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A Modified Liquid Chromatographic Method Develpoment and Validation for Simultaneous Estimation of Lamivudine and Stavudine in Bulk and Tablet Dosage Form

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GRAPHICAL ABSTRACT

In the present investigation a more accurate, precise and economically feasible reverse phase liquid chromatographic method was developed and validated for the quantification of simultaneous estimation of Lamivudine and Stavudine in pure and Marketed dosage forms. Quantification of drugs of choice was done with a C18 Kromasil Column using mobile phase of Acetonitrile and Phosphate buffer. The flow rate was adjusted to 1 ml/min and at 257 nm the column eluents were chromatographed. Both the drugs exhibit retention time at 3.3 min and 6.2 min. The established method was linear over a concentration range of 20-100 µg/ml for both the drugs. The established method proved as more reliable and reproducible with a relative standard deviation of less than 2 and have accuracy within the acceptance limits. Marketed formulation assay was determined and proved that formulation was within the percentage purity limits. The analytical method was validated in accordance with International Conference on Harmonization (ICH) and this method can be employed in the quantification of marketed tablet dosage forms also.

Keywords: Lamivudine, Stavudine, RP-HPLC and Method validation

INTRODUCTION

Lamivudine is official in Indian Pharmacopoeia. It is chemically (2R-cis)-4-Amino-1-[2-(hydroxymethyl)-1, 3-oxathiolan -5-yl] cytosine [1-7]. Lamivudine is an Anti-Retroviral agent and a nucleoside analog reverse transcriptase inhibitor (NRTI). It is used to treat HIV and AIDS and also used for the treatment of chronic Hepatitis B. It is used always as a part of a multidrug regimen. It acts by inhibiting HIV reverse transcriptase as well as hepatitis B virus (HBV) DNA polymerase and by incorporating into DNA resulting in chain termination. Systemic toxicity of Lamivudine is low.

Stavudine, synthetic thymidine analogue, is phosphorylated by cellular kinases into active triphosphate and exhibits inhibitory action against HIV-1 in vitro. Since therapy with NRTIs for treatment against HIV-1 results in rapid development of HIC strains, co-administration of other antiretroviral therapies is necessary [8].

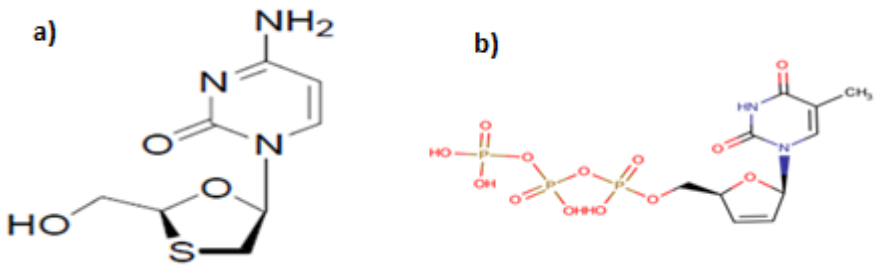


Figure 1: Chemical Structures of a) Lamivudine b) Stavudine

Extensive literature survey proved that very few methods were reported for the determination of Lamivudine and Stavudine by RP-HPLC [9-22]. In this way, in the current research, we endeavored to develop an accurate, rapid, precise, stable, sensitive and economically feasible liquid chromatographic method for the simultaneous estimation of selected drugs.

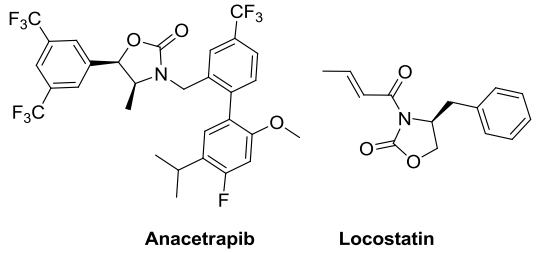
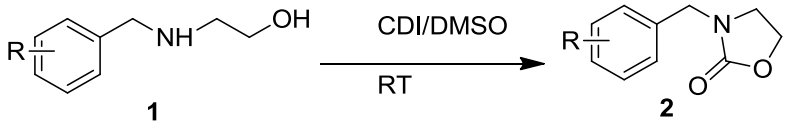


Figure 3: Examples of bioactive oxazolidinones

The most common synthetic routes reported so far for the synthesis of N-benzyloxazolidinones includes N-benzylayion of oxazolidinones [19], carboxylation of N-benzyl-β-amino alcohols [20-24]. The methods reported so far either involves use of metal catalyst or use of carbon dioxide in the presence of catalyst.

N,N-Carbonyldiimidazole (CDI) is a widely used reagent in organic synthesis. It is frequently used as a replacement for the highly toxic Phosgene in reactions with alcohols and amines [25-26] and the by- product imidazole formed after the reaction can be easily removed by dilute acid wash. Based on this, we tried to use CDI as a reagent in our synthesis of N-benzyloxazolidinones and successfully developed a catalyst free approach for the synthesis of N-benzyloxazolidinones (2) from N-benzyl-β-amino alcohol derivatives (1) using N,N-carbonyldiimidazole (CDI) in DMSO (Scheme 1).



Scheme 1: One pot synthesis of N-benzyloxazolidinones.

MATERIALS AND METHODS

Equipment used

The chromatographic separation was performed on Agilent 1220 liquid chromatographic system integrated with a variable wavelength programmable UV detector and a Rheodyne injector equipped with 20 µl fixed loop. A reverse phase C18 [Kromasil 250 mm × 4.6 mm] column was used. Lab India double beam UV visible spectrophotometer and Axis electronic balance were used for spectrophotometric determinations and weighing purposes respectively.

Reagents and chemicals

Pharmaceutical grade pure Lamivudine and Stavudine were procured as gift samples from local suppliers. Marketed tablet formulations with of 150 mg of Lamivudine and 40 mg of Stavudine were procured from local market. Acetonitrile and water of HPLC grade were procured from Merck specialties Pvt Ltd, Mumbai commercially.

Chromatographic conditions

Chromatographic separa tion was done at a detection wave length of 212 nm using Kromasil 100-5C18 column [250 mm x 4.6 mm]. Mobile phase composition of Acetonitrile and Phosphate buffer pH 5 in a ratio of 50:50 v/v was selected for elution and same mixture was used in the preparation of standard and sample solutions. 1 ml/min was the flow rate and 20 µl was the injection volume.

Preparation of Mobile phase

Phosphate buffer pH 5 was prepared by dissolving 0.136 gm of Potassium dihydrogen phosphate and 2 ml of Triethyl amine in 80 ml of HPLC grade water and adjusts the pH to 5.0 with orthophosphoric acid and volume was adjusted with water to produce 100 ml, which is then filtered through 0.45 µ membrane filter and sonicated for 20 min.

Preparation of Standard solutions

25 mg each of Lamivudine and Stavudine were accurately weighed and transferred into two 25 ml volumetric flasks respectively and dissolved in mobile phase as mentioned above and the volume was made up with the same solvent to obtain primary stock solutions A (Lamivudine) B (Stavudine) to achieve standard of concentrations of 1000 µg/ml of each drug. From the primary stock solutions, 1 ml of each solution was pipette out and transferred to a 10 ml volumetric flask and the volume was made up with the mobile phase to obtain final concentrations of 100 µg/ml of Lamivudine and Stavudine respectively and this solution is (working stock solution A).

Preparation of Sample Solution

Twenty tablets of Lamivudine and Stavudine were weighed and crushed. Tablet powder equivalent to 300 mg of Lamivudine and 100 mg of Stavudine was weighed accurately and transferred to a 25 ml volumetric flask. The contents were dissolved in 10 ml of mobile phase and sonicated for 15 min. Then the volume is made up with mobile phase and filtered through 0.45 µ membrane filter and again sonicated for 20 min. 0.1 ml of this solution was pipetted out and transferred to a 10 ml volumetric flask and the volume was made up with the mobile phase to obtain a concentration of 120 µg/ml of Lamivudine and 40 µg/ml of Stavudine (working stock solution B).

Optimization of RP-HPLC method

The HPLC method was optimized with an aim to develop a simultaneous estimation procedure for the assay of Lamivudine and Stavudine. For the method optimization, different mobile phases were tried, but acceptable retention times, theoretical plates and good resolution were observed with Acetonitrile, Phosphate buffer pH 5 (50:50 v/v) using Kromasil 100-5C18 column [250 mm x 4.6 mm].

Validation of the RP-HPLC method

The optimized method was validated according to the ICH Q2 (B) guidelines.

System suitability

System suitability was carried out with five injections of solution of 100% concentration having 100 µg/ml of Lamivudine and Stavudine of each in to the chromatographic system. A detailed report on Theoretical plates (N) and tailing factors (T) were given in Table 1.

Linearity

Linearity is determined by pipetting working stock solution A to a battery of 10 ml volumetric flasks and the volume was adjusted with the solvent to get concentration ranging from 20-100 µg/ml of lamivudine and stavudine. Each solution was injected in triplicate. Calibration curves were constructed sing observed peak areas against concentration, then regression equations were determined and correlation coefficients were calculated. The calibration curves for Lamivudine and Stavudine were shown in figure 3 and figure 4 their corresponding linearity parameters were given in Table 2.

LOD (Limit of Detection) and LOQ (Limit of Quantitation)

The LOD and LOQ were calculated form the slopes pf the calibration plot and standard deviation (SD) of the peak areas using the formulae LOD=3.3 σ/s and LOQ=10 σ/s. The results were consolidated in Table 2.

Precision

The repeatability of the method was verified by calculating the %RSD of six replicate injections of 100% concentration (100 g/µgl of Lamivudine and Stavudine respectively) on the same day and for intermediate precision, % RSD was calculated from repeated studies on different days. The results were kept in Table 3.

Accuracy

Standard addition method was adopted in order to ensure the reliability and accuracy of the method recovery studies. The percent recovery was reported by adding a known quantity of pure drug to pre-analyzed sample and contents were reanalyzed by the proposed method. The results were put in the Table 4.

Specificity

For a method, specificity was determined by testing standard substances against potential interferences. When no interferences were found because of the presence of excipients after the injection of test solution, then the method was found to be specific. The optimized chromatogram of Lamivudine and Stavudine without any interference was shown in Figure 2.

Robustness

Robustness of the method was crosschecked by altering the chromatographic conditions like mobile phase composition, wavelength detection, flow rare etc., and the % RSD should be reported. Small changes were allowed in the operational parameters were allowed and the extent of robustness of the method was determined. A deviation of ± 2 nm in the detection wave length and ±0.2 ml/min in the flow rate were tried separately. A solution of 100% test concentration with the specified changes in the operational conditions was injected to the instrument in triplicate. %RSD was reported in the Table 5.

Assay of Marketed Formulations

20 µl of sample solution containing 120 µg/ml of Lamivudine and 40 µg/ml of Stavudine was spiked into chromatographic system and the peak responses were recorded. The solution was injected thrice in to the column. The amount of drug and the percentage of purity were calculated by comparing the peak areas of the standards with that of test samples. A typical chromatogram for assay of marketed formulation was shown in figure 5 and the obtained values were presented in the Table 6.

RESULTS AND DISCUSSION

After conducting a number trials using mobile phases of varying composition, acetonitrile, phosphate buffer pH 5 in the ratio 50:50 v/v was selected as mobile phase because of better resolution and symmetric peaks. Lamivudine and Stavudine were found to show appreciable absorbance at 212 nm when determined spectrophotometrically and hence it was selected as the detection wavelength. An optimized chromatogram showing the separation of Lamivudine and Stavudine at different RTs was shown in Figure 2.

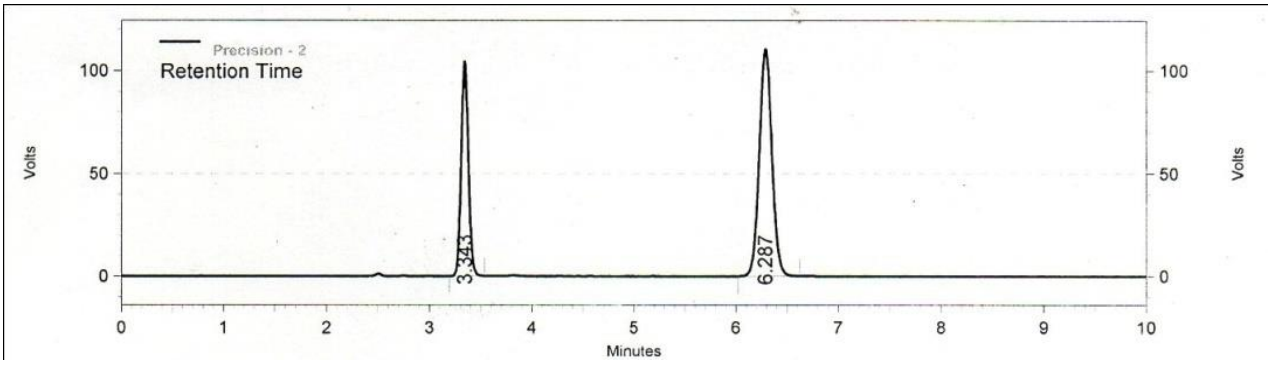


Figure 2: Optimized chromatogram of lamivudine and stavudine

System suitability was determined by injecting 5 replicate injections of 100% test concentration, Number of theoretical plates, HETP and resolution were found to be satisfactory. The chromatograms confirm the presence of Lamivudine and Stavudine at 3.3 min and 6.2 min

respectively without any interference. System conditions were given in Table 1.

Parameters	Lamivudine	Stavudine
Retention time (min)	3.3	6.29
Theoretical plates (N)	14522	11254
Tailing factor (T)	1.2	1.4
Resolution (Rs)	2.99	

∗n= No. of determinants

Table 1: System suitability parameters (n=5)

Concentration range of 20-100 µg/ml for Lamivudine and Stavudine were found to be linear with correlation coefficients 0.998 and 0.999 for Lamivudine and Stavudine respectively. The calibration curves of the respective samples were shown in Figures 3 and 4 respectively. The results were kept in Table 2.

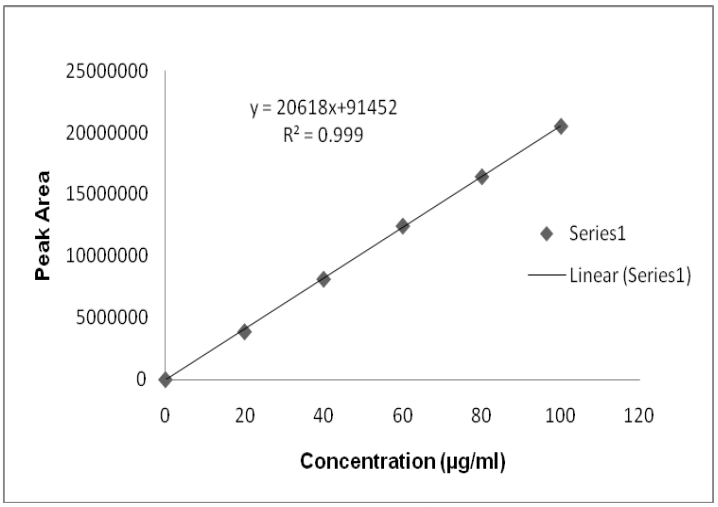


Figure 3: Calibration plot of lamivudine

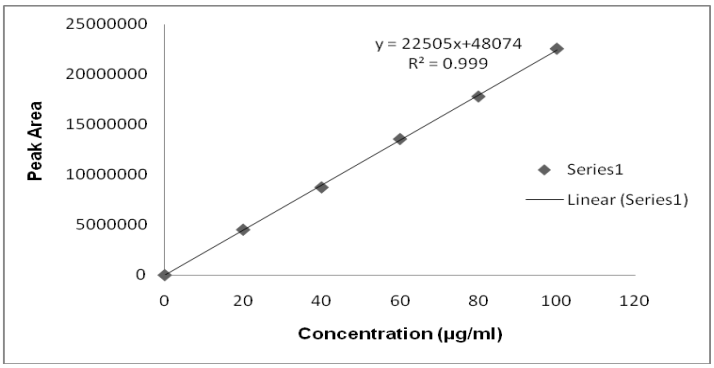


Figure 4: Calibration plot of stavudine

The limits of detection for Lamivudine and Stavudine were found to be 0.65 µg/ml and 0.54 µg/ml respectively and the limits of quantitation were 1.99 µg/ml and 1.64 µg/ml respectively. Values were represented in Table 2.

Parameter	Lamivudine	Stavudine
Linearity Range (µg/ml)	20-100	20-100
Regression Equation	y=16324x+12346	y=86676x + 37329
Slope (m)	16324	86676
Intercept (c)	12346	37329
Regression Coefficient (r2)	0.998	0.999
Limit of Detection (µg/ml)	0.75	0.91
Limit of Quantitation (µg/ml)	2.49	3.003

∗n= No. of determinants

Table 2: Results for linearity (n=3)

The proposed method was found to be precise and reproducible with %RSD of 0.42 and 0.19 for Lamivudine and Stavudine respectively. %RSD was reported in Table 3.

Drug	Intraday Precision (%RSD)	Interday Precision (%RSD)
Lamivudine	0.51	0.71
Stavudine	0.47	0.25

∗n= No. of determinants

Table 3: Results of precision (n=6)

Accuracy of the method was verified by conducting recovery studies by standard addition method. The percent recovery of the standard added to the pre-analysed sample was calculated and it was found to be 98.6% to 99.4% for Lamivudine and 98.4 to 99.3% for Stavudine. This indicates that the method was accurate. Values obtained were kept in Table 4.

Recovery level	Lamivudine				Stavudine			
	Amount Added (µg/ml)		Amount Found (µg/ml)	% Recovery	Amount Added (µg/ml)		Amount Found (µg/ml)	% Recovery
	Std	test			std	Test		
80%	20	60	79.4	99.25	20	60	79.1	98.8
100%	40	60	99.6	99.6	40	60	99.4	99.4
120%	60	60	119.1	99.25	60	60	119.6	99.66
Mean recovery	99.36				99.28			

∗n= No. of determinant

Table 4: Results for accuracy (n=3)

The robustness of the method was determined by changing the conditions like detection wavelength (± 2 nm) and flow rate (± 0.2 ml). For each variation % RSD was calculated and reported. Values attained were kept in Table 5.

Parameters (n=3)	%RSD	
	Lamivudine	Stavudine
Detection wavelength at 210 nm	0.34	0.38
Detection wavelength at 214 nm	0.52	0.87
Flow rate 0.8 ml/min	0.37	0.91
Flow rate 1.2 ml/min	0.76	0.63

*n= No. of determinant

Table 5: Results for robustness (n=3)

The method was found to be explicit for the blend of interest subsequent to checking the chromatograms demonstrating no interference of the excipients present. Hence, the method was well suitable for the estimation of the commercial formulations of the selected combination with a percentage purity of 99.4% for Lamivudine and 99.25% for Stavudine. The normal chromatogram for assay of marketed formulations was shown in figure.5 and Values obtained were kept in Table 6.

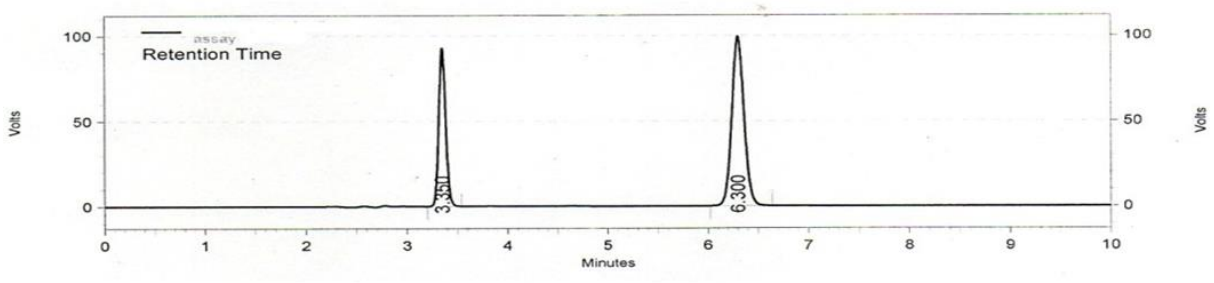


Figure 5: A typical chromatogram for assay of marketed formulation containing 120 µG/ML of lamivudine and 40 µG/ML of stavudine

Drug	Label claim (mg/tab)	Amount recovered	% Amount found in drug
Lamivudine	150	149.1	99.4
Stavudine	40	39.7	99.25

∗n= No. of determinants

Table 6: Results for assay (n=3) of marketed formulation

CONCLUSION

The RP-HPLC method developed and validated allows a simple and fast quantitative determination of Lamivudine and Stavudine from their formulations. All the validation parameters were within the limits as per ICH guidelines. The developed method was specific for the drugs of interest disregarding the excipients present and the method was found to be simple, precise, accurate, rugged and robust. Hence the developed method can be employed in the regular analysis of marketed formulations.

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