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NEW STABILITY INDICATING HPLC METHOD FOR SIMULTANEOUS ESTIMATION OF LAMIVUDINE, TENOFOVIR DF AND NEVIRAPINE IN EXTENDED RELEASE TABLETS

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ABSTRACT

New stability-indicating reverse phase LC method developed and validated for the simultaneous estimation of Lamivudine, Tenofovir DF and Nevirapine in extended release tablet dosage form. Tenofovir and Lamivudine are formulated into immediate release and Nevirapine into extended relase. The chromatographic conditions were optimized using an impurity-spiked solution and the samples generated from forced degradation studies. The chromatographic separation was achieved on a core shell technology C18 stationary phase. The method employed a linear gradient elution and the detection wavlength was set at 260 nm. The mobile phases consists of buffer and acetonitrile delivered at a flow rate of 0.7 mL·min–1. Proposed method was extensively validated as per ICH guidelines. Regression analysis shows an r value (correlation coefficient) of greater than 0.999 for individual active drug substances. The samples were assayed against a qualified reference standard and the mass balance was found to be close to 98.3%.

Keywords: Tenofovir DF, Lamivudine, Nevirapine, HPLC, Simultaneous estimation and Stability indicating.

INTRODUCTION

Lamivudine is reverse transcriptase reported to be active against HIV-1, HIV-2 and hepatitis B virus. Lamivudine, chemically 4-amino-1-[(2R, 5S)-2-(hydroxyl methyl)-1, 3-oxathiolan-5-yl]-1,2-dihydropyrimidin-2-one. It is asynthetic nucleoside analogue and is phosphorylated intracellularly to its active 5'- triphosphate metabolite, lamivudine triphosphate (L-TP). This nucleoside analogue is incorporated into viral DNA by HIV reverse transcripase an HBV polymerase, resulting in DNA chain termination [1,2]. Tenofovir disoproxil fumarate is a fumaric acid salt of the bisisopro poxycarbonyl oxymethyl ester derivative of tenofovir. The chemical name of tenofovir disoproxil fumarate is 9-[(R)-2 [[bis [[(isopropoxycarbonyl) oxy]-methoxy] phosphinyl] methoxy] propyl] adenine fumarate (1:1). Tenofovir disoproxil fumarate is a salt of an oral prodrug of tenofovir. Tenofovir disoproxil was developed to increase bioavailability because tenofovir was not well absorbed from the intestine. TDF is the first nucleotide analog approved for HIV-1 treatment [3-4]. Nevirapine falls in the non-nucleoside reverse transcriptase inhibitor (NNRTI) class of antiretrovirals [5]. Nevirapine chemically known as 11-cyclopropyl-4-methyl-5,11-dihydro-6*H*-dipyrido[3,2-*b*:2′,3′-*e*][1,4]diazepin-6-one.

A literature survey reveals that analytical methods based on HPLC [6-25], HPTLC [26-28], UV Spectrometry [29-32] are available for the determination of these drugs individually and in combination with other drugs in different dosage forms, there is no analytical method reported for the simultaneous estimation of Lamivudine, Tenofovir and Nevirapine in a Combined Dosage Form. The aim of the present work is develop and validate as per ICH [33] a new simple, precise, accurate, and rapid method for the determination of Lamivudine,

Tenofovir and Nevirapine in a Combined Dosage Form.

MATERIALS AND METHODS

Chemicals and Reagents: All the reagents were of ACS or HPLC grade unless stated otherwise. Milli-Q-water was used throughout the experiment. 1-octane sulphonic acid sodium salt (Merck, Mumbai, India) orthophosphoric acid (Merck, Mumbai, India), Methanol (J.T.Baker, Germany) and acetonitrile (J.T.Baker, Germany), were used. Lamivudine, Tenofovir disoproxil fumarate, Nevirapine standards, related impurity standards and extended release tablet dosage form were obtained from Hetero Labs Ltd (Hyderabad, India). Lamivudine resolution mixture (B) from USP, USA.

Instrumentation: The HPLC system was composed of 2695 water alliance system fitted with 2996 PDA detector with Empower2 software. Analytical column used for this method is Phenomenex Kinetex C18, (100 mm x 4.6 mm) 2.6µm particle size.

Optimization of Chromatographic conditions: The analysis was carried out on Phenomenex Kinetex, (100 mm x 4.6 mm) 2.6µm particle size, column maintained at 35°C. The mobile phase A consists of 1-octane sulphonic acid sodium salt (3gm per Litre) pH adjusted to 2.6±0.05 with dilute ortho phosphoric acid and acetonitrile is used as Mobile phase B. Flow rate was set of 0.7 mL/min in gradient elution mode. Gredient time program as set as T/%B: 0/20, 7/80, 10/80, 10.5/20 and 15/20. Before delivering the mobile phase into the system, it was degassed and filtered through 0.22 µm PVDF filter using vacuum. The injection volume was 10µL and the detection was performed at 260 nm using a photo diode array (PDA) detector. Various compositions of solution A and solution B with different ion-pairing agents were tested for this study. The typical retention times of Lamivudine, Nevirapine and Tenofovir are 3.5 minutes, 4.5minutes and 6 minutes respectively. The counter ion fumaric acid is also found to be eluting at 1.8minutes. The criticality of this method are to elute all the active ingredients with optimum separation and symmetric peak shapes with no interference due to placebo or any potential impurities arising due to degradation or during shelf life. In this dosage form Tenofovir and Lamivudine are formulated into immediate release (each 300mg per tablets) and Nevirapine as extended release (400mg per tablet). Two different duiluents are used, initially with methanol to achieve complete extraction of Nevirapine from extended release part of tablet and dilute orhto phosphoric acid for sharp and symmetric peak shapes.

Preparation of Mobile phase A: Transferred 3g of sodium 1-octane sulphonic acid into 1000mL of water. Contents were solubilized by ultra-sonication, pH of the solution adjusted to 2.6±0.05 with dilute ortho phosphosphoric acid. The resultant solution was filtered through 0.22µm membrane filter under vaccum.

Standard solution preparation: Accurately weighed and transferred about 75mg each of Lamivudine working standard and Tenofovir Disoproxil Fumarate working standard and 100mg of Nevirapine working standard into a 100mL volumetric flask, added about 60mL of methanol and sonicated to dissolve. Diluted to volume with methanol and mixed. Transferred 3.0mL of above solution into a 25mL volumetric flask, diluted to volume with
diluent and mixed.

Resolution solution Preparation: Accurately weighed and transferred about each 5 mg of Resolution mixture-B USP RS and 30 mg of Lamivudine working standard into a 50 ml volumetric flask. Added about 5 ml of methanol and sonicated to dissolve. Diluted to volume with diluent and mix.

Sample Preparation: Ten tablets were separately weighed and grounded to fine powder. An amount of powder (this powder contains 300mg each of lamivudine and Tenofovir DF and 400mg of Nevirapine) equivalent to about 30mg of Lamivdine was transferred into a 50mL volumetric flask and dissolved by ultra sonication for 20minutes in 30mL quantity of methanol. Made up the volume with methanol and mixed. A portion of the above solution was centrifuged to affect separation of colloidal materials, at about 5000rpm, then supernatant solution was filtered through 0.22µm membrane filter and discarded first few mL of the filtrate.

RESULTS AND DISCUSSION

Optimum separation between all the three active ingredients and potential degradation impurities was achieved with optimized conditions. The pharmaceutical formulation along with individual active ingredients was subjected to stress conditions of hydrolysis (acid and base), oxidation and thermal degradation as per International Conference on Harmonization (ICH) prescribed stress conditions to show the stability-indicating power of the method. It was found Tenofovir disoproxil fumarate is very

sensitive to various stress conditions and readily degrades into Mono-POC impurity. The chromatographic conditions were optimized using an impurity-spiked solution and the samples generated from forced degradation studies.

Method validation

The aim of method validation was to confirm that the present method was suitable for its intended purpose as described in ICH guidelines Q2 (R1)[33]. The described method has been extensively validated in terms of specificity, precision, linearity, accuracy and robustness. The precision was expressed with respect to the intra- and inter-day variation in the expected drug concentrations. The accuracy was expressed in terms of percent recovery of the known amount of impurities added to the sample preparation.

System suitability: System suitability tests are an integral part of a liquid chromatographic method, and they were used to verify that the proposed method was able to produce good resolution between the peaks of interest with high reproducibility. The system suitability was determined by injecting resolution solution and six replicate injections from freshly prepared standard solutions and analyzing each solute for their peak area, theoretical plates (N), resolution (R) and tailing factors (T). System suitability requirements for the proposed method are (i) the resolution (R) between Lamivudine dia steriomer and Lamivudine should not be less than 2.0, from resolution solution (ii) the theoretical pates (T) should not be less than 3000 for all peaks from standard solution, (iii) the % of RSD for peak areas of Lamivudine, Tenofovir DF and Nevirapine peaks from replicate injections of standard solutions is not more than 2.0. The results of the system suitability test in comparison with the required limits are shown in **Table 1**. According to the results presented, the proposed method fulfills these requirements within the accepted limits.

Specificity: Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Typically these might include impurities, degradants, matrix(placebo), etc. Specificity was tested by injecting the sample by spiking with appropriate levels of impurities and demonstrating the separation of these impurities individually and/or from other components in the sample matrix.

Forced degradation studies: Forced degradation studies were performed to provide an indication of the stability indicating property and specificity of the proposed method. Intentional degradation was attempted to stress conditions like acid hydrolysis (using 1 N HCl), base hydrolysis (using 0.1 N NaOH), and oxidative degradation (using 3.0% H_2O_2) to evaluate the ability of the proposed method to separate degradation products from each other and active ingredients as well. To check and ensure the homogeneity (peak purity) of peaks in the stressed sample solutions, photo diode array detector was employed. In forced degradation it was observed that tenofovir is susceptible for degradation in acid and base stress conditions, where as lamivudine susceptible for peroxide stress condition and nevirapine is found to be stable in all the three stress conditions. Results are tabulated in ***Table No.2***

Linearity: The linearity of the method was tested in order to demonstrate proportional relationship of response versus analyte concentration over the working range. It is usual practice to perform linearity experiments over a wide range of analyte. This gives confidence that the response and concentration are proportional and consequently ensures that calculations can be performed using a single reference standard/working standard, rather than the equation of a calibration line. The linearity of detector response to different concentrations of all three active ingredients was studied by preparing a series of solutions using Lamivudine and Tenofovir disoproxil fumarate and Nevirapine. The data were subjected to statistical analysis using a linear-regression model; the regression equations and coefficients (r^2) are given in ***Table 3***. The results have indicated good linearity.

Precision: Six sample solutions were prepared using single sample Lot of tablet dosage form of Lamivudine, Tenofovir DF and Nevirapine extended release tablets and the precision of the method was tested. The % RSD indicates that proposed method has got acceptable level of repeatability.

Ruggedness (Intermediate precision): Ruggedness is the intraday variation obtained at different concentration levels, and is expressed in terms of RSD calculated for each day. The RSD values were found to be below 0.45% (for all three active inredients). The intermediate precision is the interday variations calculated for six sample preparations in each set expressed in terms of % RSD values. Results indicate the proposed method has got a good intermediate precision. The ruggedness of the method was determined by analyzing the same samples in triplicate for 2 days by another instrument by a different analyst with different lots of reagents and columns. Results are tabulated in ***Table No.4***

Accuracy: Accuracy of the proposed method was established by recovery experiments. This study was employed by spiking of known amounts of active ingredients into the placebo samples of at 50%, 100% and 150% of tergetted concentration, in triplicate and injected into the chromatographic system. The resulting mixtures were analyzed as described in proposed method. Results obtained from recovery studies are given in **Table 5**.

Robustness: The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate, variations in method parameters, and provides an indication of its reliability during normal usage. In the present study, an experimental design was planned for robustness testing varying some conditions, e.g. Flow rate, column temperature, variation of buffer pH in the mobile phase and filter variability. The results are shown in **Table 6**. It can be seen that, with every employed condition, there were no dramatic changes in the chromatographic behavior. All parameters have been observed within the limits required for system suitability tests.

Stability of Analytical solutions: The stability of the resolution, standard and sample solutions is tested at regular intervals. The stability of solutions was determined by comparing results with freshly prepared standard solutions. The differences in values were within 0.2% upto 48hrs.

CONCLUSION

The validated stability-indicating HPLC method has proved to be simple, accurate, precise and reliable. The proposed method provides a good resolution between all the three active ingredients and potential degradants. The developed method reported herein was validated by evaluation of the validation parameters as described in ICH guidelines. System suitability, specificity, linearity, precision, accuracy and robustness of the proposed technique were obtained during the validation studies. The developed method is also stability-indicating and can be used for the routine analysis of combined tablet dosage form of LAmivudine, Tenofovir DF and Nevirapine and also check the purity and stability of the active substance in pharmaceutical dosage forms.

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Table 1: System suitability Data

Parameter	Result
Resolution (R) between Lamivudine Diasteriomer and Lamivudine	2.4
Theoretical Plates (T)	Lamivudine:5021
	Tenofovir :12941
	Nevirapine: 12062
%RSD	Lamivudine: 0.9
	Tenofovir :1.1
	Nevirapine: 0.8

Table 2: **Forced degradation data**

Stress condition	% Degradation observed		
	Lamivudine	Nevirapine	Tenofovir
Acid stress condition	0.3	0.2	34.73
Base stress condition	0.2	0.2	61.6
Oxidative stress condition	66.2	0.2	9.17

Table 3: **Linearity data**

Impurity Name	Correlation coefficient (r)	Range (µg/ml)
Lamivudine	0.998	45 - 135
Tenofovir DF	0.999	45 - 135
Nevirapine	0.999	60 - 180

Table 4: **Precision and Intermediate Precision data**

Impurity Name	% RSD	
	Precision data	Ruggedness data
Lamivudine	0.29	0.32
Tenofovir DF	0.21	0.45
Nevirapine	0.12	0.16

N= six sample preparations

Table 5: **Accuracy data**

Impurity Name	% Recovery¥		
	50% Level	100%Level	150%Level
Lamivudine	99.3	98.1	98.8
Tenofovir DF	98.6	98.9	101.0
Nevirapine	99.3	99.6	100.6

N= triplicate sample preparations
¥ = Average of three determinations

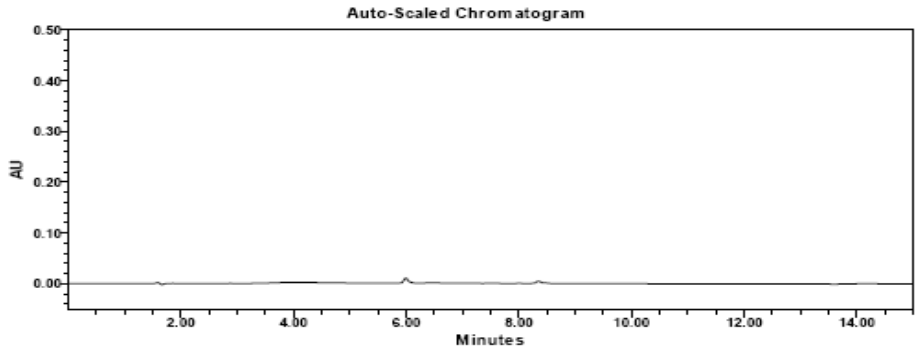
Table 6: **Robustness data**

Parameter	Deliberate change	Resolution	Minimum theoretical plates			Maximum tailing factor
			Lamivudine	Tenofovir	Nevirapine	
Flow rate (0.7mL/min)	0.6mL/min	2.3	8380	14063	11781	1.3
	0.8mL/min	1.9	8302	13980	12001	1.1
Temperature (35°C)	30°C	1.8	8255	14110	11986	1.2
	40°C	2.0	8510	14032	12164	1.0
pH of buffer (2.8)	2.7	2.1	8798	14008	12021	1.0
	2.9	1.8	8610	14180	12098	1.3

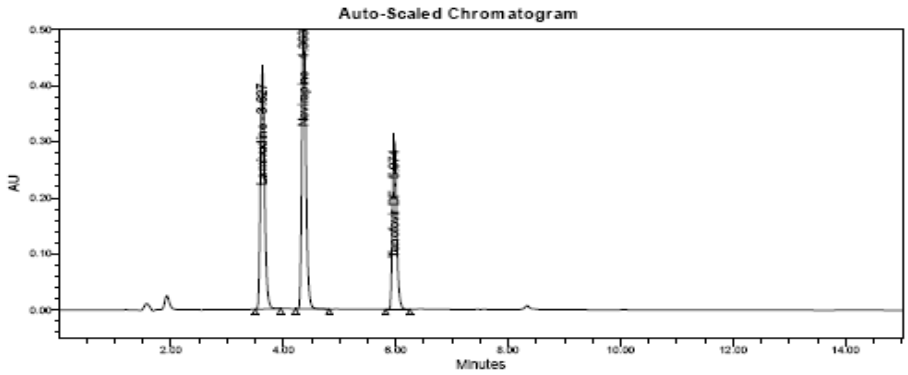
Figure 1: Chemical structure of Lamivudine

Figure 2: Chemical structure of Tenofovir disoproxil fumarate

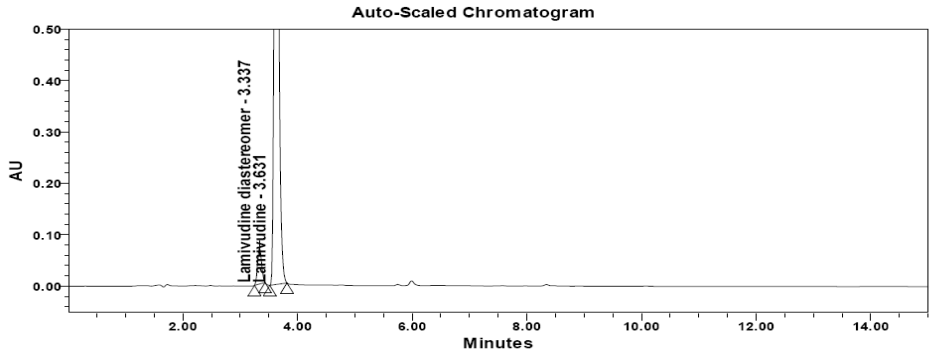
Figure 3: Chemical structure of Nevirapine



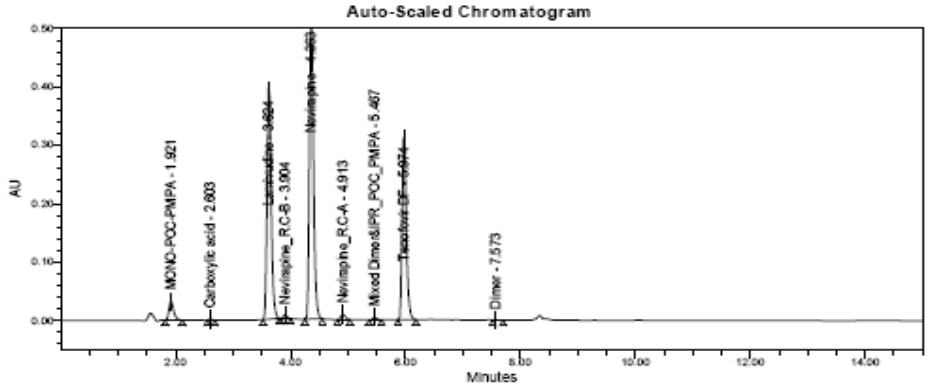
4(a). Typical chromatogram of Diluent as blank



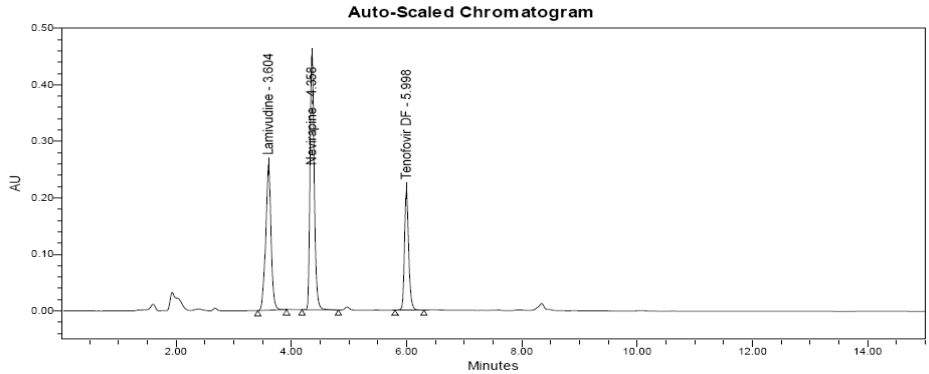
4(b). Typical chromatogram of standard



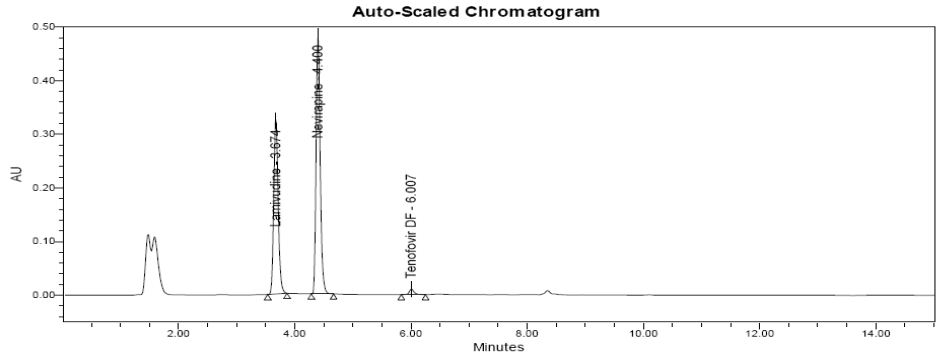
4(c). Typical chromatogram of resolution solution



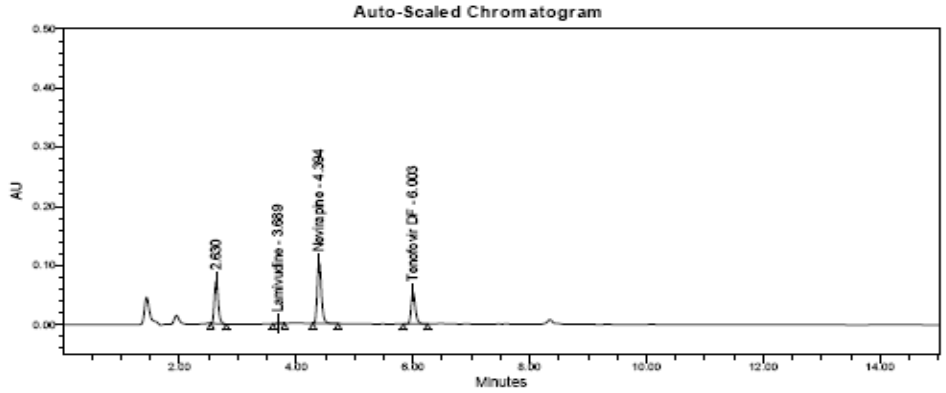
4(d). Typical chromatogram of spiked sample



5(a). Typical chromatogram of acid degradation



5(b). Typical chromatogram of base degradation



5(c). Typical chromatogram of oxidative degradation

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