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Research Article

STABILITY-INDICATING METHOD DEVELOPMENT AND VALIDATION OF LAMIVUDINE AND TENOFOVIR DISOPROXIL FUMARATE BY USING RP-HPLC

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Abstract:

A novel rapid, sensitive and reproducible stability-indicating RP-HPLC method has been developed and validated for quantitative analysis of Lamivudine (LAM) and Tenofovir disoproxil fumarate (TDF) in the bulk drug and in a pharmaceutical dosage form. Use of Thermo C_{18} analytical column (250mm×4.6 mm, 5.0µm) with 20mM KH_2PO_4: methanol 30:70% v/v as isocratic mobile phase enabled separation of the drug from its degradation products. UV detection was performed at 270nm. The method was validated for linearity, accuracy (recovery), precision, specificity and robustness. The linearity of the method was satisfactory over the range 5-25µg/ml (correlation coefficient 0.999 for LAM and 0.998 for TDF). The limits of detection and quantification of LAM and TDF were 0.45, 0.35 and1.35, 1.15µg/ml respectively. Recovery of LAM and TDF from the pharmaceutical dosage form ranged from 98.75-99.87 and 98.63-99.30% respectively. LAM and TDF were subjected to stress conditions (hydrolysis (acid, base), oxidation and thermal degradation). Samples were analyzed by this method. Extensive degradation of LAM was found under acid and alkaline hydrolysis. The degradation products were well resolved from main peak. The forced degradation study prove the stability indicating power of the method and therefore the validated method may be useful for routine analysis of LAM and TDF in bulk drug and respective dosage forms for dissolution studies and as stability indicating assay method in pharmaceutical laboratories and industries.

Keywords: Lamivudine, Tenofovir disoproxil Fumarate, RP-HPLC, Method validation, Forced degradation

Introduction

Products formulated with more than one active pharmaceutical ingredient (API), typically referred to as fixed-dose combinations (FDCs) are intended to fulfill unmet patients' needs by combining the therapeutic effects of two or more drugs in one product. These combinations products can present daunting challenges to the analytical chemist responsible for the development and validation of analytical methods [1,2]. Lamivudine/Tenofovir disoproxil fumarate formulation is one such FDC used for the management of Human Immunodeficiency Virus. Stability testing forms an important part of the process of drug product development. The purpose of stability testing is to provide evidence on how the quality of a drug substance or drug product varies with time under a variety of environmental conditions for example temperature, humidity, light, and enables storage conditions and shelf life to be recommended [3-6]. The two main aspects of study of the stability of a drug product that play an important role in shelf life determinations are assay of the active drug and the degradation products generated during stability studies.

Assay of a drug product in a stability test sample must be performed with stability-indicating method, as recommended by the international Conference on Harmonization (Q2R1 & Q2R2) [7]. Lamivudine (LAM Fig. 1B) (4-amino-1-[(2R, 5S)-2-(hydroxymethyl)-1, 3-oxathiolan-5-yl]-1, 2- dihydropyrimidin-2-one) is nucleoside analogues with a structure that consists of a pyrimidine base which is N-substituted at the 1-position with a 3'-thia derivative (1, 3-oxazolidine) of the ribose moiety that is characteristic of nucleosides. It is reverse trascriptase inhibitor and zalcitabine analog in which a sulfur atom replaces the 3' carbon of the pentose ring. It is used to treat HIV-1 and hepatitis B (HBV). This compound belongs to the class of organic compounds known as 3'-thia pyrimidine nucleosides [8]. Tenofovir disoproxil Fumarate (TDF Fig. 1A) (a prodrug of tenofovir) ({[(2R)-1-(6-amino-9H-purin-9-yl)propan-2-yl]oxy}methyl) phosphonic acid), belongs to a class of antiretroviral drugs known as nucleotide analogue reverse transcriptase inhibitors (nRTIs) which block reverse transcriptase, an enzyme crucial to viral

production in HIV-infected people. *In vivo* TDF is converted to tenofovir, an acyclic nucleoside phosphonate (nucleotide) analog of adenosine 5'-monophosphate [9]. Both the drugs are marketed as (TENVIR-L) combined dosage form in tablet formulation with ratio of 300:300mg LAM: TDF. The literature survey suggests UV method [10] and HPLC method [11, 12] for LAM and RP-HPLC method [13, 14] and HPTLC [15] for TDF. TDF is also determined in plasma [16]. This manuscript describes the development and validation in accordance with ICH guidelines (Q2B) [17] of a rapid, economical, precise and accurate stability-indicating isocratic reversed phase HPLC method for analysis of LAM and TDF in the presence of its degradation products. This paper mainly deals with the forced degradation of LAM and TDF under the stress conditions such as acidic and basic hydrolysis, oxidation, heat, and light and validation of the method for accurate quantification of LAM and TDF in the bulk drug and solid dosage form.

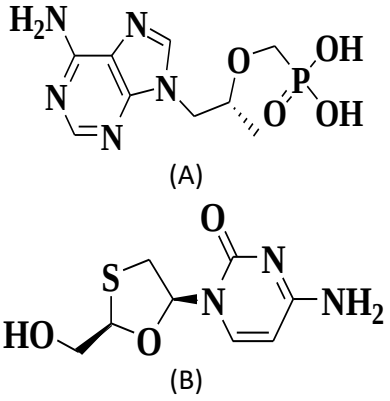

Figure 1: Chemical structure of (A) Tenofovir (B) Lamivudine

EXPERIMENTAL

Materials

Standards of pharmaceutical grade LAM and TDF were obtained as gift samples from Scan Research Laboratories, Bhopal. The tablet dosage form TENVIR – L manufactured by Cipla Limited, Mumbai, India (Label Claim: LAM 300 mg and TDF 300 mg) was procured from the local pharmacy. HPLC grade methanol, water and acetonitrile was obtained from Merck (India) limited. Potassuim Dihydrogen orthophosphate and Ortho-phosphoric acid (AR grade) was obtained from Hi Media Laboratories Private Limited, Mumbai, India. All other chemical used were of HPLC grade.

Instrument

Liquid chromatographic system from Waters model no 784 comprising of manual injector, water 515 pump for constant flow and constant pressure delivery and UV-Visible detector (Detection limit 170 nm to 700 nm) connected to software Data Ace for controlling the instrumentation as well as processing the generated data.

Chromatographic conditions

Chromatographic conditions performed at ambient temperature, the isocratic mobile phase consisted of 20mM KH_2PO_4: methanol 30:70% v/v, flowing through the column at a constant flow rate of 1.0 ml/ min. The mobile phase was filtered and degassed before use (30 min). A Thermo (C-18) Column (5 µm, 250mm x 4.60mm) was used as the stationary phase. By considering the chromatographic parameter, sensitivity and selectivity of method for drugs, 270 nm was selected as the detection wavelength for UV-Visible detector.

Selection of Diluent

Diluent used for preparation of sample were compatible with mobile phase and no any significant affect on retention and resolution of analyte. After various trials methanol was used as diluents.

Standard preparation

Standard stock solution

Accurately weighed 10 mg API of TDF and LAM was transferred into 10 ml volumetric flask separately and added 5ml of methanol as diluents, sonicated for 25 minutes and volume was made up to 10ml with methanol to get concentration of solution 1000µg/ml (Stock-A), 5ml of stock-A was taken and diluted up to 50ml with methanol to get concentration of 100µg/ml (Stock-B).

Working standard solution

Working standard solutions were prepared by taking dilutions ranging from 5-25 µg/ml for TDF and LAM.

Assay of Tablet Formulation

Assay was performed according to Sonawane et al. with slight modification [18], twenty tablets were accurately weighed and their mean weight was determined. The tablets were grinded to fine powder, an accurately weighed quantity of powder equivalent to 10 mg of

Lamivudine and 10 mg of Tenofovir was transferred to 10 ml volumetric flask containing methanol. The solution was sonicated for 25 min and the final volume was made with mobile phase. The mixture was then filtered through a 0.45 µm filter. The stock solution was further diluted sufficiently with methanol to get sample solution of drug concentration of 10 µg/mL LAM and 10 µg/mL TDF respectively. The amounts of LAM and TDF in tablets formulation were calculated by extrapolating the value of area from the calibration curve. Analysis procedure was repeated three times with formulation.

Method validation

The method was validated for linearity, specificity, limits of detection (LOD) and limits of quantification (LOQ), system suitability, accuracy, precision, robustness and stability in accordance with ICH guidelines. To assess **specificity**, peak purity was determined by use of the UV vis. detector. To check linearity, test solutions of LAM and TDF were prepared at five concentrations 5-25µg/ml. Each solution was injected in triplicate and calibration graphs were obtained by plotting peak area against concentration. **Linearity** was checked over the same concentration range on three consecutive days. RSD (%) of the slope and Y-intercept of the calibration plot were also calculated. **The limits of detection (LOD)** and **limits of quantification (LOQ)** for LAM and TDF were determined that is based on slope and standard deviation of the response using signal-to-noise ratios 3:1 and 10:1 respectively by injecting a series of dilute solutions of known concentration. **Precision**, as RSD (%) was determined by measuring the concentration of drug in the injection six times. Intermediate (inter-day) precision was evaluated by two analysts on different days in the same laboratory. The **accuracy** of the method was studied by measurement of recovery after adding known amounts of the drug (80, 100 and 120% of the label claim of known amount of LAM and TDF per injection) to the placebo. Three samples were prepared for each recovery level and results were calculated by use of the calibration plot. The **robustness** of the method was assessed by deliberate alteration of the experimental conditions and determining the effect on resolution of LAM and TDF from the main product obtained by degradation under basic conditions. The change was made by altering the pH and / or concentration of the mobile phase to check the method capacity to remain unaffected. The effect of change in pH of mobile phase, flow rate, mobile phase ratio on the retention time, theoretical plates, area under curve and percentage content of LAM and TDF was studied. During these tests all other conditions were held constant at the optimum values.

FORCED DEGRADATION STUDIES

According to ICH guidelines (Q1A) stability testing is necessary to classify the stability characteristics of active ingredients. In order to determine whether the method is stability indicating, forced degradation studies were conducted on drug powder and the analysis was carried out by RP-HPLC with a U.V. detector. 20µl of each of forced degradation samples were injected.

Acid degradation

50 mg of both the drug sample was taken into a 50 ml separate round bottom flask, 50 ml of 0.1 N HCl solution was added and contents were mixed well and kept for constant stirring for 8 h at 40ºC. Samples were withdrawn and diluted to get 10 µg/ml subjected to HPLC and calculate the percentage degradation using calibration curve of drugs.

Alkaline hydrolysis

50 mg of the drug sample was taken into a 50 ml separate round bottom flask, 50 ml of 0.1N NaOH solution was added and contents were mixed well and kept for constant stirring for 2 h at 40ºC. Samples were withdrawn and diluted to get 10 µg/ml subjected to HPLC and calculate the percentage degradation using calibration curve of drugs

Oxidative degradation

50 mg of the drug sample was taken into a 50 ml separate round bottom flask, 50 ml of 0.2% hydrogen peroxide solution was added, and contents were mixed well and kept for constant stirring for 24 hr at 40ºC. Samples were withdrawn and diluted to get 10 µg/ml subjected to HPLC and calculate the percentage degradation using calibration curve of drugs.

Thermal degradation

50 mg of the drug sample was taken in to a petri dish and kept in oven at 75ºC for 4 weeks. Samples were withdrawn and diluted to get 10 µg/ml subjected to HPLC and calculate the percentage degradation using calibration curve of drugs.

Results and Discussion

Optimization of chromatographic conditions

The primary objective in developing this stability indicating HPLC method was to achieve resolution between LAM, TDF and its degradation products. To optimize the RP-HPLC parameters, several mobile phases of different compositions were tried. A satisfactory separation, good peak symmetry and best resolution was obtained with a mobile phase consisting of 20mM KH_2PO_4: methanol 30:70% v/v, mobile phase was attempted for quantitation of LAM, TDF with acceptable system suitability parameters (RT, tailing factor, number of theoretical plates and HETP) at 270 nm as detection wavelength. The column temperature was 25°C. The tailing factor for TDF and LAM was < 2 and retention times were approximately 1.980 ± 0.2 and 8.980± 0.4min for TDF and LAM and less than 10 min for the degradation products. This low total runs time resulted in high productivity and low cost of analysis as per sample.

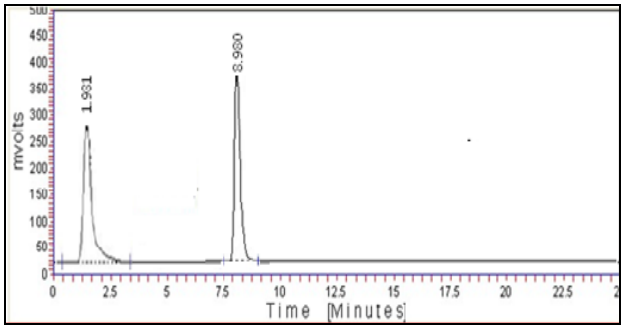

Figure 2: Standard chromatogram of TDF and LAM

Method validation

Peak purity was more than 99.9% for drug substance and drug degradation products at 270nm which showed that the analyte peaks were pure and that formulation excipients and degradation products were not interfering with analyte peaks. LOD and LOQ for LAM and TDF were 0.45, 0.35 and 1.35, 1.15µg/ml respectively for 20 µl injection volumes. Results from regression analysis are listed in table 1 with system suitability data. When precision was determined by six fold analysis of drug injection, the RSD of LAM and TDF peak area was less than 2% indicating that the method is reliable. Results from assessment of precision are listed in table 2. Results obtained from determination of recovery are listed in table 3 and results from robustness and analysis of formulation were shown in table 4 & 5 respectively.

Table 1: Results from regression analysis and system suitability data

Parameters	TDF	LAM
Retention time*	1.980 ± 0.2min	8.980± 0.4min
Tailing factor*	1.340±0.159	1.195±0.059
Theoretical plate*	2866.667±63.12862	3148.5±78.41928
Linear range (µg/ml)	5-25	5-25
LOD (µg/ml)	0.35	0.45
LOQ (µg/ml)	1.15	1.35
Linear equation	y=28.80 x+1.912	y=36.50 x+3.156
Slope	28.80	36.50
Intercept	1.912	3.156
Correlation coefficient (r^2)	0.998	0.999

*Mean of six readings

Table 2: Results of precision

	LAM	TDF
Repeatability	99.584±0.124	99.342±0.240
Intermediate precision		
Day to day precision	99.356±0.086	99.542±0.071
Analyst to Analyst	99.336±0.065	99.403±0.074

***** Value of five replicate and five concentrations

Table 3: Results of recovery study

% LEVEL	% MEAN±SD*	
	LAM	TDF
80%	98.759±2.194	98.639±2.393
100%	99.644±0.559	99.300±0.590
120%	99.873±0.156	99.259±0.485

***** Value of three replicate and three concentrations.

Table 4: Results of robustness

	TDF*	LAM *
Robustness	99.343±0.103	99.539±0.054

***** Value of five replicate and five concentrations

Table 5: Analysis of tablet sample

	TDF*	LAM *
Label Claim (mg)	300mg	300mg
% Found (mg)	299.89	299.30
% Assay	99.978	99.30
% RSD	0.125	0.225

*Average of three determination

Forced degradation study

Bakshi *et al.* [19] suggested target degradation of 20-80% when establishing the stability-indicating properties of analytical methods because even intermediate degradation products should not interfere with any stage of drug analysis. Although conditions used for forced degradation were adjusted to achieve degradation in this range, this could not be achieved for conditions other than exposure to acid, base and oxidising agent, even after long exposure. Peak purity test results confirmed that the LAM, TDF peak was homogeneous under all the stress conditions tested. Assay of unaffected LAM, TDF in the injection confirmed the stability-indicating nature of the method. The results from forced degradation studies are summarized in Table 6. No degradation peaks co-eluted with the LAM, TDF peak, suggesting the method enabled specific analysis of LAM, TDF in the presence of its degradation products.

Table 6: Results of Forced degradation studies

	LAM		TDF	
Stress conditions	**Drug recovered (%)**	**Drug decomposed (%)**	**Drug recovered (%)**	**Drug decomposed (%)**
Standard drug	99.85	0	99.95	0
Acidic hydrolysis	83.26	16.74	92.23	7.77
Alkaline hydrolysis	82.23	17.77	94.56	5.44
Oxidative degradation	90.23	9.77	85.56	14.44
Thermal degradation	94.45	5.55	83.32	16.68

Conclusion

The method developed for quantitative analysis of LAM and TDF is rapid, precise, accurate and selective. Peak purity studies under all the stress conditions showed the drug peak to be pure. The developed method can be utilized for the successful quantification of the drug in presence of its degradation product and excipients. The method was completely validated and results were satisfactory. The method can be conveniently used for routine analysis and stability of LAM and TDF in bulk drug and in respective dosage forms.

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