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A NEW DEVELOPMENT AND VALIDATION OF STABILITY INDICATING RP-HPLC METHOD FOR THE DETERMINATION OF ANTI-RETROVIRAL DRUGS IN PHARMACEUTICAL DOSAGE FORM

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Keywords:

Lamivudine, Efavirenz, UV, RP-HPLC, Stability-indicating Methods & ICH Guidelines

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ABSTRACT: In this study, a new simple, rapid and accurate, sensitive isocratic method was developed for the simultaneous determination of the Anti-retroviral drugs in pharmaceutical dosage forms. The chromatography was run through Denali C18 column (150 x 4.6 mm and 5 µm particle size). Mobile phase containing mixer of buffer 0.1% OPA (PH.4.6) acetonitrile is taken in the ratio 40:60 was pumped through column at a flow rate of 0.8ml/min and isocratic elution with a total runtime of 7 minutes. The mixer of the buffer used in this method was 0.1% OPA. The temperature was maintained at 20 °C to 30 °C, optimised wavelength selected was 258 nm. The retention time of the lamivudine and Efavirenz were found to be 2.131 min and 3.058 min as respectively. The drug was stressed under alkaline (acid, base); oxidative, thermal, photolytic degradation was analysed. The validated method was developed as per ICH Guidelines. The accuracy, linearity, precision, robustness, LOD and LOQ was within the acceptable limits. Hence, this RP-HPLC method was stability-indicating can be used for routine stability analysis of the Lamivudine and Efavirenz in pharmaceutical dosage forms.

INTRODUCTION: Lamivudineis polymerase is an inhibitor and zalcitabine analogue during which a sulphur atom replaces the three-carbon of the pentose ring. It can be usedto treat Human Immunodeficiency Virus Type 1 (HIV-1) and hepatitis B (HBV), chemically, Lamivudine is -4-amino - 1 - [(2R, 5S) - 2 - (hydroxymethyl) - 1, 3-oxathiolan – 5 - yl] - 1, 2 - dihydropyrimidin-2-one, with Chemical formula is $C_8H_{11}N_3O_3S$ and its Appearance of A white or almost white powder.

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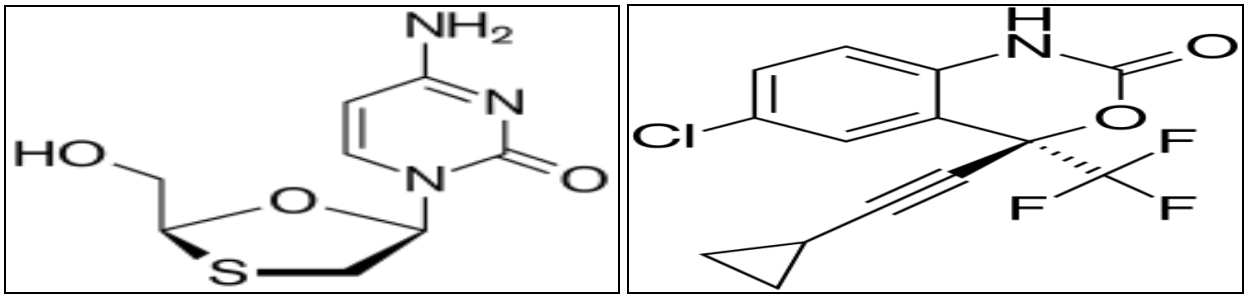
But Solubility, Soluble in water sparingly soluble in methanol, practically insoluble in acetone, the Category is Antiretroviral (Nucleoside polymerase Inhibitor). It Use of This drug is employed with other HIV medications to assist to regulate HIV infection.

It helps to reduce the amount of HIV in your body so far and its system can be work better. And Efavirenz is an HIV-1 specific, non-nucleoside, polymerase inhibitor (NNRTI). chemical Name is (4S) - 6 – chloro - 4 - (2 – cyclo -propylethynyl) - 4 - (trifluoromethyl) - 2, 4-dihydro - 1benzoxazine – 2 - one, with formula, $C_{14}H_9C_lF_3NO_2$, the Category is Anti-HIV Agents, Reverse Transcriptase Inhibitors, its Solubility, insoluble in water (< 10 micrograms/mL), Soluble in Methanol And acetonitrile, P ka: 12.52 and efavirenz inhibits the

activity of viral RNA-directed DNA polymerase (*i.e.*, reverse transcriptase). Antiviral activity of efavirenz is depended on intracellular conversion to the active triphosphorylated form [1, 2, 3, 4, 5, 6, 7]. A literature survey study reveals that analytical methods supported UV [8, 9]. Spectrometer and HPLC [10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21]. Are available for the estimation of those drugs individually and in combined with other drugs in various pharmaceutical dosage forms, there's one analytical method reported with a methanol: Acetonitrile and ammonium acetate, in the ratio of 20: 30: 50 and Mixer of buffer and acetonitrile (40:60v/v) pH 4.6 for the simultaneous determination of Lamivudine and Efavirenz during a Combined Pharmaceutical dosage form. The Aim of the present work is developing a replacement simple, precise, accurate and rapid method with less run time for the estimation of Lamivudine and Efavirenz during a combined pharmaceutical Dosage Form without lack of interference. And only few and RP-HPLC Methods were reported for the estimation of lamivudine and efavirenz. Till date one method was not reported with an honest sensitivity, economical and stability indicating RP-HPLC Method in literature for the estimation of lamivudine and efavirenz simultaneously in bulk and tablet dosage form. Hence, we've undertaken current research work to develop an efficient, sensitive, economical and stability indicating RP-HPLC Method for analysis of lamivudine and efavirenz within the drug substance and tablet dosage form. The developed method was validated as per the Q2 specification of ICH guidelines and made degradation conditions in stability-indicating studies were maintained as per the Q1A specification of ICH recommendation.

The Plan of Work is as follows: Simultaneous determination of Lamivudine and Efavirenz from formulation by UV. spectrophotometric Method. Simultaneous determination of Lamivudine and Efavirenz from formulation by RP-HPLC Method. And Stability indicating method.



1A: LAMIVUDINE **1B: EFAVIRENZ**

FIG. 1: CHEMICAL STRUCTURES OF LAMIVUDINE AND EFAVIRENZ

MATERIALS AND METHODS:

Chemicals, Reagents and Standard Solutions of Lamivudine and Efavirenz: Water HPLC [Grade], Anti-Retroviral agentsWorking Standards**,** Methanol RP-HPLC (Grade)**,** Orthophosphoric acidetc, Mobile phase, were purchased from mark (India) Ltd, WorliMumbai, India. allactive pharmaceutical ingredients (APIs) of lamivudine and efavirenz as reference standards were procured from spectrum pharma labs, Hyderabad, India.

Instrumentation: In this present study performed with waters HPLC 2695 photo diode array detectors and empower 2 software was used UV - visible spectrophotometer PG Instruments T60 with special bandwidth of 2 mm and 10 mm and matched quartz cells integrated with UV win 6 software was used for measuring absorbance of lamivudine and efavirenz. Electronic balance-Denver, P^H Meter -BVK enterprises, India, ultrasonicate-BVK enterprises.

Determination of Maximum Absorbance values: Lamivudine and efavirenz standard solution were scanned within the range of 200-400 nm against mobile phase as blank. lamivudine and efavirenz shows maximum absorbance at 246 nm. The wave length selected for the estimation of lamivudine and efavirenz is 258 nm.

Diluent of Solvents: The Mobile phase is used as a diluent, mix a mixture of buffer solution 400 ml (40%) and 600 ml of Acetonitrile HPLC (60%) and degassing in ultrasonicate water bath for ten minutes. Filter through 0.45 µ filter paper under vacuum filtration.

Standard Stock Solutions Preparation: Weigh accurately about the substance and transfer 10.0 mg of Lamivudine and 10.0 mg of Efavirenz is a working standard solution into a 10ml clean dry volumetric flask and add about the 7.0ml of Diluent solution and sonicate to dissolve it completely and make the volume up to the mark with an equivalent solvent. (Stock solution) and Further pipette out the 0.4ml of Lamivudine & Efavirenz and the above stock solution into a 10.0ml volumetric flask and dilute up to the mark with diluent solution.

Preparation of Sample stock Solution: Accurately weigh about and transfer like 10 mg of Lamivudine and Efavirenz sample into a. 10ml of clean dry volumetric flask add about 7.0ml of Diluent solution and sonicate it to dissolve it completely and make volume up to the mark with an equivalent solvent. Stock solution) and Further pipette out the 0.4ml of Lamivudine & Efavirenz of the above stock sample solution into a 10.0ml of volumetric flask and dilute up to the mark with diluent.

Preparation of Phosphate Buffer 0.1%: Weigh accurately about 7.0 grams of KH_2PO4 into a 1000ml beaker and dissolved in a diluted to 1000ml with HPLC water. Adjusted theup to pH to 2.6 with Orthophosphoric acid *etc*.

Procedure: To inject the 20 µl of the quality standard solution, and sample solution into the chromatographic system and to measure the areas for the Lamivudine and Efavirenz peaks and calculate the % Assay by the formulae.

System Suitability Data: Tailing factor for the peaks due to Lamivudine & Efavirenz Standard sample solution should not be more than 1.5, Theoretical plates for the Lamivudine & Efavirenz peaks in Standard sample solution should not be less than 2000

RESULTAND DISCUSSION:

Optimization of Chromatographic Conditions: To develop a stability indicating RP-HPLC method for estimation of lamivudine and efavirenz in Pharmaceutical dosage forms, different preliminary tests were performed and different chromatographic condition were developed which got in **Table 1**. The ultimate analysis was performed by using Column: Symmetry C18 (4.6 x 150mm, 5µm), make: X Terra) or equivalent, Flow rate: 0.8 ml per min, the sample were analysed at detector Wavelength: 258 nm, Injection volume: 20µl, Column oven: ambient, Run time: 7 min. The proposed method lamivudine and efavirenz was optimised to offer sharp peak with good resolution and minimum tailing effect for the optimized chromatogram was obtained as shown in **Table1.**

Analytical Method Validation Process: The Analytical method validation of lamivudine and efavirenz byusing HPLC was carried out with respect to the following parameters.

TABLE 1: DATA OF OPTIMIZED CHROMATOGRAPHIC CONDITIONS

Parameter	Condition
Mobile phase	Mixture of buffer 400 ml (40%) and 600 ml of Acetonitrile (60%)
Flow rate	0.8ml/min
column	Symmetry C18 (4.6 x 150mm, 5µm,)
Detector wave length	258nm
Column temperature	30 ^oC
Injection value	20 µl,
Run time	7 min
Diluent	Mixture of buffer and Acetonitrile in the ratio 40:60

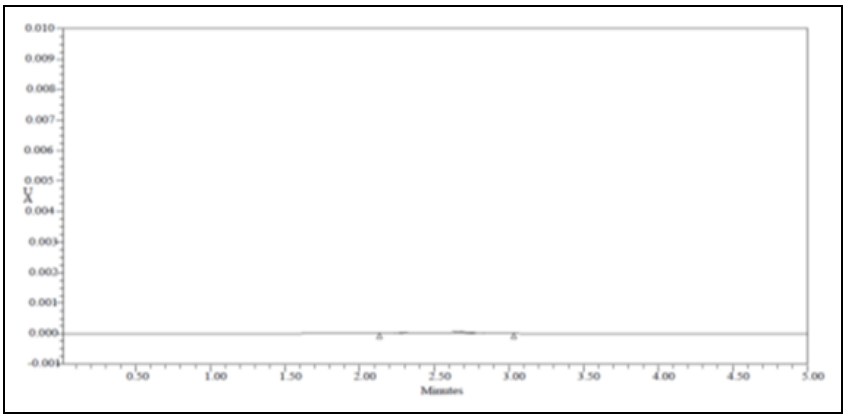


FIG. 2: BLANK OFLAMIVUDINE AND EFAVIRENZ CHROMATOGRAM

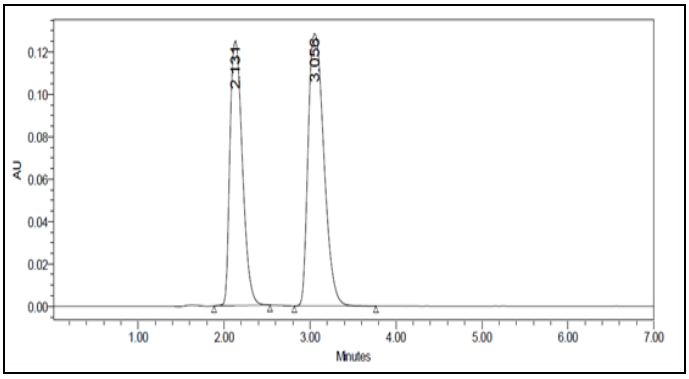


FIG. 3: STANDARD OF LAMIVUDINE AND EFAVIRENZ CHROMATOGRAM

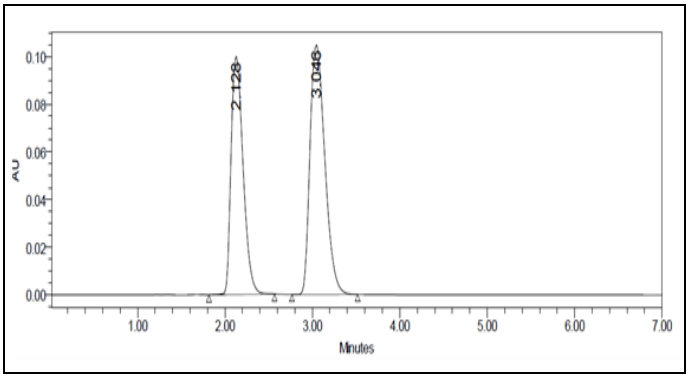


FIG. 4: FORMULATION CHROMATOGRAMS

TABLE 2: SYSTEM SUITABILITYDATAPARAMETERSFORLAMIVUDINE AND EFAVIRENZ

S. no.	Lamivudine			Efavirenz			
Injection	RT (min)	USP Plate Count	Tailing	RT (min)	USP Plate Count	Tailing	Resolution
1	0.7	2195.0	1.3	0.7	2574.4	1.3	2
2	0.8	2181.7	1.4	0.8	2589.8	1.4	2.2
3	0.9	2083.5	1.4	0.9	2382.7	1.4	2.3
Changes in Organic Composition in the Mobile Phase							
1	10% less	2103.4	1.6	10% less	2461.3	1.6	-
2	*Actual	2181.7	1.4	*Actual	2589.8	1.4	-
3	10% more	2016.6	1.3	10% more	2435.6	1.3	-

Linearity: Linearity of lamivudine and efavirenz were found by injecting five different concentrations of working standard solutions for lamivudine (20-60PPM) and efavirenz (20-60PPM). Standard calibration curves were constructed by taking mean peak area on Y-axis and concentration of drug on X-axis. The linearity equation obtained for lamivudine was y= 167890x + 65407 (R 2 =0.9994) and of efavirenz was Y= 39327x + 63509 (R 2 =0.99) correlation coefficient obtained was 0.999 for the 2 drugs. The results were shown in **Table 3**.

TABLE 3: RESULTS DATA FOR THE LINEARITY OFLAMIVUDINE AND EFAVIRENZ

	lamivudine		Efavirenz	
Linearity Level	Conc (µg/ml)	Peak Area	Conc (µg/ml)	Peak Area
1	0	0	0	0
2	20	674644	20	868569
3	30	953517	30	1240821
4	40	1216843	40	1584141
5	50	1561020	50	2039735
6	60	1841281	60	2408104

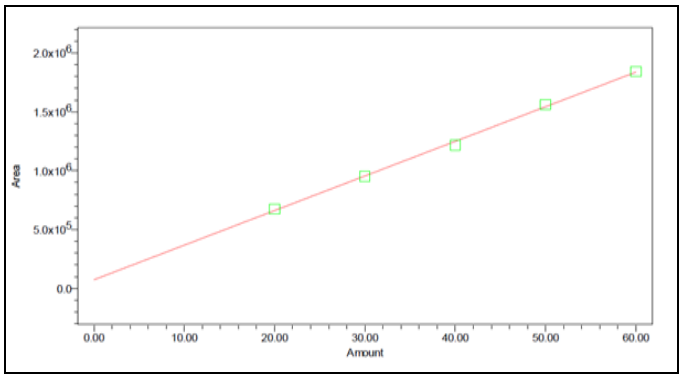


FIG. 5: CALIBRATION PLOT OF LAMIVUDINE

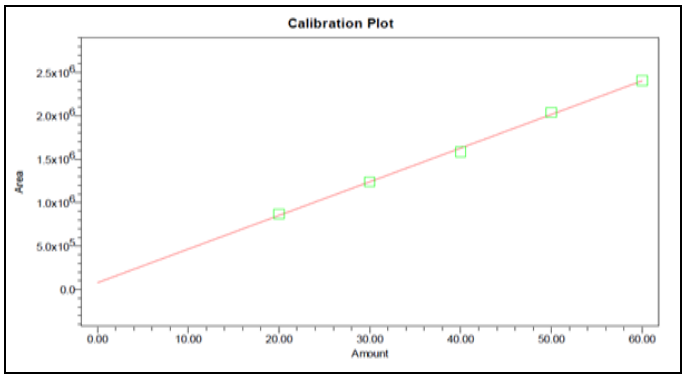


FIG. 6: CALIBRATION PLOT OF EFAVIRENZ

Precision: The system precision was established by five replicate injections of the quality solution containing analytes of interest. The worth of relative variance of lamivudine and efavirenz and

was found to be as 0.36% and 0.29% within the limit, indicating the injection repeatability of the strategy. The strategy precision was established by completing the analysis five times using the proposed method. The relative variance of lamivudine and efavirenz was found to be 0.28% and 0.28% within the limit, indicating the injection repeatability of the strategy.

TABLE 4: RESULTS DATA FOR THE PRECISION OF LAMIVUDINE AND EFAVIRENZ

-	Lamivudine	Efavirenz	Lamivudine	Efavirenz
S. no.	System Precision		Method Precision	
1	1199723	1572878	1221176	1596784
2	1208865	1576676	1213386	1588880
3	1207896	1578267	1214493	1585690
4	1207573	1580584	1216379	1588840
5	1211377	1579792	1212806	1585974
Mean	1207087	1577640	1215648	1589234
S. D	4378.4	3053.3	3377.2	4485.2
%RSD	0.36	0.29	0.28	0.28

Accuracy: To demonstrate the accuracy of the proposed method a typical addition method was used for analysing the samples. For this purpose, known amount of lamivudine and efavirenz were supplemented to the working standard sample solution which was previously analysed then compared the obtained experimental values to truth values. Each solution was injected in five times and therefore the percentage recovery was calculated. %recovery was obtained as 100.41% and 100.30% for lamivudine and efavirenz respectively.

TABLE 5: ACCURACY RESULT DATA OF LAMIVUDINE

% Concentration (at Specification Levels)	Area	Amount Added (mg)	Amount Found (mg)	% Recovery	Mean % Recovery
50%	665659	5.3	5.38	101.6%	
100%	1222077	9.9	9.88	99.8%	
150%	1851398	15.0	14.97	99.9%	100.41%

TABLE 6: ACCURACY RESULT DATA OFEFAVIRENZ

% Concentration (at Specification Levels)	Area	Amount Added (mg)	Amount Found (mg)	% Recovery	Mean % Recovery
50%	862672	5.3	5.35	100.9%	
100%	1594725	9.9	9.89	99.9%	
150%	2424504	15.0	15.03	100.2%	100.30%

Robustness: The different variations like variation of PH of the solution, flow rate, wavelength and mobile phase solution composition. The deliberate changes within the method haven't much affected the peak tailing factor, theoretical plates and therefore the percent assay. This indicated the robustness of the strategy. The robustness study results are presented in **Table 7.**

TABLE 7: ROBUSTNESS RESULT DATA FOR LAMIVUDINE AND EFAVIRENZ

S. no.	Condition	Change	% RSD of Lamivudine	% RSD of Efavirenz
1	Flow rate-1	0.7ml/min	1.6	1.6
2	Flow rate-2	0.9ml/min	1.3	1.3
3	Mobile phase-1	60:40 (%v/v)	1.4	1.4
4	Mobile phase-2	55:65 (%v/v)	1.4	1.4
5	Temperature-1	25^{o} C	1.3	1.3
6	Temperatur-2	35^{o} C	1.4	1.4

Limit of Detection and Limit of Quantification: Determination of Limit of detection and limit of Quantification was performed by variance method. Standard with low concentrations of analyte with those of blank samples and establishing the minimum concentration at which the analyte is often readily detected.

Assay of Formulation: We were prepared assay sample solution injected into the HPLC, bearing the

labels claim of Lamivudine 600 mg, Efavirenz 300 mg. assay was performed with the above formulation product. Average % assay for Lamivudine and Efavirenz obtained was 100.016 and 101.165 respectively.

TABLE 8: SENSITIVITY DATA TABLE FOR LAMIVUDINE AND EFAVIRENZ

Molecule	LOD	LOQ
Lamivudine	2.96	9.96
Efavirenz	3.0	10.0

TABLE 9: ASSAY RESULT DATA OF LAMIVUDINE AND EFAVIRENZ

Labelled Amount of Drug(mg) Efavirenz & Lamivudine	Mean (±SD) Amount (mg) Found by the Proposed Method (n=6)	Mean (± SD) Assay (n = 6)
600, 300	600.04 (±0.13) 300.53 (±0.09)	100.016 (±0.38) 101.165 (±0.44)

Forced Degradation Studies: Degradation studies were performed with the formulation and the degraded samples were injected. Assay of the injected samples was calculated and all the samples passed through the limits of degradation studies.

TABLE 10: RESULT DATA OF FORCED DEGRADATION STUDIES

	Lamivudine			Efavirenz		
Stress Conditions	**Area**	**% Recovered**	**% Degraded**	**Area**	**% Recovered**	**% Degraded**
Acidic/0.1NHCl/24h/reflux at80 °C	665659	101.6%	5.3	862672	100.9%	5.3
Basic/0.1N NaOH/24h	1222077	99.8%	9.9	1594725	99.9%	9.9
Peroxide/oxidation%80 °C	1851398	99.8%	15.0	2424504	100.2%	15.0
Thermal/dry 24hheat/80 °C/75% RH/24h	1953196	99.9%	15.7	2524691	100.3%	15.7
UV/Photolytic/24h	2031691	100.7%	15.9	2624681	100.7%	15.9
Water/Neutral	2141692	100.9%	15.9	2652691	100.8%	16.0

CONCLUSION: The proposed newly stability indicating RP-HPLC method were developed and validated for the simultaneous estimation of Lamivudine and Efavirenz within the pharmaceutical dosage form.

The proposed method was validated following ICH Guidelines by testing its parameters include linearity, precision, accuracy, robustness, LOD and LOQ. The Stress-induced studies proves the effectiveness of the proposed validated stability-indicating method.

So, the strategy developed was simple and economical which will be adopted in regular internal control test in pharmaceutical industries.

The share %RSD for all parameters was found to be but ± 2, which indicates the validity of the methods and the assay results obtained by this method are in elegant agreement.

And therefore, the Sample recoveries altogether pharmaceutical formulations were in the good treaty with their respective label claims.

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