



HPLC METHOD DEVELOPMENT AND VALIDATION FOR SIMULTANEOUS ESTIMATION OF LAMIVUDINE AND STAVUDINE IN BULK AND COMBINED TABLET DOSAGE FORM

G.KUMARASWAMY^{1*}, J.M.RAJENDRA KUMAR², J.V.L.N. SHESHAGIRI RAO³, D.VINAY KUMAR⁴, D.PRABAKAR⁴, U.ASHOK KUMAR¹

¹Department of Pharmaceutical Analysis, Trinity College of Pharmaceutical sciences, Peddapalli, Karimnagar (dist) - 505172.A.P, ²Holy Mary Institute of Technology and Science (HITS), Ghatkesar, Hyderabad, ³University College of Pharmaceutical Sciences, Andhra University, Visakhapatnam-A.P.India, ⁴School of Pharmacy, JNTU Kakinada.A.P *Email: kumaraswamy.gandla@gmail.com

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ABSTRACT

A Simple, accurate and precise HPLC method was developed for the simultaneous estimation of Lamivudine and Stavudine in bulk and pharmaceutical dosage forms. The quantification was carried out using symmetry C-18, Thermo (100×4.6), i.d. and 3.5µm particle size in isocratic mode, with mobile phase comprising of Phosphate buffer pH 2.5: Methanol (80:20v/v). The flow rate was 0.8 ml/min and the detection was carried out by UV detector dual i.e. wavelength of 262nm for Stavudine and 271nm for Lamivudine was selected. The retention time for Lamivudine and Stavudine was found to be 2.16 and 4.32 min, respectively. The method was found to be linear in the range of 25-125 µg/ml for Lamivudine and Stavudine. The developed method was validated in terms of accuracy, precision, LOD, LOQ, robustness and solution stability. The percentage recovery was found to be 98.73 and 99.15 % for Lamivudine and Stavudine respectively. The proposed method can be successfully used for the estimation of Lamivudine and Stavudine in bulk and combined dosage forms.

Keywords: HPLC- UV detector, Lamivudine, Stavudine, Tablet dosage form

INTRODUCTION

Lamivudine¹ (fig.1) is chemically 4-amino-1-[(2R, 5S)-2-(hydroxymethyl)-1, 3-oxathiolan-5-yl] pyrimidin-2(1H)-one. Lamivudine is compound with a molecular formula C₈H₁₁N₃O₃S g mol⁻¹ and is a white to off-white crystalline solid. Lamivudine is practically insoluble in acetone, very soluble in Soluble in water and methanol.

Lamivudine is an analogue of Cytidine. It can inhibit both types (1 and 2) of HIV reverse Transcriptase and also the reverse transcriptase of Hepatitis B. It needs to be phosphorylated to its

triphosphate form before it is active. 3TC-triphosphate also inhibits cellular DNA polymerase. **Stavudine**² (fig.2) is chemically 1-[(2R, 5S)-5-(hydroxymethyl)-2, 5-dihydrofuran-2-yl]-5-methyl pyrimidine- 2, 4(1H, 3H) - dione. Stavudine is compound with a molecular formula C₁₀H₁₂N₂O₄ g mol⁻¹ and is a white to almost white powder. Stavudine is Soluble in water and sparingly soluble in ethanol (~750 g/l) TS (ethanol (95 per cent)).

Stavudine is a nucleoside analogue of Thymidine. It possesses virustatic activity against human immunodeficiency virus type 1 (HIV - 1) and type 2 (HIV - 2). It inhibits the replication of HIV in human cells in vitro.

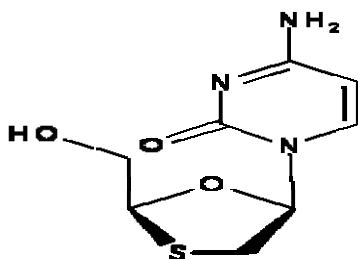


Fig. 1: structure of Lamivudine

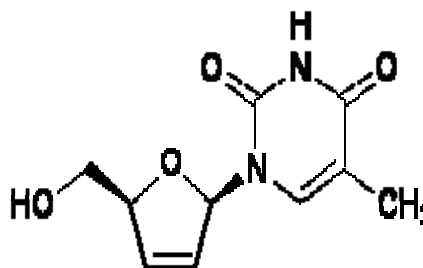


Fig. 2: structure of Stavudine

Pharmacology of Lamivudine and Stavudine³

Lamivudine was rapidly absorbed after oral administration and maximum plasma concentrations are attained within 1 hour of administration. The area under the plasma concentration versus time curve (AUC) and C_{max} increased in proportion to oral dose over the range from 0.25 to 10 mg/kg.

The accumulation ratio of Lamivudine in HIV-1-positive asymptomatic adults with normal renal function was 1.50 following 15 days of oral administration of 2 mg/kg twice daily. Stavudine is a nucleoside analogue of Thymidine. It possesses virustatic activity against human immunodeficiency virus type 1 (HIV - 1) and type 2 (HIV - 2). It inhibits the replication of HIV in human cells in vitro.

Stavudine (Zerit) is a nucleoside analogue antiviral used in combination with other medicines to manage Human Immunodeficiency Virus (HIV) infection.

Literature review⁴⁻¹⁰ shows that there are developed methods including developed Simultaneous quantification of Lamivudine, Stavudine and Nevirapine by U.V spectroscopy, RP-HPLC and HPTLC in tablets. In RP-HPLC method the drugs were separated using C18-ODS-Hypersil (5µm,250mm×46mm) with mobile phase of 20mm sodium phosphate buffer pH 3.5: Acetonitril (4:1v/v) at flow rate of 1.0ml/min and detection wavelength of 265nm. The retention time of Lamivudine and Stavudine was found to be 4.33 and 2.85min and the recovery values was in the range of 99.16-101.89%. But till this date no simultaneous method has been published anywhere for the simultaneous estimation of both drugs. So the aim of our study is to

develop simple, fast, accurate and specific HPLC with UV detection method for simultaneous estimation of Lamivudine and Stavudine in bulk and combined dosage formulations.

EXPERIMENTAL¹¹⁻¹⁹

Materials and instruments

Reference standards of Lamivudine and Stavudine were obtained as gift samples from Pharmatrain Research centre, Hyderabad. Market formulation of this combination Generic Epivir and Zerit (Lamivudine + Stavudine) were procured from the local market. HPLC grade Methanol was obtained from Merck (India). Analytical grade potassium hydrogen phosphate buffer and potassium hydroxide were purchased from SD Fine chemicals, India. Water obtained from Millipore with milli Q system, filtered through 0.45 μ nylon-66 membrane was used for the HPLC work. The LC system consisted of isocratic pump, auto sampler and UV detector. The output signal was monitored and integrated using Empower Software (Prominence HPLC, Water's) was employed for the present study.

Chromatographic conditions²⁰⁻²³

The analysis was carried out with UV detection at 266 nm using a 20 μ l Injection volume. Assay was performed using a C18 reversed-phase column eluted with Phosphate buffer pH 2.5: Methanol (80:20 v/v), at a flow rate of 0.8 ml/min. Chromatography was carried out at ambient temperature. The solvents were mixed, filtered through a membrane filter of 0.45 micron pore and degassed in ultrasonic bath prior to use.

Preparation of phosphate buffer pH 2.5²⁴⁻²⁶

Weigh accurately 3.5 grams of KH₂PO₄ into a 500ml beaker, dissolved and diluted to 500ml with HPLC water. Adjusted the pH to 2.5 with orthophosphoric acid and degassed in ultrasonic water bath for 5 minutes. Filter through 0.45 μ filter under vacuum filtration.

Preparation of mobile phase

The mobile phase is prepared by mixing a mixture of above buffer 80ml of pH 2.5 phosphate buffer and 20ml of Methanol (HPLC grade) in 100ml of volumetric flask.

Preparation of standard solution

Weigh Accurately and transfer 25 mg of Lamivudine & 25mg of Stavudine working standard into a 100 ml volumetric flask add about 70 ml of diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent (Stock solution). Further pipette 2 ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent. Mix well and filter through 0.45 μ m filter. (Fig.No.3)

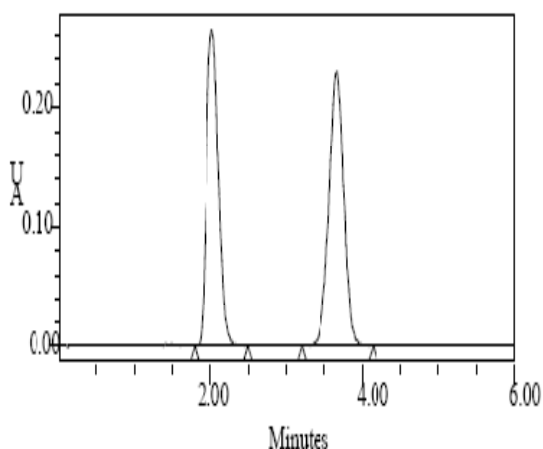


Fig. 3: Standard chromatogram of Stavudine and Lamivudine

Preparation of sample solution

Twenty tablets were weighed accurately and powdered. A quantity of tablet powder equivalent to

Weigh 20 tablets of Lamivudine & Stavudine and calculate the average weight. Weigh accurately and transfer the sample equivalent to 25 mg of Lamivudine and 25mg of Stavudine into a 100 ml volumetric flask. Add about 70 ml of diluent and sonicate to dissolve it completely and make volume up to the mark with diluent. Mix well and filter through 0.45 μ m filter. Further pipette 2ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent. After setting the chromatographic conditions and stabilizing the instrument to obtain a steady baseline, the tablet sample solution was injected, chromatogram was obtained and the peak areas were recorded. The injections were repeated six times and the amount of each drug present per tablet was estimated from the respective calibration curves.

Method validation

The method was validated for linearity, accuracy, intra-day and inter-day precision and robustness, in accordance with ICH guidelines

Selection of wavelength

From the UV-visible Spectrophotometric results, a detection wavelength of 262nm for Stavudine and 271nm for Lamivudine was selected. Because at this wavelength they shows maximum absorbance and then 266nm was selected as common wavelength for simultaneous estimation of both the drugs, as these are eluting in the same mobile phase with good absorbance. The maximum absorbance with good peak intensity, good peak shape and height was observed at 266nm (Fig. 4).

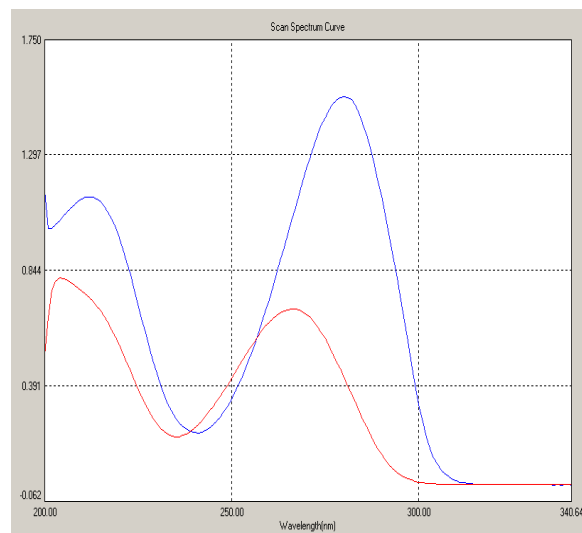


Fig. 4: Overlay spectra between Lamivudine and Stavudine

Linearity

A series of standard curves were prepared over a concentration range of 25 -125 μ g/ml by diluting the standard stock solution of Lamivudine and Stavudine with mobile phase. The data from peak area versus drug concentration plots were treated by linear least square regression analysis and r^2 was found 0.9998 of Lamivudine and Stavudine (fig.5). The standard curves were evaluated for intra-day and inter-day reproducibility. Each experiment was repeated in triplicate (Table.1).

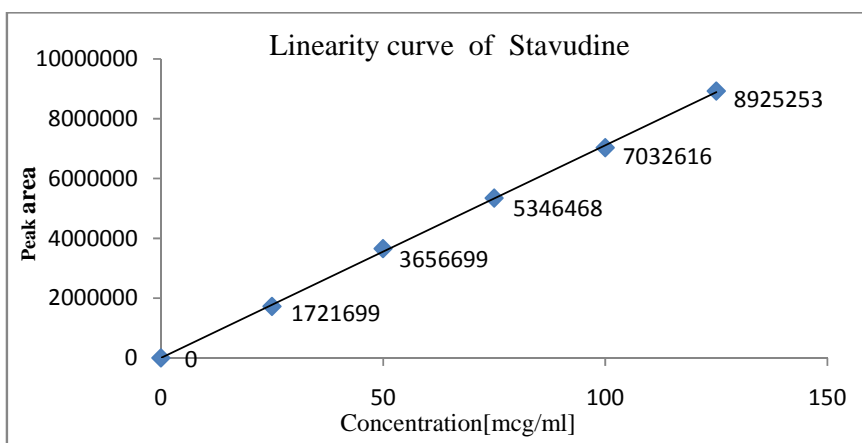
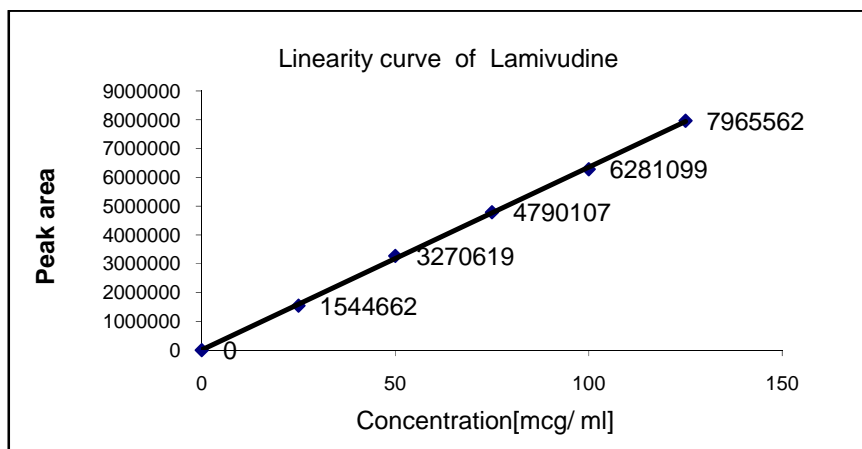


Fig. 5: Calibration curve of Lamivudine and Stavudine

Table 1: Calibration curve of Lamivudine and Stavudine

S.No	Parameter	Lamivudine	Stavudine
1	Correlation coefficient	0.9998	0.9998
2	Slope (m)	63493.3	71141.1
3	Intercept (c)	7012.2	780.7
4	LOD ^a (µg/ml)	0.8927	0.0887
5	LOQ ^b (µg/ml)	2.070	0.2688

^aLOD = Limit of detection; ^bLOQ =Limit of quantitation;

Precision

One set of three different concentrations of mixed standard solutions of Lamivudine and Stavudine were prepared. All the solutions were analyzed thrice, in order to record any intraday variations in the results. For Inter day variations study three different concentrations of the mixed standard solutions in linearity range were analyzed on three consecutive days (fig.6). The peak areas were recorded and Relative standard deviation (RSD) was calculated for both series of analyses Table.2.

Accuracy

Recovery studies by the standard addition method were performed with a view to justify the accuracy of the proposed method. Previously analyzed samples Lamivudine & Stavudine were spiked with known amount of standard so as to get three different levels (50%, 100% and 150%) and the mixtures were analyzed by the proposed method (Table.3). The experiment was performed in triplicate. Recovery and % RSD was calculated for each

concentration. The results of accuracy (Table1) revealed that the method was more accurate. (Fig.7)

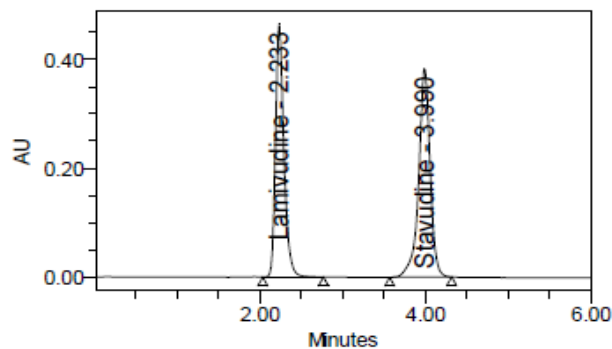


Fig. 6: Precision of Lamivudine & Stavudine

Table 2: Method precision for Lamivudine & Stavudine in combined dosage form

Parameter	Intraday Precision		Interday Precision	
	Lamivudine	Stavudine	Lamivudine	Stavudine
Average	3284765	3675650	3103171	3458791
SD	13312.8	3724.3	34255.5	15284.9
%RSD	0.41	0.10	1.09	0.44

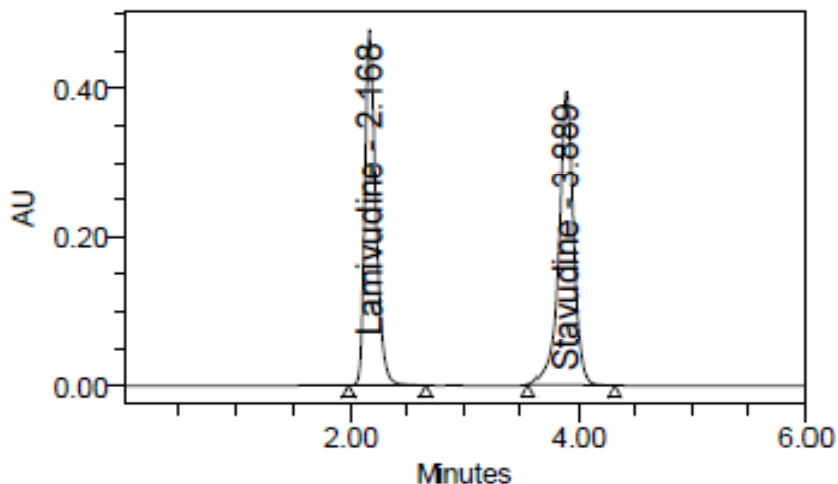


Fig. 7: Accuracy of Lamivudine and Stavudine

Table 3: Recovery studies for Lamivudine and Stavudine

Inject sample	Spike level	Mean area	Amount present	Amount recovered	% recovered	Mean recovery
Lamivudine	50 %	1499303	11.8mg	11.6mg	98.3%	98.7%
	100 %	3207970	25.12mg	24.83mg	98.8%	
	150 %	4706055	36.85mg	36.43mg	98.8%	
Stavudine	50 %	1672538	11.9mg	11.71mg	98.41%	99.1%
	100 %	3566497	25.15mg	24.97mg	99.3%	
	150 %	5196283	36.53mg	36.38mg	99.5%	

Limit of detection (LOD) and limit of quantification (LOQ)

LOD and LOQ were calculated as $3.3 \sigma / S$ and $10 \sigma / S$ respectively; where σ is the standard deviation of the response (y-intercept) and S is the slope of the calibration plot.

Specificity

No interference of peaks were found in the chromatogram indicating that excipients used in the tablet formulation did not interfere with the estimation of the drugs by the proposed method for the simultaneous determination of Lamivudine & Stavudine in the combined dosage form, hence the method is specific.

Ruggedness

The ruggedness of the method was demonstrated by analysis of the samples as for precision study by a second analyst. The RSD of the two sets of data indicates the ruggedness of the method. Further, the t-test was performed on the data and the difference was found to be not significant.

Robustness

The robustness of the method was determined to assess the effect of small but deliberate changes of the chromatographic conditions on the determination of Lamivudine and Stavudine. The different variations are in flow rates by ± 0.1 ml/min, in wavelength by ± 2 nm and in temperature by ± 5 °C. The concentration of the solution analyzed was 15 µg/ml. (shown as fig.8).

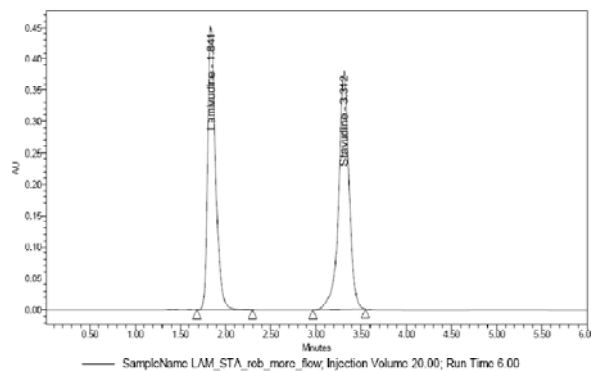


Fig. 8: Robustness of Lamivudine and Stavudine

System suitability

The chromatographic systems used for analyses must pass the system suitability limits before sample analysis can commence. The capacity factor (K), injection repeatability, tailing factor (T), 1.24 and 0.97 for Lamivudine and Stavudine theoretical plate number (N) 2772 and 2687 Lamivudine and Stavudine. Resolution (Rs) for the principal peaks was the parameters tested on a 10 µg/ml samples of Lamivudine and Stavudine to assist the accuracy and precision of the developed HPLC system Results were represented in Table 4.

Table 4: System suitability parameters for HPLC method

S.No	System suitability parameters	Lamivudine	Stavudine
1	Tailing factor (T)	1.24	0.97
2	Number of theoretical plate(n)	2772	2687
3	Retention time (Rt)	2.16	4.32
4	%RSD	0.41	0.10

RESULTS AND DISCUSSION

A simple, selective, accurate and Precise HPLC method for simultaneous estimation of Lamivudine and Stavudine in bulk and pharmaceutical formulation has been developed and validated. The linearity range was determined by external standard calibration method in the concentration range of 25-125µg/ml. The correlation co-efficient was found to be 0.9998 and 0.9998 indicated that the concentrations of Lamivudine and Stavudine had good linearity. The LOD and LOQ were found to be 0.8927, 0.0887 and 2.0705, 0.2688 µg/ml of Lamivudine and Stavudine respectively. The system suitability parameters like capacity factor, asymmetric factor, tailing factor, HETP and number of theoretical plates were calculated and it was observed that all the values are within the limits. Further the precision of the method was confirmed by the repeatable analysis of formulation. The % RSD was found to be 0.8077. It indicated that the method has good precision. The percentage recovery of Lamivudine and Stavudine present in formulation was found to be 98.7±1.25 and 99.1± 1.351 the percentage RSD value was found to be 0.8700. The low percentage RSD value indicated that there is no interference due to excipients used in formulation. Hence, the accuracy of the method was confirmed.

CONCLUSION

An HPLC method has been developed for the simultaneous estimation of Lamivudine and Stavudine in tablet dosage forms, using UV-detector. The developed method was validated as per ICH guidelines²⁷⁻²⁹ and specificity, linearity & range, accuracy, precision and robustness was performed. Specificity was determined by comparing the results obtained by running the placebo solution with that of standard and method was found to be specific due to no interference between placebo peaks and drugs peaks. Estimation of the drugs was performed by developed method in market formulations, Lamivudine and Stavudine. The assay was found to be 98.7% & 99.1% for Lamivudine and Stavudine tablets respectively. It is evident from the study that the developed method is simple, specific, precise and accurate. This newly developed method can be used for routine analysis as method for the simultaneous estimation of Lamivudine and Stavudine in bulk and tablet dosage forms.

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