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Method Development and Validation for the Simultaneous Estimation of Lamivudine and Stavudine by RP-HPLC

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ABSTRACT

The proposed method was found to be simple, sensitive, rapid and economical for the determination of Lamivudine and Stavudine in combined tablet formulation. The developed method was checked for the performance characteristics and has also been validated. The method was found to be linear (r>0.999), precise (RSD: 0.41 for Lamivudine, 0.10 for Stavudine) and accuracy (mean percentage recovery fields 98.7% for Lamivudine, 99.1% for Stavudine). The proposed HPLC method was simple, precise because of commonly used buffer and shorter run time. The mean percentage recovery above 95% indicates the reproducibility and accuracy of new developed method compared. The result of study include the proposed method is highly accurate, simple, precise and specific. The sample recoveries in all formulations were in good agreement with their respective label claims and they suggest non-interference of formulation excipients in the estimation. After validating proposed method as per ICH guidelines and correlating obtained values with the standard values, satisfactory results were obtained. Hence the method can easily and conveniently adopted for the estimation of combined dosage form of Lamivudine and Stavudine.

INTRODUCTION

The HPLC method was considered the choice of estimation since this method is the most powerful of all chromatographic than other separative methods. The HPLC method has enabled analytical chemist to attain great success in solving analytical problems. The HPLC is the method of choice in the field of analytical chemistry since this method is specific, robust, linear, precise, and accurate and the limit of detection is low. HPLC is a form of column chromatography used frequently in biochemistry and analytical chemistry to separate, identify, and quantify compounds. HPLC utilizes a column that holds chromatographic packing material (stationary phase), a pump that moves the mobile phase(s) through the column, and a detector that shows the retention times of the molecules. Retention time varies depending on the interactions between the stationary phase, the molecules being analyzed and the solvent(s) used.

Normal phase chromatography is also known as Normal Phase HPLC (NP-HPLC) or adsorption chromatography, separate analytes based on adsorption to a stationary surface chemistry and by polarity. It was one of the first kinds of HPLC that chemists developed. In this type stationary phase used is polar in nature and the mobile phase used is non-polar and nonaqueous in nature depending on the nature of the analyte and stationary phase. If the affinity between the stationary phase and the analyte increases the selection time (R_t) of the analyte also increases and *vice versa*. The interaction strength depends not only on the functional groups in the analyte molecule but also on steric factors. The effect of steric on interaction strength allows this method to resolve (separate) structural isomers. In reverse phase technique, a non-polar stationary phase is used and the mobile phase is polar in nature. Hence polar components get eluted first and non-polar compounds are retained for a longer time. Since most of the drugs and pharmaceuticals are polar in nature, they are not retained for a longer time and eluted faster, columns used in the mode of chromatogram are ODS (Octadecyl silane) or C_{18}, C_8, C_4, etc.

Partition chromatography was the first kind of chromatography that chemists developed. The partition coefficient principle has been applied in paper chromatography, thin layer chromatography, gas phase and liquid-liquid applications. Partition chromatography uses a retained solvent, on the surface or within the grains or fibers of an "inert" solid supporting matrix as with paper chromatography; or takes advantage of some additional columbic and/or hydrogen donor interaction with the solid support. Molecules equilibrate (partition) between a

liquid stationary phase and the eluent separate analytes based on the polar differences is known as Hydrophilic Interaction Liquid Chromatography (HILIC). Partition HPLC has been used historically on unbonded silica or alumina supports. Each works effectively for separating analytes by relative polar differences. However, HILIC has the advantage of separating acidic, basic and neutral solutes in a single chromatogram.

Validation by definition is an act of proving that any procedures, process, equipment, materials, activity or system performs as expected under a given set of conditions basically validation is proving that the performance is as intended when extended to an analytical procedure, depending upon the application, it means that a method works reproducibly when carried out by some different persons, in same or different laboratories using different reagents, different equipments etc.

MATERIALS AND METHODS

MATERIALS REQUIRED

Table No. 1: List of Instruments Used

INSTRUMENTS	NAME OF THE COMPANY
HPLC	Water's model 2695
U.V detector	Dual λ absorbance detector model 2487
Analytical balance	Ascoset
Ultrasonicate water-bath	Ultrasonicate SE-COUS
pH meter	ADWA Model AD1020

DRUGS AND CHEMICALS REQUIRED:

Table No. 2: List of Chemicals and Reagents

CHEMICALS	NAME OF THE COMPANY
Lamivudine	Pharmatrain Research centre**.** Hyderabad
Stavudine	Pharmatrain Research centre**.** Hyderabad
Potassium dihydrogen phosphate	E.Merck
Methanol	E.Merck
Orthophosphoric acid	E.Merck
Water	E.Merck

OPTIMIZED CHROMATOGRAPHIC CONDITIONS:

Table No. 3: Optimized Chromatographic Conditions

Mode of separation	Isocratic elution
Mobile phase	Phosphate buffer pH 2.5**:**Methanol (80:20)
Column	C-18, Thermo (100×4.6, 3.5µm)
Flow rate	0.8 mL/ min
Detection Wavelength	266 nm
Injection volume	20 µl
Column oven	Ambient
Run time	6 min

Preparation of Phosphate buffer pH 2.5:

Weigh accurately 3.5 grams of KH_2PO_4 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 0.45µ 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:

Accurately weigh 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 0.45µm filter.

Preparation of Sample Solution:

Weigh 4 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 0.45µm filter. Further pipette 2ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent. Mix well and filter through 0.45µm filter.

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 266 nm.

OVERLAID SPECTRA OF LAMIVUDINE AND STAVUDINE

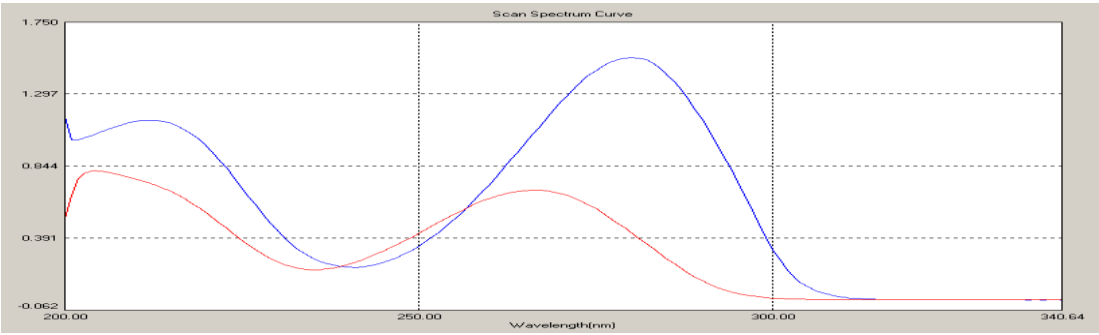

Figure No. 1: Maximum absorbance occur at 266nm in the Overlaid spectra of Lamivudine and Stavudine

Accuracy:

Preparation of standard stock solution:

Accurately weigh and transfer 25mg of Lamivudine and 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).

Preparation of 50µg/ml 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 0.45µm filter.

Preparation 50% sample solution:

Accurately weigh and transfer 12.5mg of Lamivudine and 12.5 mg of Stavudine Active Pharmaceutical Ingredient (API) sample 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 2ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent. Mix well and filter through 0.45µm filter.

Preparation of 100% solution:

Accurately weigh and transfer 25mg of Lamivudine and 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 0.45µm filter.

Preparation of 150% solution:

Accurately weigh and transfer 37.5 mg of Lamivudine and 37.5 mg of Stavudine API sample 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 2ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent. Mix well and filter through 0.45µm filter.

Procedure:

Inject the standard solution, Accuracy -50%, Accuracy -100% and Accuracy -150% solutions into HPLC. Now calculate the amount obtained and amount added API for Lamivudine and Stavudine samples. Calculate the individual recovery and mean recovery values.

Precision:

Procedure:

The standard solution of 50 µg/ml was injected for five times and area is calculated. The %RSD for the area of five replicate injections was found to be within the specified limits. The precision was done for Intraday and Interday.

Intraday Precision:

To evaluate the intraday precision the injections are carried out at mean time interval of for five times.

Intermediate Precision:

To evaluate the intermediate precision (also known as Ruggedness) of the method, Precision was performed on different day by using different make column of same dimensions.

Preparation of stock solution:

Accurately weigh and transfer 25mg of Lamivudine and 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).

Preparation of 50µg/ml 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 0.45µm filter.

Procedure:

The standard solution50 µg/ml was injected for five times and area is the %RSD for the area of five replicate injections was found to be within the specified limits.

Linearity:

Preparation of stock solution:

Accurately weigh and transfer 25 mg of Lamivudine and 25 mg of Stavudine API sample 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).

Preparation of (25, 50, 75, 100 and 125 µg/ml) sample solutions:

From the above stock 25,50,75,100,125 µg/ml of sample solutions has been prepared by pipetting out of 1,2,3,4,5ml in 10ml of volumetric flask with buffer solution pH 2.5.

Procedure:

Inject each level into the chromatographic system and measure the peak area. Plot a graph of peak area versus concentration (on X-axis concentration and on Y-axis Peak area) and calculate the correlation coefficient.

RESULTS

Accuracy:

Accuracy 50%:

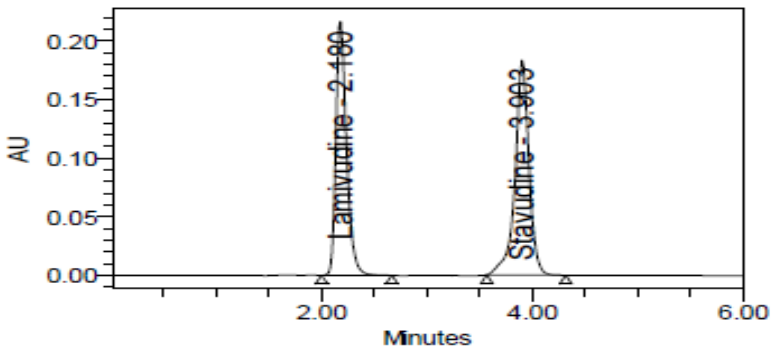

Figure No. 2: Chromatogram of accuracy 50% solution

Accuracy 100%:

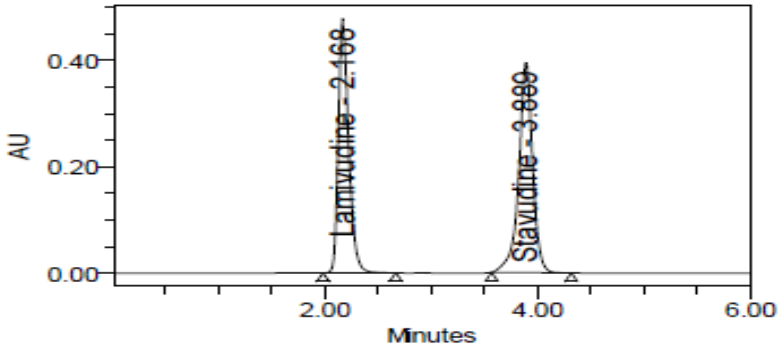

Figure No. 3: Chromatogram of accuracy 100% solution

Accuracy 150%:

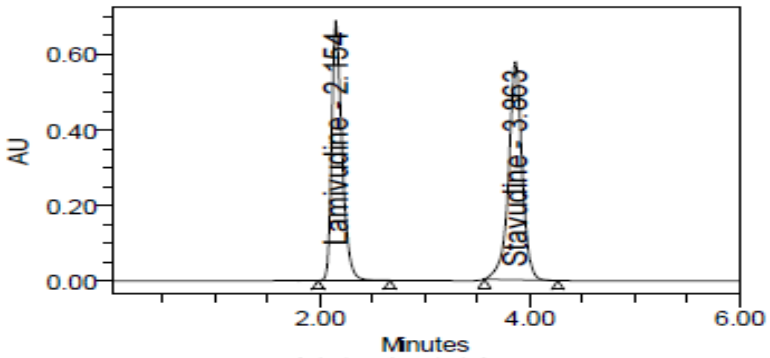

Figure No. 4: Chromatogram of accuracy 150% solution

Table No. 4: Recovery studies for Lamivudine and Stavudine

Injection 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%	

Precision:

Intraday Precision:

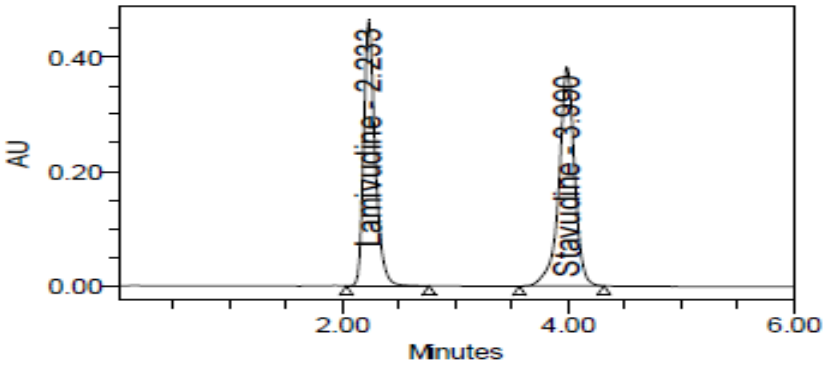

Figure No. 5: Chromatogram for Precision

Interday Precision:

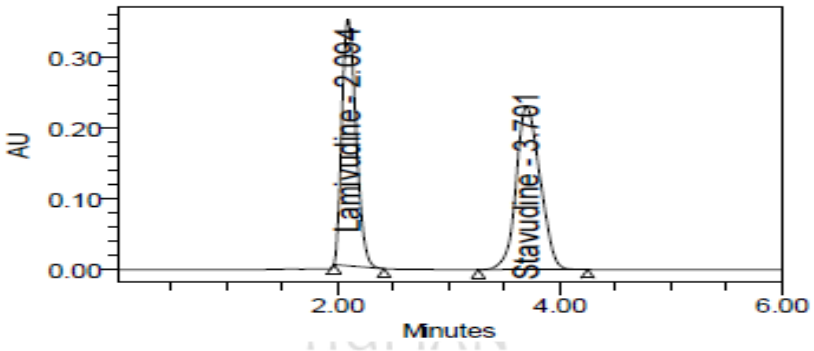

Figure No. 6: Chromatogram for Interday Precision

Table No. 5: Chromatographic parameters for Intraday and Interday Precision

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

n = 5; n = no. of injections

Linearity:

Lamivudine:

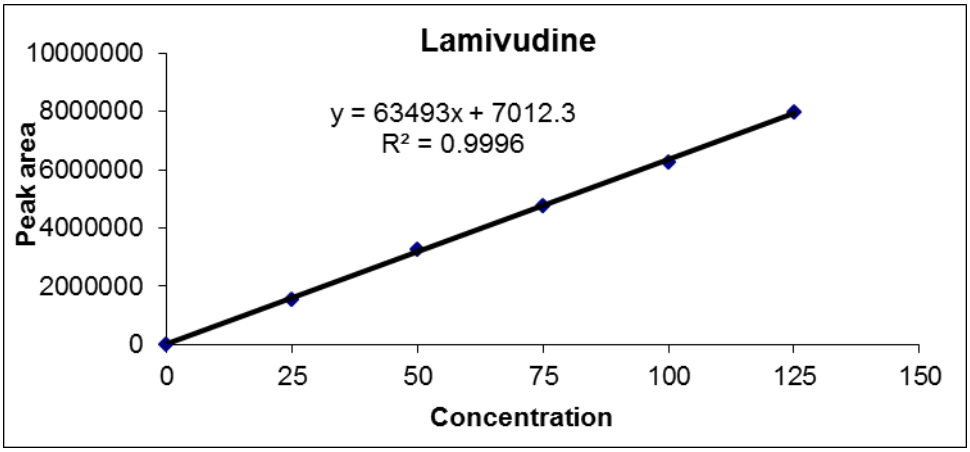

Figure No. 7: Chromatogram for linearity curve of Lamivudine

Table No. 6: Linearity of Lamivudine Stavudine:

S. No.	Linearity Level	Concentration	Area
1	I	25 µg/ml	1544662
2	II	50µg/ml	3270619
3	III	75µg/ml	4790107
4	IV	100 µg/ml	6281099
5	V	125 µg/ml	7965562
Correlation Coefficient			0.999

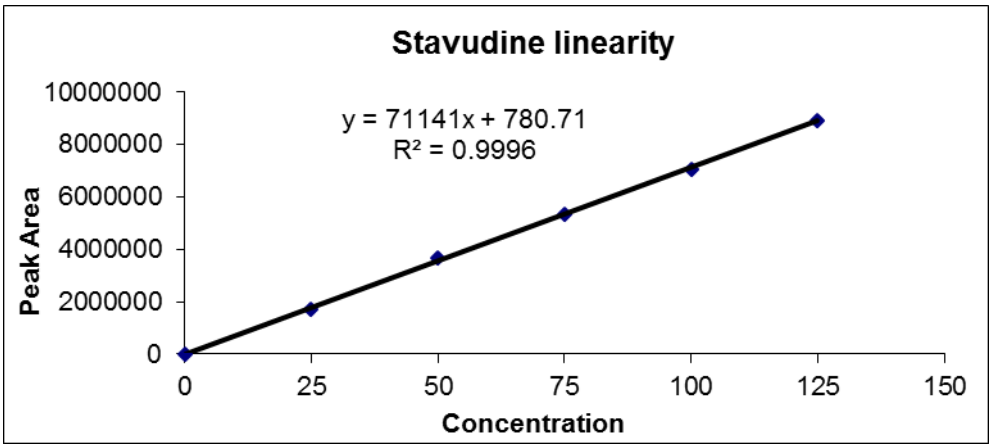

Figure No. 8: Chromatogram for linearity curve of Stavudine

Table No. 7: Linearity of Stavudine

Sr. No.	Linearity Level	Concentration	Area
1	I	25 µg/ml	1721699
2	II	50µg/ml	3656699
3	III	75 µg/ml	5346468
4	IV	100 µg/ml	7032616
5	V	125 µg/ml	8925253
Correlation Coefficient			0.999

DISCUSSION

An effort has been made to identify a simple, precise, specific and accurate method for the estimation of Lamivudine and Stavudine in formulations by using RP-HPLC method.

During the selection of mobile phase, several solvents were tried at various levels and finally selected mobile phase system was methanol and phosphate buffer of pH 2.5 at ratio 80:20.

The solution of 10µg/ml of Lamivudine and Stavudine in mobile phase (methanol: phosphate buffer pH 2.5) was prepared and the solution was scanned in the range of 200-400nm. At 266nm the drugs shows maximum absorbance overlapping spectrum. Hence this was selected as a detection wavelength of U.V. spectrum shown in figure. After considering all the system suitability parameters methanol and phosphate buffer pH 2.5 (80:20) was selected for analysis at optimized flow rate of 0.8ml/min. The retention time of Lamivudine and Stavudine was found to be 2.017min and 3.66 min.

The calibration was done with the optimized chromatographic conditions, stock solution of Lamivudine and Stavudine using mobile phase and various concentration ranges of 25 to 125µg/ml were prepared. From this 20µl of each solution were injected individually and the chromatogram was recorded at 266nm. The linearity graph was plotted using concentration against peak area. The correlation coefficient for both drugs was found to be 0.995 and 0.996 indicates that the concentration of Lamivudine and Stavudine had given good linearity as shown in figures.

Accuracy was confirmed by recovery studies by proposed method and their chromatograms were recorded as shown in the Figures 3, 5 & 6. The percentage recovery of Lamivudine and

Stavudine was found to be 98.7% and 99.1% which are within the limits as shown in table. The high percentage of recovery indicates that there are no interfere will be produced. Hence the developed method was found to be accurate.

The precision has done in two ways i.e. Intraday and Interday precision. 20µl of 5 injection samples are injected for each precision. For Intraday precision, the %RSD values were found to be 0.41 and 0.10 for Lamivudine and Stavudine respectively. For the Inter day precision, the %RSD values were found to be 1.09 and 0.44 for Lamivudine and Stavudine respectively as shown in table 5.

All the above parameters combined with the simplicity and ease of operation ensures that the RP-HPLC method can be applied for the Simultaneous estimation of Lamivudine and Stavudine in tablet dosage form.

CONCLUSION

The proposed method was found to be simple, sensitive, rapid and economical for the determination of Lamivudine and Stavudine in combined tablet formulation. The developed method was checked for the performance characteristics and has also been validated. The method was found to be linear (r>0.999), precise (RSD: 0.41 for Lamivudine, 0.10 for Stavudine) and accuracy (mean percentage recovery fields 98.7% for Lamivudine, 99.1% for Stavudine). The proposed HPLC method was simple, precise because of commonly used buffer and shorter run time. The mean percentage recovery above 95% indicates the reproducibility and accuracy of new developed method compared. The result of study include the proposed method is highly accurate, simple, precise and specific. The sample recoveries in all formulations were in good agreement with their respective label claims and they suggest non-interference of formulation excipients in the estimation. After validating proposed method as per ICH guidelines and correlating obtained values with the standard values, satisfactory results were obtained. Hence the method can easily and conveniently adopted for the estimation of combined dosage form of Lamivudine and Stavudine.

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I humbly present this work to the eternal almighty. Indeed my final work is done with the help of primitive persons at heart. So it is my bounded duty to promulgate them individually.

I wish to extend my sincere thanks to my beloved guide Dr. Anil Kumar V, Associate Professor, Department of Pharmaceutical Analysis, Sahasra Institute of Pharmaceutical Sciences, Warangal for his encouragement, which made me to finish this work.

I would like to thank Pharmatrain Research Centre**,** Hyderabad for providing Lamivudine and Stavudine API as a gift samples.

ABBREVIATIONS

HPLC	High performance Liquid Chromatography
%	Percent
PDA	Photodiode Array
ICH	International Conference for Harmonization
GR	General reagent
C18	Octadecyl
UV	Ultraviolet
ml	Milliliter
Min	Minute
MeOH	Methanol
µl	Micro Liter
µ	Micron
µg	Micro gram
ppm	Parts per million
nm	Nanometer
RSD	Relative Standard Deviation
Fig	Figure

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