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Development and Validation of RP-HPLC Method for Simultaneous Estimation of Zidovudine and Lamivudine in Pharmaceutical Dosage Form

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ABSTRACT
A simple, reproducible, feasible and innovative reversed-phase high performance liquid chromatographic method was developed with UV-Visible detector and validated for the Simultaneous estimation of Zidovudine and Lamivudine in Pharmaceutical dosage form. The method was carried out using C18 column [Primsil column, 4.6 mm x 250 mm ; 5µm Hypersil Gold] with mobile phase comprised of Acetonitrile : Water (50:50) with 0.5 ml/min flow rate. The optimum wavelength for detection was 271 nm at which better detector response for the drug was obtained. The run time was set at 15 min. The method was validated for Linearity, Precision, Accuracy, Specificity, Limit of detection(LOD), Limit of quantification (LOQ), Robustness, and Stability. LOD of Zidovudine and Lamivudine were found 0.4336 and 0.221183 µg/ml respective and LOQ of Zidovudine and Lamivudine were found 1.3140 and 0.6702 µg/ml respectively. The recovery studies were also carried out and mean % recovery was found to be 99% – 101%. The % RSD was found to be less than 2 %. The proposed method was successfully applied for Simultaneous estimation of Zidovudine and Lamivudine in Pharmaceutical dosage form.

Key Words : Lamivudine, Zidovudine, RP-HPLC, Method Development, Method Validation

I. INTRODUCTION

Zidovudine (Azidothymidine/AZT) is a thymidine analogue. Its chemical name is 1-[(2R,4S,5S)-4-azido-5-(hydroxymethyl)oxolan-2yl]-5methyl-1,2,3,4-tetrahydropyrimidine-2,4-dione. It works by selectively inhibiting HIV's reverse transcriptase, the enzyme that the virus uses to make a DNA copy of its RNA. Reverse transcription is necessary for production of HIV's double-stranded DNA, which would be subsequently integrated into the genetic material of the infected cell. At very high doses, AZT's triphosphate form may also inhibit DNA polymerase used by human cells to undergo cell division, but regardless of dosage AZT has an approximately 100-fold greater affinity for HIV's reverse transcriptase. The selectivity has been proven to be due to the cell's ability to quickly repair its own DNA chain if it is broken by AZT during it's formation, whereas the HIV virus lacks that ability. Thus AZT inhibits HIV replication without affecting the function of uninfected cells.

Lamivudine is an analogue of Cytidine. Its chemical name is 4-amino-1-[(2R,5S)-2-(hydroxymethyl)-1,3-oxathiolan-5-yl]-1,2-dihydropyrimidin-2-one. It can inhibit both types (1 & 2) of HIV reverse transcriptase and also the reverse transcriptase of hepatitis B virus. It is phosphorylated to active metabolites that compete for incorporation into viral DNA. They inhibit the HIV reverse transcriptase enxyme competitively and act as a chain terminator of DNA Synthesis. The lack of a 3'-OH group in the incorporated nucleoside analogue prevents the formation of the 5' to 3' phosphodiester linkage essential for DNA chain elongation, and therefore, the viral DNA growth is terminated.

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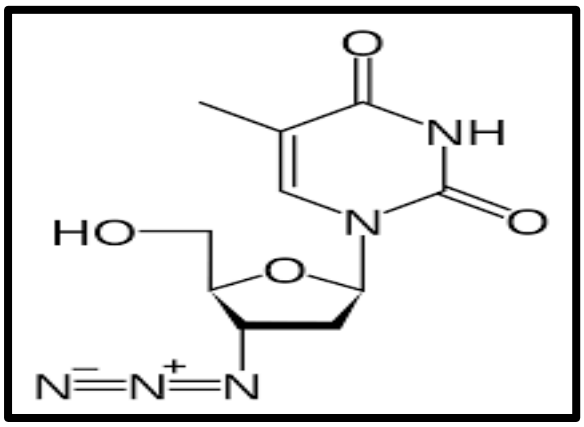

Figure no. 1 : Structure of Zidovudine

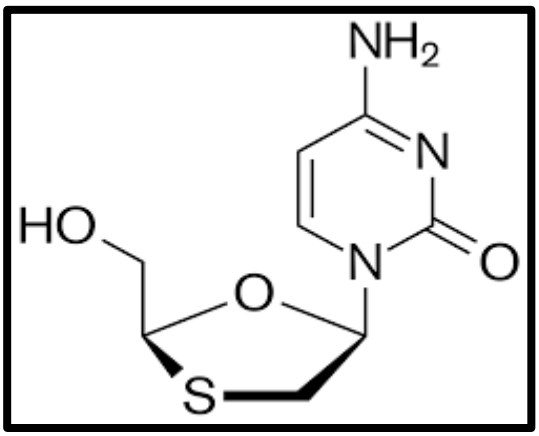

Figure no. 2 : Structure of Lamivudin

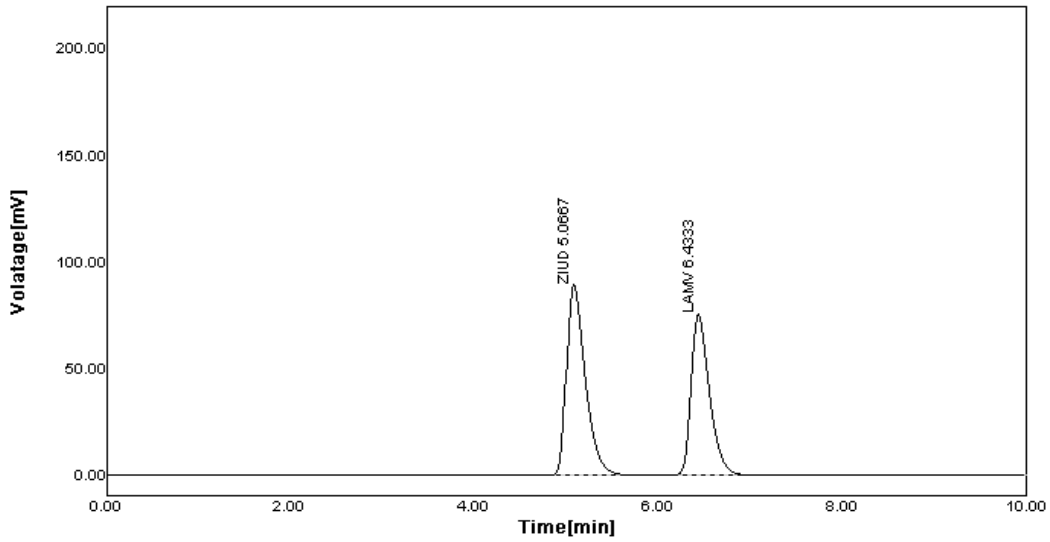

Figure No. 3 : Chromatogram of Lamivudine + Zidovudine (10+20µg/ml)

II. EXPERIMENTAL WORK

2.1Chemicals and Reagents : The Zidovudine and Lamivudine (API) marketed preparations and other required chemicals used for the investigation were procured from Cipla Pharmaceuticals. The marketed preparationi.e. Duovir Tablet containing Zidovudine 300 mg + Lamivudine 150 mg were supplied by Cipla Pharmaceuticals. Other reagents like Methanol, Acetonitrile, HPLC Water, Buffer, Orthophosphoric acid, Triethylamine, 1N HCL, 1N NaOH and 3% H2O2 were purchased from Rankem Ltd. And all the reagents were of analytical grade.

2.2 Instrumentationand conditions The analysis was carried out on a HPLC Younglin (S.K.) system equipped with Gradient system UVdetector, pressure controlled by P-3000-M Reciprocating pump (40MPa) and operated by Autochrome-3000 Software. C18 column (250mm × 4.6mm i.d., particle size 5 μm) was used for separation. Mobile phase used for separation was Acetonitrile : Water (50:50). The flow rate was kept at 0.5 mL/min, column temperature was ambient (25∘C), eluents were detected by UV detector at 271 nm, and the injection volume was 20 μL.

2.2.1 Preparation of Mobile Phase. Mobile phase was prepared by mixing 50 volumes of acetonitrile and 50 volumes of Water and was adjusted to pH 3.2 with orthophosphoric acid. The mobile phase was ultrasonicated, filtered through 0.45 μm nylon

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filter, and degassed and this mobile phase was also used as diluent.

2.2.2 *Preparation of Standard Solution :*
For HPLC Method :

Standard Solution was prepared by weighing accurately 10mg of Lamivudinne and 20 mg of Zidovudine individually and dissolve in 0 ml of mobile phase in 10 ml clean and dry volumetric flask and sonicated to dissolve it completely and make volume up to the mark with diluents.

For UV Spectrophotometric method : The standard solutions of Lamivudine and zidovudine was prepared by dissolving 10 mg of lamivudine and 20 mg of zidovudinein 10 ml of volumetric flask of methanol. The standard solutions were diluted appropriately to obtain a concentration of 1000µgm/ml Lamivudine and 2000µgm/ml zidovudine. Concentrations of 5-25µg/ml of Lamivudine, 10-50µg/ml of zidovudine were prepared.

2.2.3 Preparation of Sample Solution :
For HPLC Method :
Sample Solution was prepared by weighing accurately 10mg of Lamivudinne and 20 mg of Zidovudine individually and dissolve in 10 ml of methanol and diluted with standard solution of lamivudine and zidovudine.
For UV Spectrophotometric method : The powder equivalent to 10 mg lamivudine and 20 mg of zidovudine was weighed accurately transferred into 10 ml of standard volumetric flask. Suitable aliquot of the solution was diluted to produce the Concentrations of 5-25µg/ml of Lamivudine, 10-50µg/ml of zidovudine were prepared.

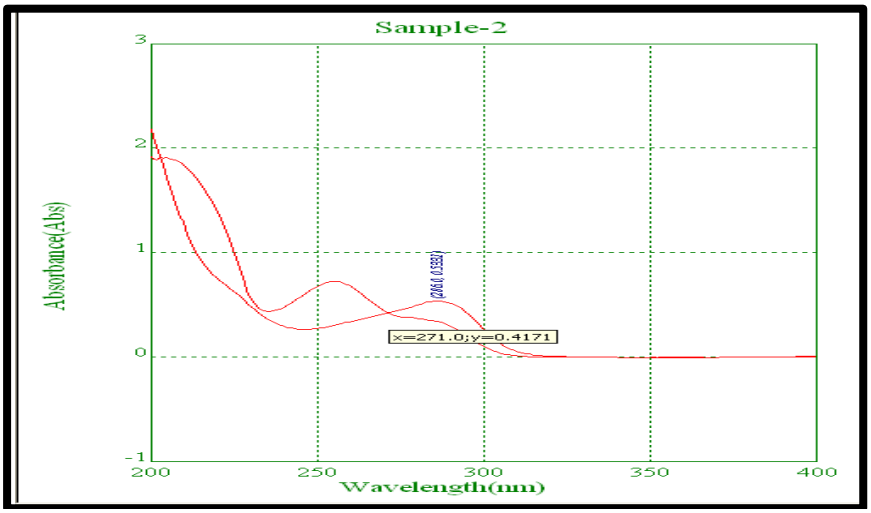

Figure no. 4 : Spectrum of Lamivudine and Zidovudine

III. METHOD VALIDATION

***3.1. Linearity*:** The linearity was performed by diluting standard stock solution to give final concentration in the range of 5-25 μg/ml for Lamivudine and 10-50 μg/ml for zidovudine for 5 μg/ml concentration injected and calibration curve was constructed.

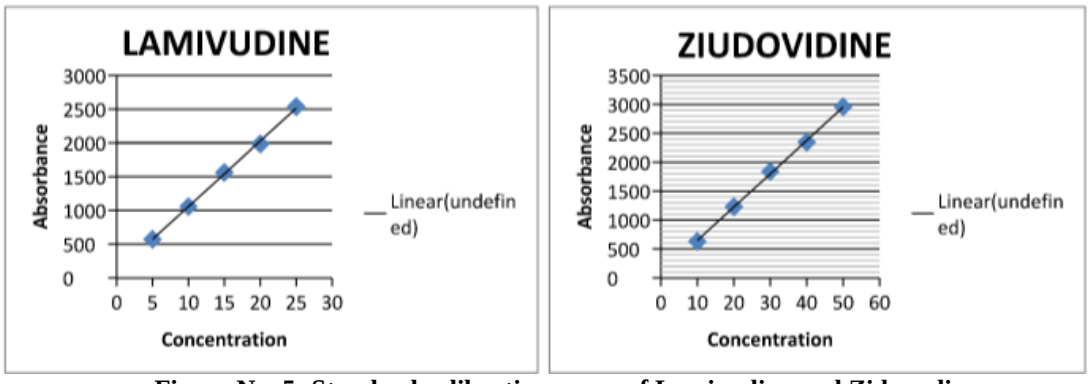

Figure No. 5: Standard calibration curve of Lamivudine and Zidovudine

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3.2.Precision

Repeatability : Precision of the method was studied by making repeated injections of the mixture of drugs on the same day for intraday precision. The coefficient of variation after five determinations was determined at 5-25 μg/ml for Lamivudine and 10-50 μg/ml for zidovudine .

Intermediate Precision : Intermediate precision was carried out by injecting three replicates of standard concentration by different analysts. The % RSD was calculated.

Table No.1 : Results of Precision

Drug	Lamivudine	Zidovudine
Intra-day precision (% RSD)	0.29	0.25
Inter-day precision (% RSD)	0.32	0.26

3.3. Accuracy : Sample solutions were prepared at three different concentrations 80%, 100% and 120% and known amount of sample was added to this solutions and recovery of added sample was studied.

Table No. 2 Results of Accuracy

Concentration added	Lamivudine mean % recovery	Zidovudine mean % recovery
80%	1.18 %	0.75%
100%	0.44%	0.69%
120%	0.54%	0.52%

3.4. Robustness: Influence of small changes in chromatographic conditions such as change in flow rate, that is, ±0.1mL/mins and wavelength of detection ±1nm, mobile phase ±1ml, was studied to determine the robustness of the method for the development of RP-HPLCmethod for the simultaneous estimation Zidovudine and Lamivudine and their %RSD was determined.

Table No. 3 Results of Robustness

Parameter	Lamivudine (% RSD)	Zidovudine (%RSD)	Inference
Flow rate (±0.1mL/mins)			
0.4ml/min	1.53	0.55	
0.6ml/min	0.64	1.86	
Wavelength (±0.1nm)			% RSD was found to be < 2
270 nm	0.9	1.91	
272 nm	0.53	1.38	
Mobile Phase (±0.1ml)			
51:49 ml	1.18	1.06	
49:51 ml	0.69	0.41	

3.5. Specificity: To check the specificity placebo, standard and sample solutions were injected, verified that there no interference of impurities and also no change in the retention time.

3.6. Limit of Detection (LOD) : LOD was determined using the signal-to noise ratio, and then comparing the test results from the samples with known concentrations.

Table No. 4 Results of LOD

Sr. No.	Peak	LOD
1	Lamivudine	0.2211
2	Zidovudine	0.4336

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3.7. Limit of quantification (LOQ) : LOQ is defined as the lowest concentration of the analyte that can be determined with acceptable precision and accuracy under the stated experimental conditions

Table No. 5 Results of LOQ

Sr. No.	Peak	LOQ
1	Lamivudine	0.6702
2	Zidovudine	1.3140

Table 6: System suitability parameters.

Parameters	Proposed Method	
	LAM	ZID
Retention Time	3.850	1.28
Resolution	4	5
Therotical Plates	2978	8650

3.8. System Suitability. The stock solution containing 5 mg of lamivudine and 10 mg of zidovudine was injected and repeated five times and the chromatograms were recorded. The resolution, number of theoretical plates, and peak asymmetry were calculated to determine whether the result complies with the recommended limit.

IV. RESULT AND DISCUSSION

4.1 Optimization of Chromatographic Conditions *:* To develop suitable RP-HPLC method for simultaneous estimation of Zidovudine and Lamivudine, different chromatographic conditions were applied and optimized chromatographic conditions were developed.

Optimized chromatographic conditions are as follows:
instrument: HPLC Younglin (S.K.) system equipped with gradient system UV detector, pressure controlled by P-3000-M Reciprocating pump (40MPa) and operated by autochrome-3000 software, mobile phase: acetonitrile: water (50 : 50)
column: C18 (250mm × 4.6 mm, 5 μm),
injection volume: 20 μL,
flow rate: 0.5 mL/min,
detection wavelength : 271 nm,
run time : 15 min,
temperature : Ambient (25∘C).

4.2. Validation

4.2.1. Linearity. The linearity of an analytical method is its ability to elicit test results which are directly proportional to the concentration (amount) of
analyte in the sample within the given range. (Figure no. 5)

4.2.2. Precision and Intermediate Precision. To demonstrate agreement among results, a series of measurements are done with Lamivudine and Zidovudine, triplet injections of the specific standard at various time intervals on same day were injected into the chromatograph and the value of % RSD of Intra-day precision of lamivudine and Zidovudine are 0.29 and 0.25 respectively. In inter-day precision same standard was injected on different days and the found % RSD of lamivudine and zidovudine are 0.32 and 0.26 respectively (See table no.1)

4.2.3. Accuracy. Accuracy of the method was calculated by recovery studies at three levels by standard addition method. The mean percentage recoveries obtained for Lamivudine and zidovudine were 0.72 % and 0.65% respectively (see Table no.2).

4.2.4. Robustness. The method for the development of RP-HPLC method for the simultaneous estimation of Lamivudine and Zidovudine was found to be robust as the % RSD was found to be less than 2 (see Table no.3).

4.2.5. Specificity. Specificity is ability to asses unequivocally the analyte in the presence of components that may be expected to be present. Typically these might be include impurities, degradents, matrix etc.Specificity may often be expressed as the degree of bias of the results obtained by analysis of samples containing added impurities, degradation products, related chemical compounds or placebo ingredients when compared to test results without added substances. No peak is observed and the result shows that the method is specific for estimation.

4.2.6 Limit of Detection (LOD) of Lamivudine and Zidovudine were determined by calibration curve method. Solutions of Lamivudine and Zidovudine were prepared and injected in triplicate (see table no.4).

4.2.7.Limit of Quantitation (LOQ) of Lamivudine and zidovudine were determined by calibration curve method. Solutions of were prepared and injected in triplicate (see Table no 5).

4.2.8. System Suitability. The resolution, number of theoretical plates, and peak asymmetry were calculated for the standard solutions. The stock solution was injected and repeated five times and the chromatograms were recorded. The resolution, number of theoretical plates, and peak asymmetry were calculated to determine whether the result complies with the recommended limit (see Table no.6).

V. CONCLUSION

The proposed method is precise, simple, sensitive, accurate, rugged and rapid and can be applied successfully for the estimation of Zidovudine and Lamivudine in pharmaceutical dosage form.

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