should not exceed 0.2 % at various storage conditions.

As per the USP monograph, the specification limits for tenofovir monoester and tenofovir isopropyl impurities are 1.0 % and 0.3 %, respectively. Under various environmental conditions, a change in the purity of these drug substances is common and it results in change in the amount of impurities. In order to maintain the quality of these drug substances, degradation study of combined dosage forms is of prime importance for the determination of the various degraded impurities.

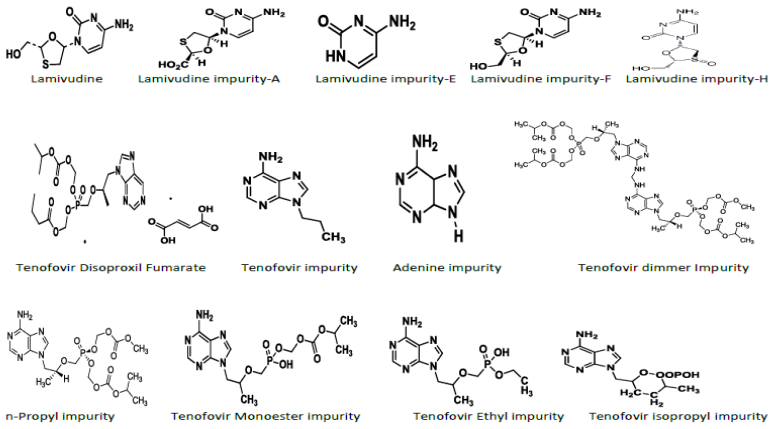

Fig. 1: Chemical structures of lamivudine, tenofovir and its impurities

The literature review revealed that most efforts were put in developing analytical methods for estimating the lamivudine and tenofovir drug substances, either individually or in combination with other drugs in different dosage forms or biosamples which includes spectrophotometric method [3-6], high performance liquid chromatography [7-18], ultra-performance liquid chromatography [19, 20] and mass spectrometry [21-23]. Rao *et al.* [18], reported a stability indicating a method for the simultaneous estimation of lamivudine, tenofovir and dolutegravir in bulk and their dosage forms. But, no any analytical method was reported for the simultaneous estimation of related substances in a combined dosage form of lamivudine and tenofovir. Therefore, the present study is aimed to develop a stability indicating high performance liquid chromatographic method for the determination of 11 impurities including two drug substances in the dosage forms. The structures of both the drug substances and related substances are presented in fig. 1 and chemical names are given in table 1. The method was validated as per the guidelines are given by food and drug administration (FDA) and international conference on harmonization (ICH) with respect to the limit of detection, limit of quantification, linearity, precision, accuracy, specificity and stability studies.

Table 1: Chemical names of lamivudine, tenofovir disoproxil fumarate and their impurities

S. No.	Name of the compounds	IUPAC name
1	Lamivudine	4-Amino-1-[(2R,5S)-2-(hydroxymethyl)-1,3-oxathiolan-5-yl]-1,2-dihydropyrimidin-2-one
2	Impurity-A	(2*RS*,5*SR*)-5-(4-amino-2-oxopyrimidin-1(2*H*)-yl)-1,3-oxathiolane-2-carboxylic acid
3	Impurity-E	4-aminopyrimidin-2(1*H*)-one
4	Impurity-F	pyrimidine-2,4(1*H*,3*H*)-dione
5	Impurity-H	4-Amino-1-[(2R,3R,5S)-2-(hydroxymethyl)-1,3-oxathiolan-5-yl] pyrimidin-2(1H)-one S-oxide
6	Tenofovir disoproxil fumerate	Bis{[(isopropoxycarbonyl)oxy]methyl} ({[(2R)-1-(6-amino-9H-purin-9-yl)-2-propanyl]oxy}methyl)phosphonate.
7	Adenine impurity	6-Amino purine
8	Mono ester impurity	[2-(6-amino-purin-9-yl)-1-methyl-ethoxymethyl]-phosphonic acid monoisopropoxycarbonyloxymethyl ester.
9	Ethyl impurity	Isopropyloxycarbonyloxy methy (ethoxy)-(R)-9-[2 phosphono methoxy) propyl adinine
10	Isopropyl impurity	Isopropyl ether (R)-9-(2-mono isopropoxy carbonyl oxy methyl phosphine methyl) propyladenine
11	n-propyl impurity	[2-(6-amino-purin-9-yl)-1-methyl-ethoxymethyl]-phosphonic acid isopropoxycarbonyloxymethyl ester-methoxycarbonyloxymethyl ester, fumarate.
12	Tenofovir impurity	(R)-1-(6-Amino-9H-purine-9-yl) propane-2-yloxy)methyl phosphonic acid
13	Dimer impurity	[2-[6-[[[9-(Bis-isopropoxycarbonyloxy methoxy phosphonyl-methoxy)propyl]-9Hpurin-6-ylamino]methyl]amino]-purin-9-yl]-1-methyl-ethoxymethyl]-phosphonic acid diisopropoxycarbonyloxy methyl ester.

MATERIALS AND METHODS

Chemicals and reagents

Reference standards of drug substances and related compounds are supplied by GSN Pharmaceuticals private limited, Hyderabad as gift samples. Lamivudine and tenofovir tablet dosage forms were purchased from local market. HPLC grade acetonitrile was procured from Qualigens, India. Ammonium acetate and orthophosphoric acid were purchased from Merck, India. All other chemicals and solvents of analytical grade were supplied by Merck, India. Water used in the HPLC analysis was purified by the water purifier (Milli-Q Millipore). The mobile phase and all the solutions were filtered through a 0.45 mm nylon filter (Millipore).

Instrumentation

The HPLC system was composed of 2695 waters alliance system fixed with 2996 PDA detector with Empower 2 software. Analytical column used for this method is Waters x-terra RP18 (150 mm x 4.6 mm, 3.5 µm).

Operating conditions of HPLC

Initially, several trial runs were carried out to select suitable a solvent system for accurate and precise analysis and to achieve a proper resolution of analytes. Various parameters like columns, solvent type, solvent and buffer ratio, the flow rate of the mobile phase and wavelength were tried to establish the chromatographic conditions for the separation of related compounds in short run time. Finally, optimum parameters were found on X-terra RP18 (150 x 4.6 mm, 3.5 µm) column and the mixture of mobile phase A consisting of mixed ammonium acetate (pH adjusted to 5.0±0.05 with dilute OPA) and mobile phase B consisting of a mixture of methanol and above ammonium acetate buffer in the ratio of 20:80 (v/v) at a flow rate of 1 ml/min in gradient elution mode with column temperature at 35 °C. Gradient time program as set as a time in minutes/% of mobile phase B composition 0/0, 45/90, 53/90, 54/0, 60/0. Before delivering the mobile phase into the system, it was degassed and filtered through 0.45 µm nylon filter using the vacuum. The injection volume was 10 µl and the detection was performed at 260 nm using a photodiode array (PDA) detector. The typical retention times of lamivudine and tenofovir are 7.85 min and 30.11 min respectively. The criticality of this method is to elute the entire active compounds as well as their related impurities with optimum separation and symmetric peak shapes in short run time.

Preparation of mobile phases

Mobile phase A prepared by taking 1.53 g of ammonium acetate into a beaker containing 1000 ml of water (0.02 N) and adjusted the pH of the resultant solution to 5.0±0.05 with diluted orthophosphoric acid and filtered through a 0.45 µm filter. Mobile phase B prepared by mixing of methanol and ammonium acetate buffer in the ratio of 20:80 (v/v). Diluent is prepared mixing of methanol and ammonium acetate buffer in the ratio of 80:20 (v/v).

Stock and standard solution preparation

Stock solution (100 µg/ml) was prepared by dissolving about 10 mg of each lamivudine and tenofovir into 100 ml volumetric flask, added 75 ml of diluent and sonicated to dissolve and diluted to volume with diluent and mixed well. Standard solution (1 µg/ml) was prepared by transferring 1.0 ml of the above stock solution into a 100 ml volumetric flask and diluted with diluent up to the mark.

Sample preparation

10 tablets were separately weighed and ground into fine powder by using mortar and pestle. An amount equivalent to 100 mg of lamivudine and tenofovir was transferred into a 200 ml volumetric flask, added 150 ml of diluent and sonicated to extract all the active compounds and their related impurities completely and made up to the volume with diluent and mixed well. A portion of the above solution was filtered through 0.45 µm nylon syringe filter and discarded first 2 ml of the filtrate followed by collecting the remaining filtrate into the 2 ml HPLC vial.

RESULTS AND DISCUSSION

Separation of lamivudine, tenofovir and related substances was achieved with the above optimized conditions and the method was validated as per ICH Q2 (R1) guideline and current industrial practices.

Method validation

The aim of method validation is to confirm that the present method is suitable for its intended use purpose. The described method has been extensively validated in terms of specificity, limit of detection (LOD) and quantification (LOQ), linearity, accuracy, precision, robustness and solution stability. The precision was expressed in terms of intraday and interday variation in the expected drug concentrations. The accuracy was expressed in terms of percent recovery by adding a known amount of impurities to the sample preparation.

System suitability

The system suitability was evaluated by a series of six injections of the standard solution with the concentration of 1 µg/ml. The system suitability criteria like % RSD, USP tailing factor and USP theoretical plates summarized in table 2. The corresponding standard chromatogram is shown in fig. 2. As per the results tabulated in table 2 system suitability parameters are fixed as % RSD of all the injections should be less than 5 and tailing factor should not more than 2.0 and the limit for theoretical plates should more than 5000 for both the peaks.

Table 2: System suitability results

S. No	Lamivudine				Tenofovir			
	Retention time (min)	Peak area	Tailing factor	Theoretical plates	Retention time (min)	Peak area	Tailing factor	Theoretical plates
1	7.85	27222	1.18	12579	30.11	37119	1.18	13213
2	7.84	27121	1.17	12614	30.10	37108	1.18	13196
3	7.87	27037	1.18	12678	30.08	37155	1.18	13233
4	7.82	27069	1.18	12689	30.14	37160	1.17	13254
5	7.80	27175	1.18	12632	30.18	37110	1.18	13252
6	7.81	27268	1.17	12589	30.12	37083	1.18	13264
Mean		27149				37120		
SD		89.323				30.773		
%RSD		0.33				0.08		

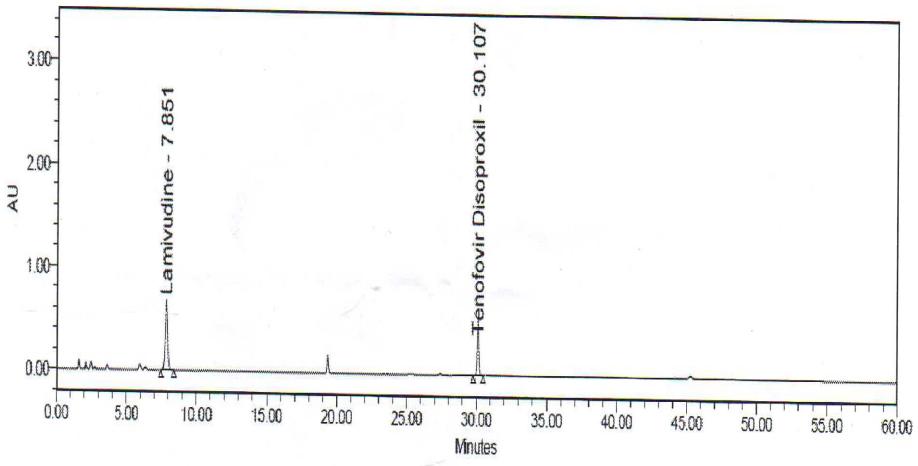

Fig. 2: Typical chromatogram of standard solution

Table 3: Specificity results of spiked sample

S. No	Name	RT	RRT	Purity angle	Purity threshold	Peak purify
1	Fumaric acid	1.62	0.21	12.501	14.254	Pass
2	Impurity-E	2.12	0.27	10.124	15.587	Pass
3	Impurity-F	2.49	0.32	0.584	2.157	Pass
4	Impurity-A	2.78	0.36	5.687	6.789	Pass
5	Impurity-H	3.63	0.46	1.242	3.610	Pass
6	Adenine	5.96	0.76	3.547	4.587	Pass
7	Tenofovir impurity	6.35	0.81	9.125	13.524	Pass
8	Lamivudine	7.81	1.00	14.254	16.245	Pass
9	Mono ester impurity	19.36	2.48	0.554	1.021	Pass
10	Di ehyl impurity	25.39	3.25	3.014	3.541	Pass
11	Isopropyl impurity	27.37	3.50	6.647	6.847	Pass
12	Tenofovir disoproxil	30.11	3.85	1.201	1.542	Pass
13	n-Propyl impurity	30.66	3.93	3.125	4.852	Pass
14	Tenofovir dimer impurity	44.17	5.65	2.325	2.854	Pass

RT= Retention time, RRT= Relative retention time

Specificity

The specificity of the present method was checked by injecting blank, placebo preparations and samples by spiking with appropriate levels of impurities and demonstrated the separation of these impurities individually and/or from other components in the sample matrix of tenofovir, lamivudine. The chromatograms of the blank and spiked sample for the specificity study are included in fig.

3 and fig. 4. The results of the specificity in terms of retention time (RT) and relative retention time (RRT), purity angle, purity threshold and peak purity of all analytes are included table 3. The chromatograms of blank and placebo in fig. 3 and fig. 4 showed that no any peak was found at the retention times of impurities. The spiked chromatogram presented in fig. 5 showed that all the peaks are well separated with each other with resolution more than 2.0 confirmed the specificity of the present method is good. The results presented in table 3 indicate that there is no interference between the peaks of impurities with main drug substances and the specificity of the method is confirmed by their peak purities i. e purity angle is less than purity threshold. Hence, the chromatographic system used for the estimation of related substances in tenofovir and lamivudine is very selective and specific. Rao *et al.* [18], presented a stability indicating a method for three drug combination formulations without impurity profile study. But, the present method is able to quantify all the individual impurities that arise during storage of both active pharmaceutical ingredient and tablet dosage forms.

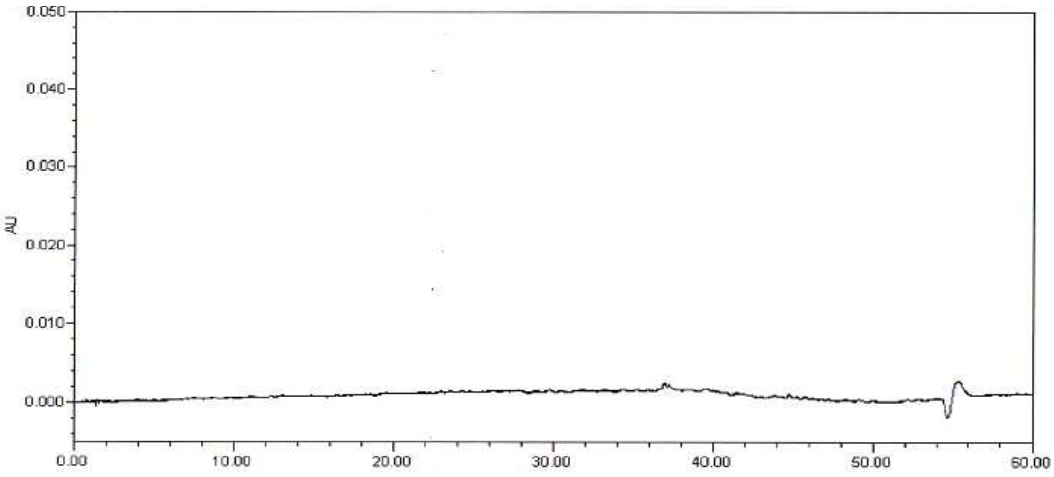

Fig. 3: Chromatogram of blank solution

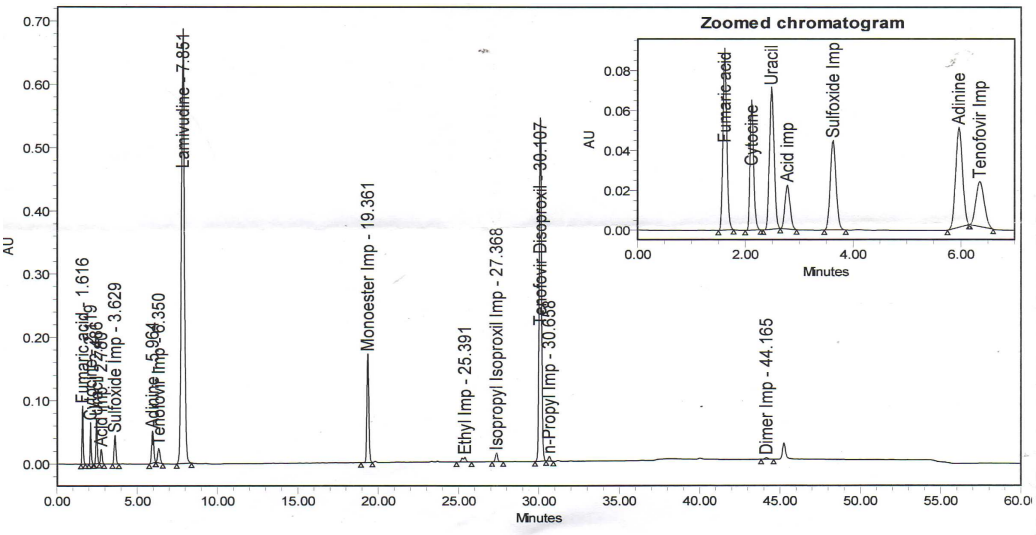

Fig. 4: Typical chromatogram of impurity spiked sample

Forced degradation studies

Intentional degradation was attempted at various stress conditions like the thermal sample (at 60 °C for 7 d), photolytic sample (1.2 million lux h), humidity (at 90 % related humidity for 7 d), acid hydrolysis (using 0.5 N hydrochloric acid, 1h at room temperature), base hydrolysis (using 0.5 N sodium hydroxide, 1h at room temperature) and oxidative degradation (using 10 % hydrogen peroxide, 1h at room temperature) to evaluate the ability of the proposed method to separate degradation products from each other and active ingredients as well and to provide an indication of stability indicating property of the method. To check and ensure the homogeneity (peak purity) of all peaks in the stressed sample solutions, photodiode array detector was employed. The results of forced degradation study presented in table 4 indicate that tenofovir is susceptible for degradation in acid, base and high humidity stress conditions, whereas lamivudine is susceptible to peroxide and high humidity stress conditions.

Limit of detection and quantification

Limit of detection (LOD) and limit of quantification (LOQ) for related substances are determined by injecting a series of solutions of known concentration till the signal-to-noise ratio became as 3:1 and 10:1, respectively, and the corresponding values are summarized in table 5. The found LOQ values are sufficient to quantify these impurities below the 0.2 % of the drug concentration as per the limits defined by pharma regulating agencies. The LOD and LOQ values are determined as 0.048 µg/ml and 0.160 µg/ml for lamivudine and 0.035 µg/ml and 0.115 µg/ml for tenofovir respectively, which are lower values when compared to earlier methods [18].

Table 4: Forced degradation results

Sample details	Lamivudine			Tenofovir disoproxil			Peak purity of both peaks
	% of degradation	% of assay	Mass balance	% of degradation	% of assay	Mass balance	
As such sample	0.23	100.6	-	0.85	100.1	-	Pass
Thermal sample	0.25	100.4	99.8	0.91	98.2	99.0	Pass
Photolytic sample	0.17	100.5	100.1	1.12	97.8	98.8	Pass
Humidity sample	2.5	97.2	99.1	0.51	98.1	98.5	Pass
Acid degradation	2.18	97.7	99.5	8.5	91.9	100.3	Pass
Base degradation	1.5	98.3	99.4	11.5	89.8	101.2	Pass
Oxidative degradation	14.2	84.9	98.8	1.5	99.2	100.6	Pass

Table 5: LOD and LOQ concentration of analytes along with S/N ratios

S. No	Name	LOQ conc. (µg/ml)	s/n ratio	LOD conc. (µg/ml)	s/n ratio
1	Impurity-E	0.095	10.3	0.029	3.1
2	Impurity-F	0.110	11.5	0.033	3.8
3	Impurity-A	0.045	10.6	0.014	3.0
4	Impurity-H	0.105	9.8	0.032	2.7
5	Adenine	0.140	12.2	0.042	3.8
6	Tenofovir impurity	0.060	10.4	0.018	3.2
7	Lamivudine	0.160	11.1	0.048	3.6
8	Mono ester impurity	0.085	10.3	0.026	3.1
9	Di ethyl impurity	0.105	11.5	0.032	3.8
10	Isopropyl impurity	0.065	10.6	0.020	3.0
11	Tenofovir disoproxil	0.115	9.8	0.035	2.7
12	n-propyl impurity	0.170	12.2	0.052	3.0
13	Tenofovir dimer impurity	0.045	10.4	0.014	3.2

n=1, LOD = Limit of detection and LOQ = Limit of quantification and Linearity, range and relative response factor

A series of solutions of lamivudine, tenofovir and all impurities solutions with concentrations ranging from LOQ to 150 % of specification level (impurities specification not more than 0.2 %) were prepared and injected into the HPLC system. The linearity of the method was established by plotting a graph between the concentration and response of lamivudine, tenofovir and all impurities. The results of the linearity study are presented in table 6. The results in table 6 indicate that the detector response was found to be linear from LOQ to 150 % of specification level and squared correlation coefficient (r^2) is more than 0.990 confirms the linearity the method. The relative retention factor (RRF) is critical for quantification of impurities. Establishment of the RRF is required to avoid the stability issues with impurity standards, to reduce the cost on the preparation of impurity standards, to reduce maintenance of impurity standards due to the lack of donation of impurity standards and difficulty in the synthesis and isolation of impurity standards. RRF is used to correct the difference in detector response of impurities with analyte peak. RRF is established by the linearity slope method with a linear range of solutions and corresponding RRF values of this method is summarized in table 6.

Table 6: Linearity and RRF values of lamivudine, tenofovir and all impurities

S. No	Name	Correlation coefficient	Y-intercept at 100% level	RRF
1	Impurity-E	0.999	-1.2	1.02
2	Impurity-F	0.992	0.8	1.12
3	Impurity-A	0.995	-2.5	0.98
4	Impurity-H	0.991	1.8	1.15
5	Adenine	0.999	-2.1	1.31
6	Tenofovir impurity	0.998	1.8	1.21
7	Lamivudine	0.995	3.1	1.00
8	Mono ester impurity	0.996	-1.8	1.12
9	Di ethyl impurity	0.995	2.5	0.95
10	Isopropyl impurity	0.993	1.3	0.92
11	Tenofovir disoproxil	0.999	3.6	1.00
12	n-propyl impurity	0.994	-3.8	1.16
13	Tenofovir dimer impurity	0.996	-4.1	1.01

n=1, RRF = Relative retention factor

Accuracy

The accuracy of an analytical method is an expression of the agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found. The accuracy of the method is established in terms of recovery. Sample solutions for accuracy study were prepared in triplicate by spiking all impurities at the specification level (Not more than 0.2 % of each impurity) to the test sample at LOQ, 50 %, 100 % and 150 % of the specification level and injected into the HPLC system. Individual % recovery, mean % recovery and % RSD at each level are presented in table 7. The recovery of samples was found to be within the range of 90 % to 110 % and agreement with the ICH guidelines.

Table 7: Accuracy results of lamivudine, tenofovir and all impurities

S. No	Name	% recovery of triplicate samples				% mean of all levels±SD	% RSD of all levels
		LOQ level	50% level	100% level	150% level		
1	Impurity-E	98.5	102.6	100.8	96.2	99.5±2.8	2.8
2	Impurity-F	105.6	99.8	103.6	101.2	102.6±2.6	2.5
3	Impurity-A	108.2	103.2	101.9	100.9	103.6±3.2	3.1
4	Impurity-H	104.6	99.6	107.5	105.1	104.2±3.3	3.2
5	Adenine	108.2	105.3	104.9	100.5	104.7±3.2	3.0
6	Tenofovir impurity	95.6	100.6	100.9	106.4	100.9±4.4	4.4
7	Lamivudine	99.3	102.1	103.1	103.1	101.9±1.8	1.8
8	Mono ester impurity	103.5	101.6	102.3	104.9	103.1±1.4	1.4
9	Di ethyl impurity	106.8	99.1	106.1	100.2	103.1±4.0	3.8
10	Isopropyl impurity	108.3	103.8	97.2	103.5	103.2±4.6	4.4
11	Tenofovir disoproxil	96.1	105.1	99.2	102.3	100.7±3.9	3.9
12	n-propyl impurity	94.6	101.5	103.9	106.2	101.6±5.0	4.9
13	Tenofovir dimer impurity	102.6	105.2	101.1	100.3	102.3±2.2	2.1

n=3 at each level, SD = Standard deviation and % RSD = % Relative standard deviation

Method precision and intermediate precision

To evaluate the method precision for related substances, six replicates test preparations (n=6) of lamivudine and tenofovir tablets were prepared and spiked all individual known impurities at the specification level (0.2 % of drug substance) and analyzed as per method. The % of individual known impurities and % of total impurities were calculated and reported in table 8. The % RSD values were found to be below 8.9 for all impurities and found to be more precise as per the ICH guidelines. The intermediate precision of the method was evaluated by adopting the same method by using a different HPLC system, the different column of the same make at different days and the results are tabulated in table 9. The % RSD values were found to be below 7.9 for all impurities and found to be method is more ruggedness.

Table 8: Method precision results

S. No.	Name	Impurity (µg/ml)						% mean ±SD	% RSD
		Spl-1	Spl-2	Spl-3	Spl-4	Spl-5	Spl-6		
1	Impurity-E	1.05	1.00	0.90	1.10	0.95	1.05	1.01±0.07	7.3
2	Impurity-F	0.95	1.10	0.95	0.90	1.00	1.10	1.00±0.08	8.4
3	Impurity-A	1.05	0.95	1.00	1.05	0.95	1.00	1.00±0.04	4.5
4	Impurity-H	1.10	1.00	1.05	0.95	1.05	1.00	1.03±0.05	5.1
5	Adenine	1.05	1.00	1.10	1.10	1.00	1.05	1.05±0.04	4.3
6	Tenofovir impurity	1.00	0.95	1.00	0.95	0.90	0.95	0.96±0.04	3.9
7	Monoester impurity	5.10	5.50	5.25	5.30	4.95	5.40	5.25±0.20	3.8
8	Di ethyl impurity	1.05	1.00	0.90	1.00	0.95	1.05	0.99±0.06	5.9
9	Isopropyl impurity	1.60	1.50	1.55	1.45	1.40	1.65	1.53±0.09	6.1
10	n-Propyl impurity	1.10	1.05	1.00	1.05	1.00	0.95	1.03±0.05	5.1
11	Tenofovir dimer impurity	1.05	0.95	1.00	0.95	0.90	1.15	1.00±0.09	8.9
12	Total impurities	16.10	16.00	15.70	15.80	15.05	16.35	15.83±0.45	2.8

Table 9: Intermediate precision results

S. No.	Name	Impurity (µg/ml)						% mean ±SD	% RSD
		Spl-1	Spl-2	Spl-3	Spl-4	Spl-5	Spl-6		
1	Impurity-E	1.10	0.90	1.10	1.05	1.00	1.05	1.03±0.08	7.1
2	Impurity-F	1.05	0.95	1.00	1.10	0.95	1.10	1.03±0.07	7.3
3	Impurity-A	1.05	1.00	1.10	0.95	0.90	1.00	1.00±0.07	5.6
4	Impurity-H	0.95	1.10	0.95	1.00	1.10	1.05	1.03±0.07	5.7
5	Adenine	1.00	1.15	0.95	1.10	0.95	1.10	1.04±0.09	6.3
6	Tenofovir impurity	1.05	1.10	1.00	1.05	0.90	1.15	1.04±0.09	7.7
7	Mono ester impurity	5.50	5.40	5.60	5.25	5.50	6.05	5.55±0.27	5.1
8	Di ethyl impurity	0.90	1.00	1.05	0.90	0.95	1.00	0.97±0.06	5.9
9	Isopropyl impurity	1.65	1.55	1.60	1.55	1.50	1.60	1.58±0.05	5.0
10	n-Propyl impurity	1.05	1.05	0.95	1.00	1.15	1.05	1.04±0.07	5.6
11	Tenofovir dimer impurity	1.05	1.15	1.05	1.00	1.10	0.95	1.05±0.07	7.9
12	Total impurities	16.35	16.35	16.35	15.95	16.00	17.10	16.35±0.41	2.5

Spl = Sample

Robustness

The robustness of the method was studied by injecting the blank and standards with deliberate changes in flow rate of the mobile phase (±0.1 ml/min), column temperature (±5 °C) and pH of the mobile phase buffer (±0.2). The results of the robustness are presented in table 10. Results tabulated in table 10 showed that method is not affected by intentional changes in the method parameters which was proved by tailing factors of standards are less than 2 and % RSD of six replicate standards is less than 2.

Table 10: Robustness results

S. No.	Robustness parameter	Lamivudine			Tenofovir		
		% RSD	Tailing factor	Theoretical plates	% RSD	Tailing factor	Theoretical plates
1	Low flow (0.9 ml/min)	1.2	1.0	12451	0.8	1.1	13512
2	High flow (1.1 ml/min)	1.0	1.1	10147	1.1	1.0	13602
3	Low buffer pH (4.8)	0.8	1.0	11458	0.2	1.1	12915
4	High buffer pH (5.2)	0.4	1.2	10987	0.1	1.0	13025
5	Low column temp. (30 °C)	1.1	1.0	12142	1.5	1.0	12854
6	High column temp. (40 °C)	0.5	1.1	11411	1.2	1.1	12965

n = 1

Stability of analytical solutions

To evaluate the stability, standard solution and the spiked sample solution were prepared and kept on the bench top (25 °C) and refrigerator (2-8 °C) conditions for 24 h. Similarity factor was calculated for a standard solution with a freshly prepared standard solution and the % of the difference of individual impurities and total impurities were calculated with the initial results. It was concluded that the standard solution is stable up to 24 h in bench top and refrigerator conditions. Sample solutions were unstable at bench top and refrigerator. Hence, samples were prepared freshly and injected immediately.

CONCLUSION

A specific, linear, precise and more accurate stability indicating high performance liquid chromatographic method has been developed for the quantification of 11 related substances including the tenofovir dimer impurity. The method has been validated for specificity, linearity, accuracy, precision, robustness and stability. This method is able to quantify all the impurities in the presence of other impurities and the main drug with less than 0.03 % of the drug concentration which is far times less than the specification level concentration (0.2 %). The method is linear in the range of LOQ to 150 % of the specification concentration for all the impurities with a correlation coefficient not less than 0.992. The accuracy of the method is in the range of 94.6 % to 108.3 % for all impurities. As the method is validated according to the International council of harmonization (ICH) guidelines, it could be adopted for the analysis of all the related substances in the dosage forms of lamivudine and tenofovir in quality control labs, pharmaceutical industries and research laboratories.

CONFLICTS OF INTERESTS

Declared none

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