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Development and Validation of a Simple, Accurate, and Precise RP-HPLC Method for Simultaneous Estimation of Lamivudine and Stavudine in Combined Pharmaceutical Dosage Form

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ABSTRACT

Objective: Combination of Lamivudine and Stavudine was approved by FDA for the treatment of human immunodeficiency virus (HIV). The objective was to develop a simple, accurate and precise Related Substance RP-HPLC method for simultaneous estimation of Lamivudine and Stavudine in combined pharmaceutical dosage form

Experimental Work: A simple, rapid, economical, precise and accurate Related Substance RP-HPLC method for simultaneous estimation of Lamivudine and Stavudine In Their Combined Dosage Form has been developed. The separation was achieved by LC- 20 AT C_{18} (250mm x 4.6 mm, 5 µm) column and Buffer (Potassium Dihydroden Phosphate, pH 4.0) : Acetonitrile (85:15) as mobile phase, at a flow rate of 1 ml/min. Detection was carried out at 212 nm.

Result and Discussion: Related Substance HPLC method was developed and validated. Retention time of Lamivudine and Stavudine were found to be 5.77 min and 2.81 min respectively and the retention time of Lamivudine impurity and Stavudine impurity were found to be 6.14 min and 3.25 min respectively The method has been validated for linearity, accuracy and precision. Linearity observed for Lamivudine impurity 2.5-7.5 µg/ml and for Stavudine impurity 10-30 µg/ml. The LOD were 0.071µg/ml and 0.035µg/ml for Lamivudine impurity and Stavudine impurity respectively. The LOQ were 0.215µg/mL and 0.108µg/mL for Lamivudine impurity and Stavudine impurity respectively. The proposed method was successfully applied for the simultaneous estimation of both the drugs and their related impurity in commercial Combined dosage form.

Conclusion: The Related Substance RP-HPLC methods were found to be simple, accurate, robust and reproducible.

Keywords: Lamivudine, Stavudine, Related Substance RP-HPLC Method, ICH Q2 (R1) guidelines.

I. INTRODUCTION

Literature review reveals that numbers of individual analytical methods available for estimation of Lamivudine and Stavudine in their individual dosage forms and combined dosage form. But no Related Impurities method has been reported for simultaneous estimation of Lamivudine and Stavudine in combined pharmaceutical dosage form by RP-HPLC. So it is thought to develop Related Impurities method for simultaneous estimation of Lamivudine and Stavudine in Combined Dosage Form by RP-HPLC. So Aim of present work is to develop simple, accurate, precise, rapid, specific, sensitive and selective Related Impurities Reverse Phase HPLC method for simultaneous estimation of Lamivudine and Stavudine in combined pharmaceutical dosage.

RATIONAL

- Lamivudine is a Nucleoside reverse transcriptase inhibitors (NRTIs), used as a monotherapy for hepatitis B virus (HBV) and in conjunction with other medications as an antiviral treatment for human immunodeficiency virus type-1 (HIV-1).
- Stavudine is a Nucleoside reverse transcriptase inhibitors (NRTIs) work against HIV-1, also known as the human immunodeficiency virus. Phosphorylation of stavudine produces active metabolites that vie with one another to be incorporated into viral DNA.

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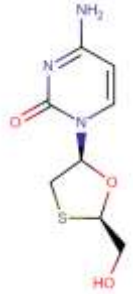
- Analytical method development and validation plays an important role in drug discovery and manufacture of pharmaceuticals. These methods are used to ensure the identity, purity, potency and performance of drug products. Spectrophotometry and HPLC methods are considered to be most suitable for estimation of drugs present in pharmaceutical dosage form.
- The Literature survey reveals that these drugs have been analyzed individually and in combination by many analytical methods like HPLC, UPLC and spectroscopic method, but no method have been reported for the Estimation of Lamivudine and Stavudine for Related Impurities HPLC Method in combined pharmaceutical dosage form.
- So here attempt will be made to develop and validate Related Impurities method for estimation of Lamivudine and Stavudine in combined dosage form by RP-HPLC.

OBJECTIVE OF WORK

- To develop Related Impurities HPLC method for simultaneous estimation of, Lamivudine and Stavudine in pharmaceutical dosage form.
- Applying the newly developed, validated analytical method for the estimation of Lamivudine and Stavudine in formulations.

DRUG PROFILE

DRUG PROFILE OF LAMIVUDINE

INTRODUCTION	
Name	**Lamivudine**
Official in	Not Official in any Pharmacopoeia
Description	Lamivudine is a nucleoside reverse transcriptase inhibitor (NRTI) indicated for the treatment of HIV infection in adults.
Structure	
Chemical Formula	$C_8H_{11}N_3O_3S$
Mol. Weight	229.256 g/mol
IUPAC Name	(-)-1-((2R,5S)-2-(Hydroxymethyl)-1,3-oxathiolan-5-yl)cytosine
Categories	Anti-HIV Agents
Solubility	With a solubility in water
PHARMACOLOGY	
Classes	Nucleoside reverse transcriptase inhibitor (NRTI)

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Mechanism of Action	Lamivudine is an artificial nucleoside analogue, and phosphorylated intracellularly to produce lamivudine triphosphate (L-TP), which is the active 5'-triphosphate metabolite. HIV reverse transcriptase and HBV polymerase integrate this nucleoside analogue into viral DNA, causing DNA chain termination.
PROPERTIES	
State	Solid.
CAS NO.	134678-17-4
Melting point	170-175°C
pK_a	2.7
Water solubility	70 mg/mL
Log P	-0.42

Table 1:Description of Lamivudine

Related Impurities Of Lamivudine

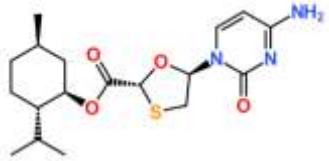
Name	Structure	Chemical name
Lamivudine EP impurity I		(1R,2S,5R)-2-isopropyl-5-methylcyclohexyl(2R,5R)-5-(4-amino-2-oxopyrimidin-1(2H)-yl)-1,3-oxathiolane-2-carboxylate

Table 2: Name and Structure of Impurities of Lamivudine

Drug Profile Of Stavudine

INTRODUCTION	
Name	**Stavudine**
Official in	Not Official in any Pharmacopoeia
Description	Stavudine is a nucleoside reverse transcriptase inhibitor (NRTI) indicated for the treatment of HIV infection

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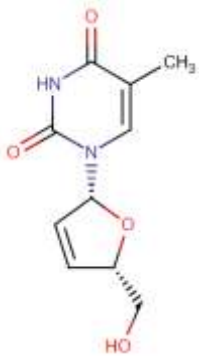
Structure	
Chemical Formula	$C_{10}H_{12}N_2O_4$
Mol. Weight	224.213g/mol
IUPAC Name	1-(2,3-Dideoxy-beta-D-glycero-pent-2-enofuranosyl)thymine
Categories	Nucleoside reverse transcriptase inhibitor (NRTI)
Solubility	Soluble in methanol and sparingly soluble in water
PHARMACOLOGY	
Classes	Nucleoside reverse transcriptase inhibitor (NRTI)
Mechanism of Action	Stavudine inhibits the activity of HIV-1 reverse transcriptase (RT) both by competing with the natural substrate dGTP and by its incorporation into viral DNA.
PROPERTIES	
State	Solid.
CAS NO.	3056-17-5
Melting point	130.134^0C
pK_a	2.7
Water solubility	20 mg/mL
Log P	0.16

Table 3: Description of Stavudine

Related Impurities Of Stavudine

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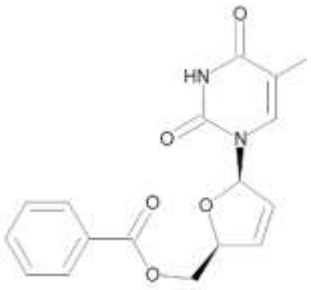
Name	Structure	Chemical name
Stavudine impurity		2',3'-Didehydro-3'-deoxythymidine 5'-benzoate, 5'-O-Benzoyl-2',3'-didehydro-3'-deoxythymidine(as per EP)

Table 4: Name and Structure of Impurities of Stavudine

Literature review reveals that numbers of individual analytical methods available for estimation of Lamivudine and Stavudine in their individual dosage forms and combined dosage form. But no Related Impurities method has been reported for simultaneous estimation of Lamivudine and Stavudine in combined pharmaceutical dosage form by RP-HPLC. So it is thought to develop Related Impurities method for simultaneous estimation of Lamivudine and Stavudine in Combined Dosage Form by RP-HPLC. So Aim of present work is to develop simple, accurate, precise, rapid, specific, sensitive and selective Related Impurities Reverse Phase HPLC method for simultaneous estimation of Lamivudine and Stavudine in combined pharmaceutical dosage.

- Lamivudine is a Nucleoside reverse transcriptase inhibitors (NRTIs), used as a monotherapy for hepatitis B virus (HBV) and in conjunction with other medications as an antiviral treatment for human immunodeficiency virus type-1 (HIV-1).
- Stavudine is a Nucleoside reverse transcriptase inhibitors (NRTIs) work against HIV-1, also known as the human immunodeficiency virus. Phosphorylation of stavudine produces active metabolites that vie with one another to be incorporated into viral DNA.
- Analytical method development and validation plays an important role in drug discovery and manufacture of pharmaceuticals. These methods are used to ensure the identity, purity, potency and performance of drug products. Spectrophotometry and HPLC methods are considered to be most suitable for estimation of drugs present in pharmaceutical dosage form.
- The Literature survey reveals that these drugs have been analyzed individually and in combination by many analytical methods like HPLC, UPLC and spectroscopic method, but no method have been reported for the Estimation of Lamivudine and Stavudine for Related Impurities HPLC Method in combined pharmaceutical dosage form.
- So here attempt will be made to develop and validate Related Impurities method for estimation of Lamivudine and Stavudine in combined dosage form by RP-HPLC.

Combination Product

Brand Name	Content	Marketed By	Dosage Form	Dose
Lamistar	Stavudine + Lamivudine	Hetero Healthcare Ltd	Tablet	Stavudine (30mg) and Lamivudine (150mg)

Table 5: Combination Brand Available in Market

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Figure 1: Marketed Formulation of Stavudine and Lamivudine

II. MATERIALS AND METHODS

In present research work, an attempt was made for development and validation of Related Substance method for simultaneous estimation of Lamivudine and Stavudine in pharmaceutical dosage form by RP-HPLC.

Instruments

Instruments Name	Manufacturer
HPLC	Shimadzu LC-20 AT
UV Visible spectrophotometer	Systronic 119
Electronic balance	Shimadzu ATX-240
Sonicator	Frontline Ultrasonic Cleaner
Hot air oven	Thermolab Mumbai, India
pH meter	Analab Scientific Pvt Ltd

Table 5.1: List of Instruments

Apparatus

Components	Description
Volumetric flasks	Borosilicate glass
Pipettes	Borosilicate glass
Measuring cylinder	Borosilicate glass
Beaker	Borosilicate glass
Whatman Filter	Filter Paper No.42

Table 5.2: List of Apparatus

Reagents

Chemicals	Grade	Manufacturer
Acetonitrile	HPLC	Merck, Rankem
Potassium Dihydrogen Phosphate	AR	Merck, Rankem
Water	HPLC	HPLC Grade
Orthophosphoric Acid	AR	Merck, Rankem
Methanol	HPLC	Merck, Rankem
Lamivudine and its Related Impurities	Rivan pharmaceutical pvt Ltd.	
Stavudine and its Related Impurities	Arrina lifescience pvt Ltd.	

Table 5.3: List of Reagents

Drug Identification

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The identification of standard API for experimental work had done for confirmation of its identity, standard, quality and purity. The identification was done by taking IR and UV spectra, Solubility and Melting point determination.

Melting Point Determination

Melting point of Lamivudine and Stavudine have been determined using Capillary Method. Drug was taken in capillary and injected into melting point apparatus. Result of determination is shown in Table 6.1.

Solubility Study

The solubility of Lamivudine and Stavudine were practically determined as per Indian pharmacopoeia. Solubility was determined by taking 10 mg of Lamivudine and 10 mg of Stavudine in 100 ml volumetric flasks, adding required quantity of solvent at room temperature and shaken for few minutes. Solubility data for each study was observed and recorded in Table 6.2.

IR spectra and Structure Interpretation

IR spectra of drugs were taken for structure interpretation from % transmission at specified wave numbers. Diffuse reflectance method was used. Potassium Bromide (KBr) was preheated at 105 ºC for 1 hour. Then it was triturated to convert crystalline form into amorphous powder. Then a suitable amount was filled in sample holder and background spectra were taken. After the drug was mixed with KBr in a ratio of 1:100. It was triturated and spectra were taken as shown in figure 6.2 and 6.4.

METHOD DEVELOPMENT

Selection and Detection of Wavelength

The sensitivity of HPLC method that uses UV detection depends upon proper selection of detection wavelength. An ideal wavelength is the one that gives good response for the drugs that are to be detected. At 212 nm both drug give good response. So 212 nm was selected as detection wavelength for estimation of Lamivudine and Stavudine in tablet dosage form by RP-HPLC.

Selection of Chromatographic Condition

Proper selection of the HPLC method depends upon the nature of the sample (ionic or neutral molecules), its molecular weight, pK_a and solubility. RP-HPLC was selected for the initial separation based on literature survey and its simplicity and suitability. To optimize the chromatographic conditions the effect of chromatographic variables such as mobile phase, pH, flow rate and solvent ratio were studied. Finally the chromatographic condition was chosen that give the best resolution, symmetry and capacity factor for estimation of both drugs and its related impurities.

Selection of Column

For RP-HPLC method, various columns are available and pure drugs chromatogram was developed in different mobile phase, different columns (e.g. $C_{8,}$ $C_{18,}$ phenyl etc) with different dimensions. The retention time and tailing factor was calculated for each drugs and its related impurities and for each chromatogram. Sharp peak and good resolution was found in C_{18}. Finally BDS Hypersil C_{18} (250mm X 4.6mm, 5µm) column was chosen for method development.

Procedure for Solution Preparation

Preparation of Standard Stock Solution

- **Standard Stock Solution of Lamivudine (500 ppm):**

Take 50 mg of Lamivudine into a 100ml volumetric flask and dissolve with methanol upto the mark to get 500 µg/ml of Lamivudine Standard Stock Solution.

- **Standard Stock Solution of Lamivudine Impurity (50 ppm):**

Take 5 mg of Lamivudine into a 100ml volumetric flask and dissolve with methanol upto the mark to get 50 µg/ml of Lamivudine Impurity Standard Stock Solution.

- **Standard Stock Solution of Stavudine (2000 ppm):**

Take 200 mg of Stavudine into a 100ml volumetric flask and dissolve with methanol upto the mark to get 2000 µg/ml of Stavudine Standard Stock Solution.

- **Standard Stock Solution of Stavudine Impurity (200 ppm):**

Take 20 mg of Stavudine into a 100ml volumetric flask and dissolve with methanol upto the mark to get 200 µg/ml of Stavudine Impurity Standard Stock Solution.

5.5.4.2. Preparation of Working Standard Solution

- **Working Standard Solution of Lamivudine (50 ppm):**

From above Lamivudine Standard Stock solution 1 ml was taken in to 10 ml volumetric flask and was made up to the mark with the mobile phase to get

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50 µg/ml of Lamivudine Working Standard Solution.

- **Working Standard Solution of Lamivudine Impurity (5 ppm):**

From above Lamivudine Impurity Standard Stock solution 1 ml was taken in to 10 ml volumetric flask and was made up to the mark with the mobile phase to get 5 µg/ml of Lamivudine Impurity Working Standard Solution.

- **Working Standard Solution of Stavudine (200 ppm):**

From above Stavudine Standard Stock solution 1 ml was taken in to 10 ml volumetric flask and was made up to the mark with the mobile phase to get 200 µg/ml of Stavudine Working Standard Solution.

- **Working Standard Solution of Stavudine Impurity (20 ppm):**

From above Stavudine Impurity Standard Stock solution 1 ml was taken in to 10 ml volumetric flask and was made up to the mark with the mobile phase to get 20 µg/ml of Stavudine Impurity Working Standard Solution.

Preparation of Mobile Phase

Prepare 0.05M Potassium Dihydrogen Phosphate by dissolving 6.8 gm of Potassium Dihydrogen Phosphate in 1000 ml water; adjust pH 3.5 with Ortho phosphoric acid (OPA). This solution was sonicated for 5 min for degassing and filtered through 0.45µ Millipore filter. Prepare the ratio of Buffer (pH 4.0): Acetonitrile (85:15).

Preparation of Test Solution

The average weight of 10 tablets was determined and was ground in a mortar. Test solution was prepared by dissolving tablet powder equivalent to 50 mg of Lamivudine or 200 mg of Stavudine was transferred to 100ml volumetric flask. Then 60 ml mobile phase was added and sonicated for 15 mins to ensure complete solubilization of drug. Further dilute 5ml of above solution and make up with 100 ml of mobile phase, After sonication, volume was made up to the mark with mobile phase. Filter the solution with 0.45 micron membrane filter and the final filtrate is collected as test solution.

Chromatographic Separation

Standard solutions of Lamivudine and Stavudine along with its related impurities were injected in column with 20 µl micro-syringe. The chromatogram was run for appropriate minutes with mobile phase. The detection was carried out at wavelength 212 nm. The chromatogram was stopped after separation achieved completely. Data related to peak like area, height, retention time, resolution etc. were recorded using software.

Chromatographic Trials

Mobile Phase	Ratio (v/v)	Retention Time	Remarks
Lamivudine and Stavudine in Water: Methanol	50:50	4.13	One Peak Observed
Lamivudine in Water: Methanol	50:50	4.13	Peak shape is not good
Stavudine in Water: Methanol	50:50	-	Peak is not observed
Lamivudine and Stavudine in Water: Methanol	70:30	5.67	Still second peak did not find
Lamivudine and Stavudine in Water : Acetonitrile	50:50	3.24	Still second peak did not find
Lamivudine and Stavudine in Water: Acetonitrile	70:30	3.81(Stavudine) 6.61(Lamivudine)	Both peak has not a good resolution
Lamivudine and Stavudine in buffer (pH 4.5): Acetonitrile	50:50	3.65(Stavudine) 4.32(Lamivudine)	Both peak has not a good resolution
Lamivudine and Stavudine in Buffer(pH 3.5): Acetonitrile	50:50	4.62(Lamivudine) 3.85 (Stavudine)	Still peak shape is not good

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Lamivudine and Stavudine in Buffer(pH 3.5): methanol	60:40	4.19 (Stavudine) 6.84(Lamivudine)	Not good resolution
Lamivudine and Stavudine in Buffer(pH 3.5): Acetonitrile	70:30	3.95 (Stavudine) 6.32 (Lamivudine)	Still no good resolution
Lamivudine and Stavudine in Buffer(pH 3.5): Acetonitrile	80:20	2.96 (Stavudine) 6.04 (Lamivudine)	Peak shape are good but resolution of peak are not good
Lamivudine and Stavudine buffer(pH 3.5): Acetonitrile	85:15	2.81 (Stavudine) 5.77 (Lamivudine)	Peak shape are good and resolution of peak are good
Lamivudine and Stavudine in Impurity + impurity buffer(pH 3.5): Acetonitrile	85:15	2.81 (Stavudine) 5.77 (Lamivudine) 3.25 (Stavudine imp) 6.14 (Lamivudine imp)	All peak shape are good and as well as show good resolution

Table 5.4: List of Chromatographic Trials

Chromatogram of trials is shown in Fig 6.6 – 6.20.

Chromatographic Conditions

Table 5.5: Chromatographic Conditions of HPLC

Components	**Description**
Column	C_{18} (25 cm × 0.46 cm) Hypersil BDS
Mobile Phase	Impurity Buffer (pH 3.5): Acetonitrile (85:15)
Flow Rate	1.0 ml/min
Detection Wavelength	212 nm
Run time	10 min
Injection volume	20.0 µl

Table 5.5: Chromatographic Conditions of HPLC

VALIDATION OF RP-HPLC METHOD

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System Suitability Test

It is an integral part of chromatographic method. These tests are used to verify that the resolution and reproducibility of the system are adequate for the analysis to be performed. System suitability tests are based on the concept that the equipment, electronics, analytical operations and samples constitute an integral system that can be evaluated as a whole. System suitability testing provides assurance that the method will provide accurate and precise data for its intended use.

Acceptance criteria

- Theoretical Plates for the analyte peak should not be less than 2000.
- Tailing factor for the analyte peak should not be more than 2.0.

Linearity and Range

The linearity for Lamivudine and Stavudine were assessed by analysis of combined standard solution in range of 2.5-7.5 µg/ml and 10-30 µg/ml respectively, 0.5,0.75,1,1.25 and 1.5 ml solutions were pipette out from the Stock solution of Lamivudine and Stavudine and transfer to 100 ml volumetric flask and make up with mobile phase to obtain 2.5,3.75,5,6.25 and 7.5µg/ml and 10,15,20,25 and 30µg/ml for Lamivudine and Stavudine respectively. In term of slope, intercept and correlation co-efficient value. The graph of peak area obtained verses respective concentration was plotted.

Acceptance criteria: Value of r^2 should be nearer to 1 or equal to 1.

Precision

- **Repeatability**

Standard solution containing Lamivudine (5 µg/ml) and Stavudine (20 µg/ml) was injected six times and areas of peaks were measured and % R.S.D. was calculated.

Acceptance criteria: % RSD of Area should not be more than 5.0%

- **Intraday Precision**

Standard solution containing (0.15,5.0,7.5µg/ml) of Lamivudine and (0.25,20.0,30.0µg/ml) of Stavudine were analyzed three times on the same day and % R.S.D was calculated.

Acceptance criteria: % RSD of Area should not be more than 5.0%

- **Interday Precision**

Standard solution containing (0.15,5.0,7.5µg/ml) of Lamivudine (0.25,20.0,30.0)µg /ml) of Stavudine were analyzed three times on the different day and % R.S.D was calculated.

Acceptance criteria: % RSD of Area should not be more than 5.0%

5.7.4. Accuracy

For Lamivudine

5 µg/ml drug solutions was taken in three different flask label A, B and C. Spiked 80%, 100%, 120% of standard solution in it and diluted up to 10ml. The area of each solution peak was measured at 212 nm. The amount of Lamivudine was calculated at each level and % recoveries were computed.

For Stavudine

20 µg/ml drug solutions was taken in three different flask label A, B and C. Spiked 80%, 100%, 120% of standard solution in it and diluted up to 10ml. The area of each solution peak was measured at 212 nm. The amount of Stavudine was calculated at each level and % recoveries were computed.

Acceptance criteria

% Recovery (individual) at each level should be between 98.00% and 102.00%

Limit of Detection and Limit of Quantitation

The LOD was estimated from the set of 3 calibration curves used to determination method linearity. The LOD may be calculated as,

LOD = 3.3 × (SD/Slope)

Where, SD = Standard deviation of Y-intercepts of 3 calibration curves.

Slope = Mean slope of the 3 calibration curves.

The LOQ was estimated from the set of 3 calibration curves used to determine method linearity. The LOQ may be calculated as,

LOQ = 10 × (SD/Slope)

Where, SD = Standard deviation of Y-intercepts of 3 calibration curves.

Slope = Mean slope of the 3 calibration curves.

Robustness

Following parameters were changed one by one and their effect was observed on system suitability for standard preparation.

1. Flow rate of mobile phase was changed (± 0.2 ml/min) 0.8 ml/min and 1.2 ml/min.
2. pH of Mobile phase was changed (± 0.2) 3.7 and 3.3

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3. Ratio of Mobile phase was changed (±2) Buffer: Acetonitrile (83:17) and Buffer: acetonitrile (87:13).

Acceptance criteria

- Number of theoretical plates for the analyte peak should not be less than 2000.
- Asymmetry value for the analyte peak should not be more than 2.0
- % RSD for the analyte peak should not be more than 5.0%

Calculation of Known Impurities of Lamivudine and Stavudine

Analyzed test solution for three times and calculate % of each known impurities in comparison with standard preparations of Lamivudine and Stavudine.

The amount of known related impurities presents in the formulation of Lamivudine and Stavudine is calculated by using the formula given below.

For each known impurities of Lamivudine and Stavudine:

% of each known impurities = (Cu/Cs) X (Ru/Rs) X 100

Where,

Cu= Concentration of each impurity in standard preparation

Cs= Concentration of each impurity in test preparation

Ru= Area of each impurity in test preparation

Rs= Area of each impurity in standard preparation

Results for % of each known impurities of Lamivudine and Stavudine. were shown in Figure 12 and Table 20.

III. RESULT AND DISCUSSION

Drug Identification

The identification of drugs was carried out by performing melting point determination, solubility study and taking IR and UV spectra as preliminary work which showed into following results.

Melting Point Determination

Melting point of Lamivudine and Stavudine have been determined using Capillary Method.

Drug Name	Reported (°C)	Observed (°C)
Lamivudine	160-162 ^{o}C	159-162 ^{o}C
Stavudine	165-166 ^{o}C	165-167 ^{o}C

Table 1: Melting Point of Drugs

Observation: Melting point of Lamivudine and Stavudine was found to be in the range of acceptance criteria as shown in above table.

Solubility Study

Table 6.2: Solubility Data of Lamivudine and Stavudine

Solvent	Solubility	
	Lamivudine	Stavudine
Water	soluble	Freely soluble
0.1 N HCl	Freely soluble	Freely soluble
0.1 N NaOH	Slightly Soluble	Slightly Soluble
Acetonitrile	Freely soluble	Freely soluble
Methanol	Slightly Soluble	Slightly Soluble

Table 2: Solubility Data of Lamivudine and Stavudine

Identification by IR Spectroscopy

1) Lamivudine

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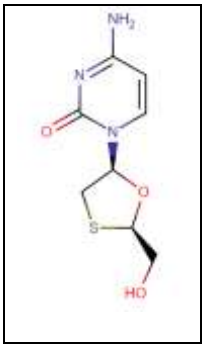

Figure 1: Structure of Lamivudine

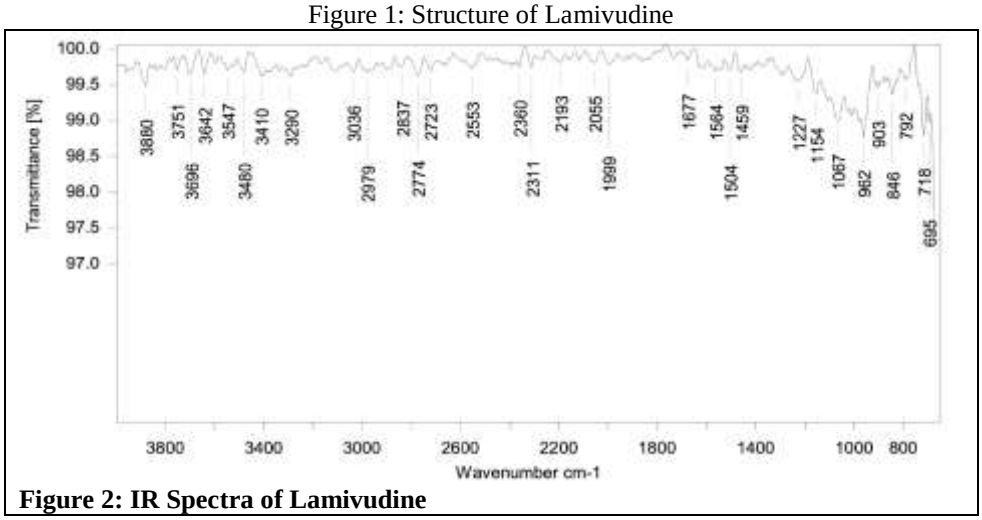

Figure 2: IR Spectra of Lamivudine

Functional Group	Frequency (cm^{-1})
C=C stretching	1459-1677
C=o stretching	1999
C-N stretching	1154

Table 3: IR Interpretation of Lamivudine

2) Stavudine

Figure 3: Structure of Stavudine

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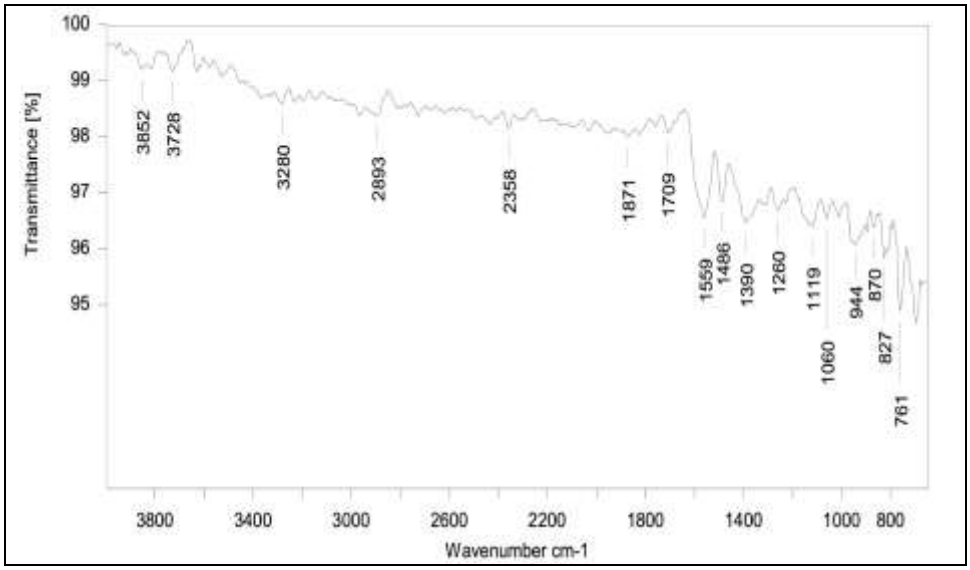

Figure 4: IR Spectra of Stavudine

Functional Group	Frequency (cm^{-1})
C-N stretching	1060
C-O stretching	1709
C=C stretching	1486-1559
C-O stretching	1119

Table 4: IR Interpretation of Stavudine

Conclusion

From the IR interpretation data it can be concluded that major functional group peak are observed in IR spectra of both the drug samples. So it reveals that the given sample is of Lamivudine and Stavudine drug.

METHOD DEVELOPMENT

Wavelength Determination

UV spectra of Lamivudine and Stavudine were taken in Methanol and λmax was observed using Systronic 119

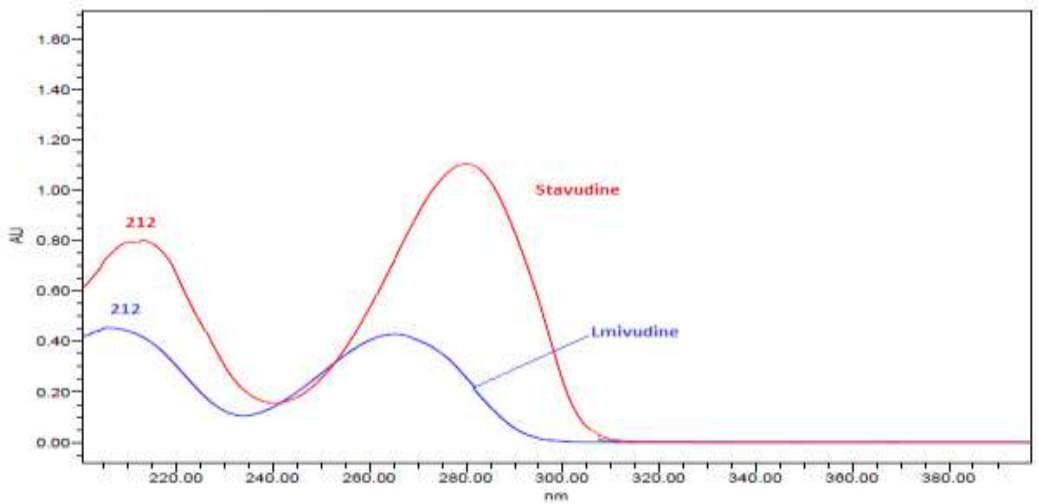

Figure 5: Overlay UV Spectrum of Lamivudine and Stavudine showing Selection of Wavelength Detection

Observation

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Lamivudine and Stavudine both drug give higher absorbance at 212 nm.
So 212 nm has been selected as detection wavelength.

Note: All the chromatograms are shown at wavelength of 212 nm. So, 212 nm is shown in final optimized method.

Chromatographic Trials

Mobile Phase	Ratio (v/v)	Retention Time	Remarks
Lamivudine and Stavudine in Water: Methanol	50:50	4.13	One Peak Observed
Lamivudine in Water: Methanol	50:50	4.13	Peak shape is not good
Stavudine in Water: Methanol	50:50	-	Peak is not observed
Lamivudine and Stavudine in Water: Methanol	70:30	5.67	Still second peak did not find
Lamivudine and Stavudine in Water : Acetonitrile	50:50	3.24	Still second peak did not find
Lamivudine and Stavudine in Water: Acetonitrile	70:30	3.81(Stavudine) 6.61(Lamivudine)	Both peak has not a good resolution
Lamivudine and Stavudine in buffer (pH 4.5): Acetonitrile	50:50	3.65(Stavudine) 4.32(Lamivudine)	Both peak has not a good resolution
Lamivudine and Stavudine in Buffer(pH 3.5): Acetonitrile	50:50	4.62(Lamivudine) 3.85 (Stavudine)	Still peak shape is not good
Lamivudine and Stavudine in Buffer(pH 3.5): methanol	60:40	4.19 (Stavudine) 6.84(Lamivudine)	Not good resolution
Lamivudine and Stavudine in Buffer(pH 3.5): Acetonitrile	70:30	3.95 (Stavudine) 6.32 (Lamivudine)	Still no good resolution
Lamivudine and Stavudine in Buffer(pH 3.5): Acetonitrile	80:20	2.96 (Stavudine) 6.04 (Lamivudine)	Peak shape are good but resolution of peak are not good
Lamivudine and Stavudine buffer(pH 3.5): Acetonitrile	85:15	2.81 (Stavudine) 5.77 (Lamivudine)	Peak shape are good and resolution of peak are good
Lamivudine and Stavudine in Impurity + impurity buffer(pH 3.5): Acetonitrile	85:15	2.81 (Stavudine) 5.77 (Lamivudine) 3.25 (Stavudine imp) 6.14 (Lamivudine imp)	All peak shape are good and as well as show good resolution

Table 5: Effect of different mobile phase compositions on the separation of Lamivudine and Stavudine

After considering the varying combinations of various mobile phases, Buffer (pH 3.0): Acetonitrile (85:15), [Buffer (0.05 M KH_2PO_4, pH 4.0) Take 6.8 gm. KH_2PO_4 in to a

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1000 ml beaker, add 800 ml water and dissolve, adjust pH 3.5 with Orthophosphoric acid(OPA), Make up Volume 1000 mL with water] was finalized as it was showing good peak shapes and a significant amount of resolution.

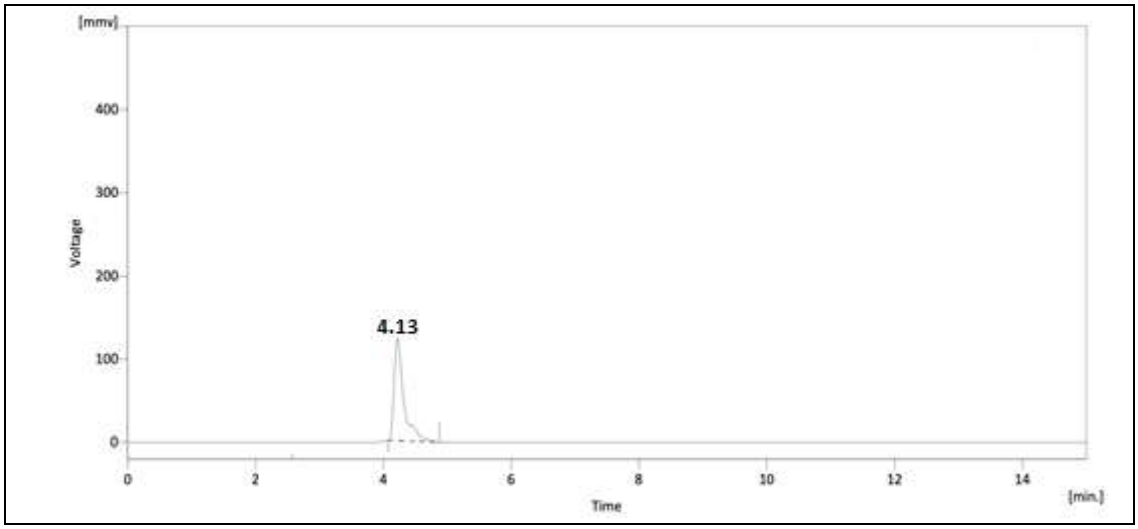

Figure 6: Chromatogram of Lamivudine and Stavudine Water: Methanol (50:50v/v).

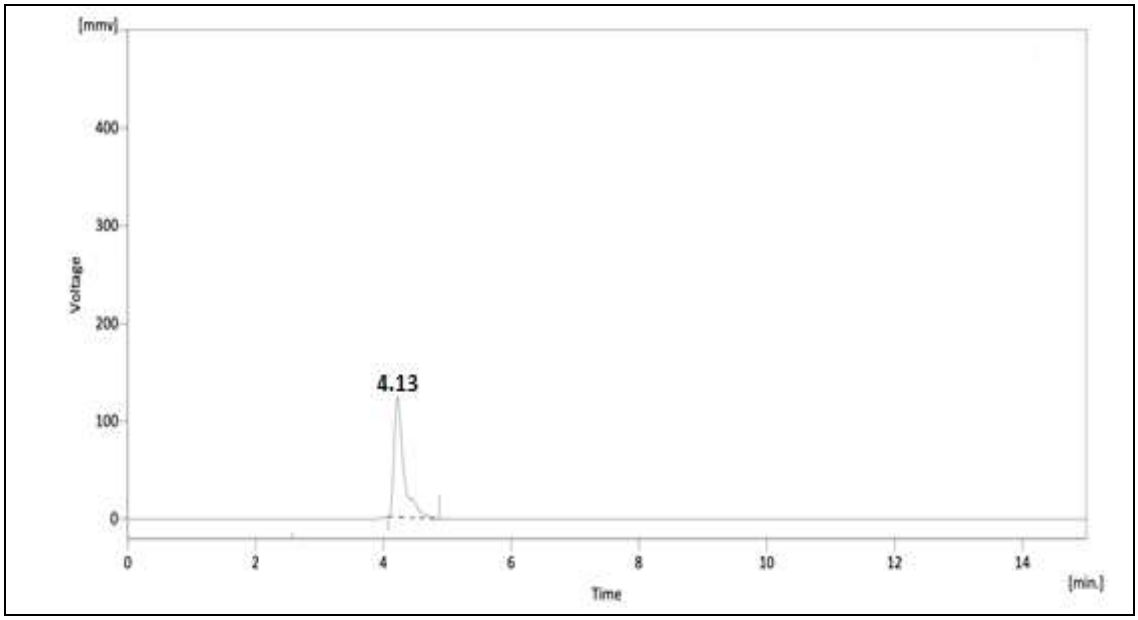

Figure 7: Chromatogram of Lamivudine in Water: Methanol (50:50v/v).

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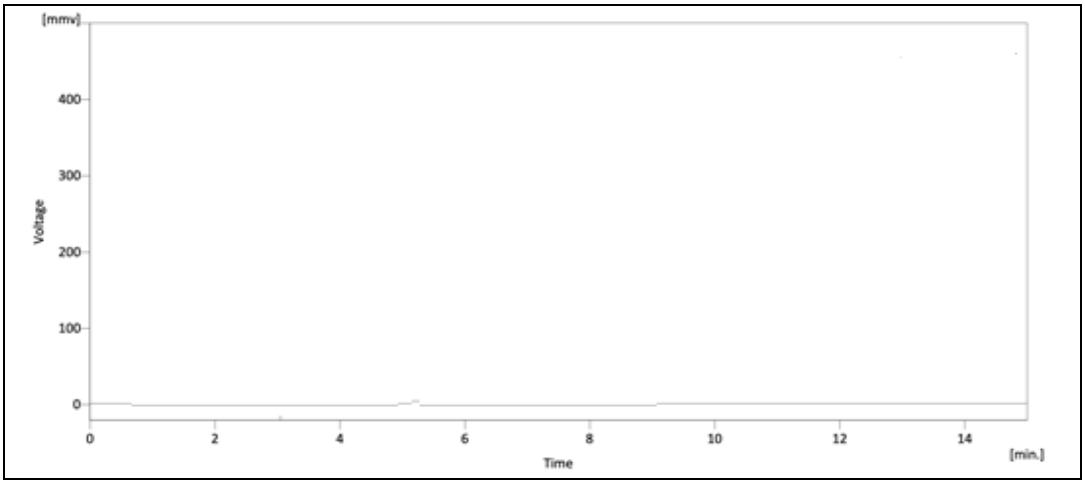

Figure 8: Chromatogram of Stavudine Water: Methanol (50:50 v/v)

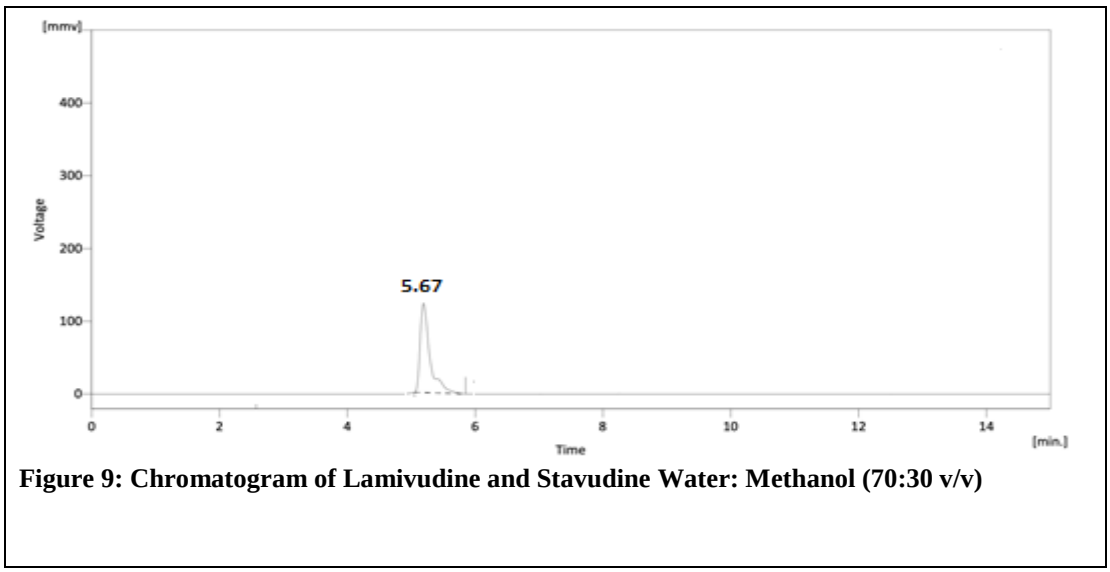

Figure 9: Chromatogram of Lamivudine and Stavudine Water: Methanol (70:30 v/v)

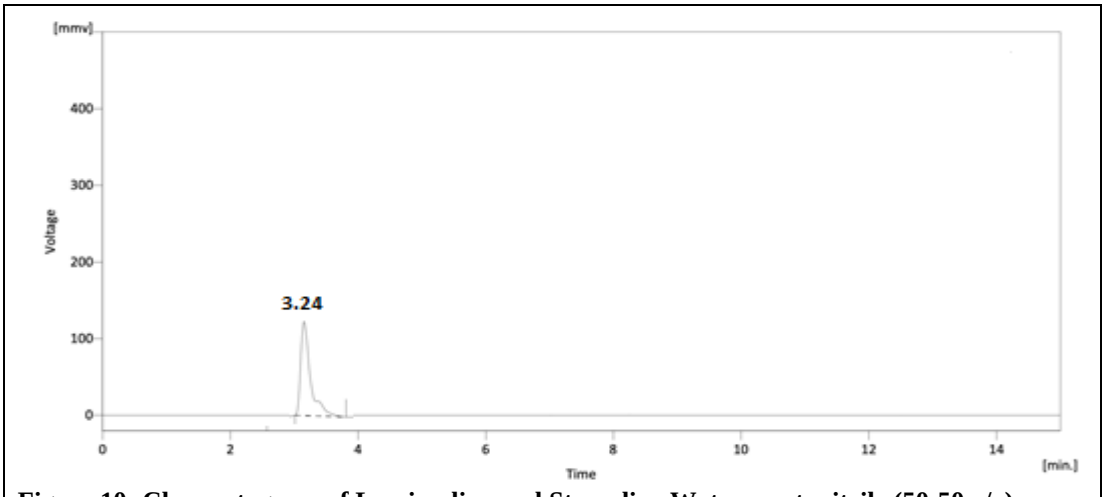

Figure 10: Chromatogram of Lamivudine and Stavudine Water: acetonitrile (50:50 v/v)

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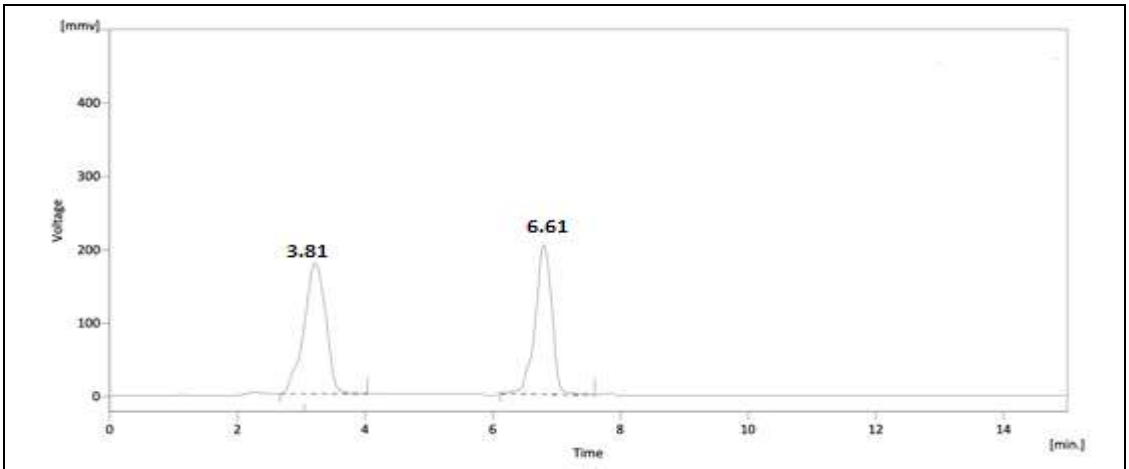

Figure 11: Chromatogram of Lamivudine and Stavudine Water: Acetonitrile (70:30v/v)

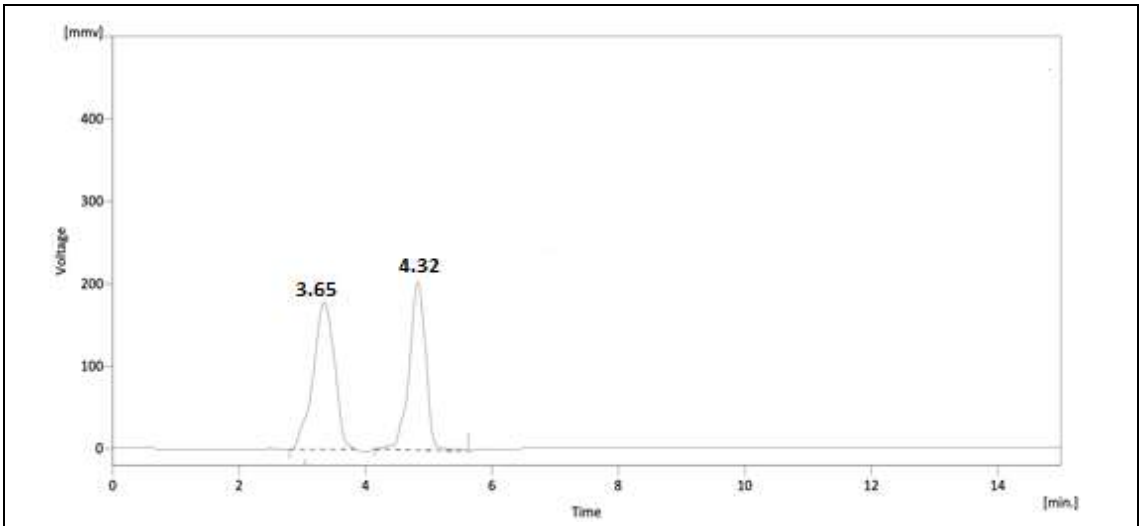

Figure 12: Chromatogram of Lamivudine and Stavudine buffer(pH 4.5) : Acetonitrile (50:50 v/v)

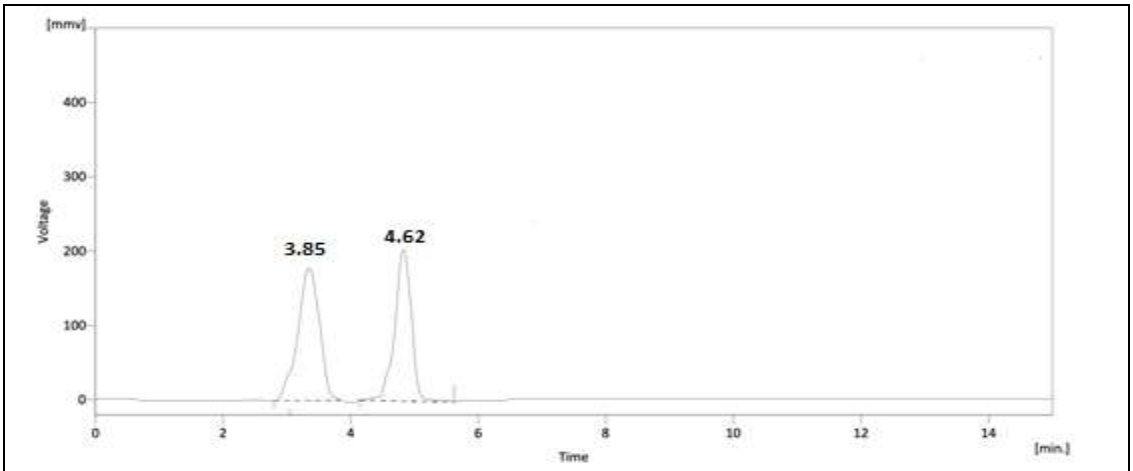

Figure 14: Chromatogram of Lamivudine and Stavudine in buffer (pH 3.5): methanol(50:50 v/v)

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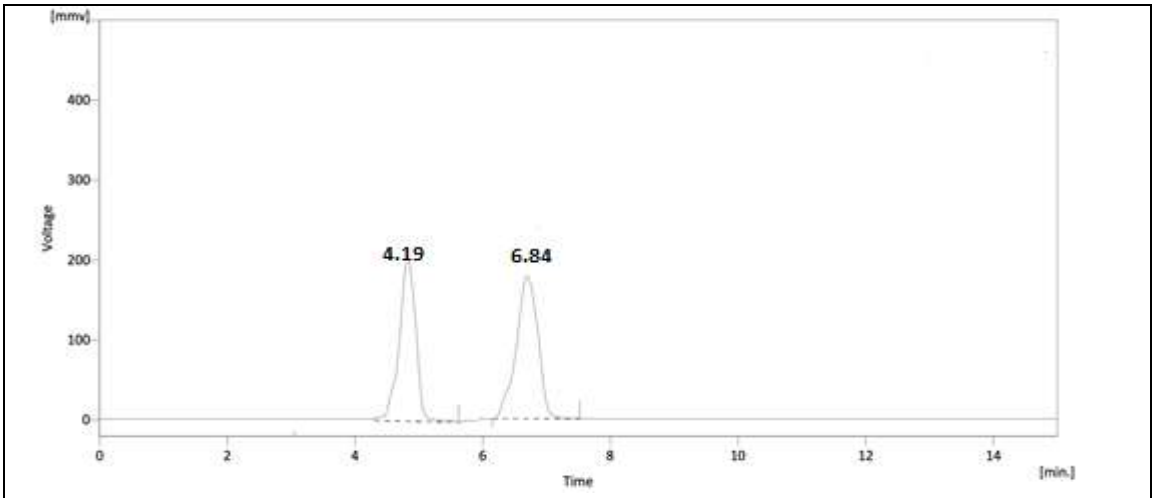

Figure 15: Chromatogram of Lamivudine and Stavudine in buffer (pH 3.5): acetonitrile(60:40 v/v)

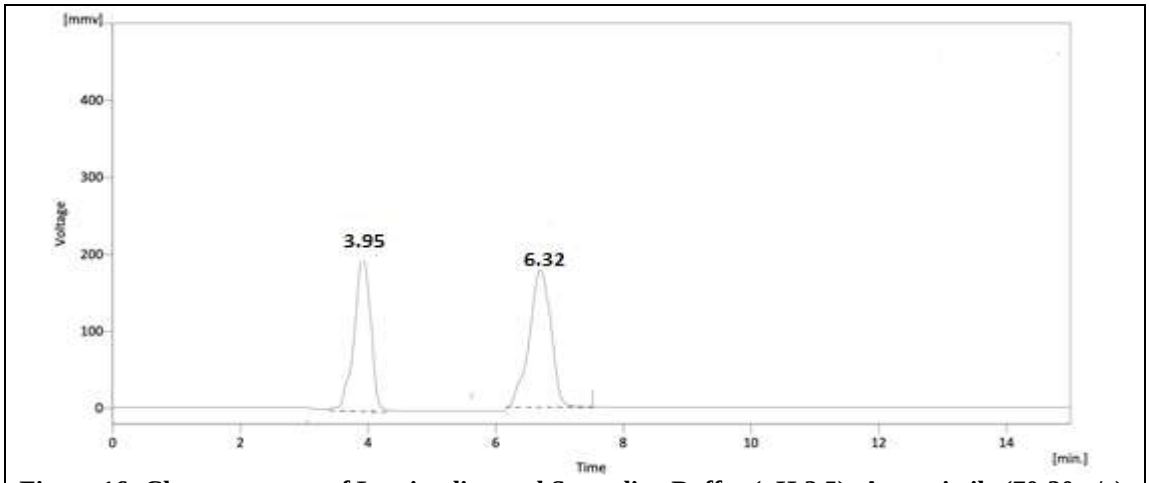

Figure 16: Chromatogram of Lamivudine and Stavudine Buffer (pH 3.5): Acetonitrile (70:30 v/v).

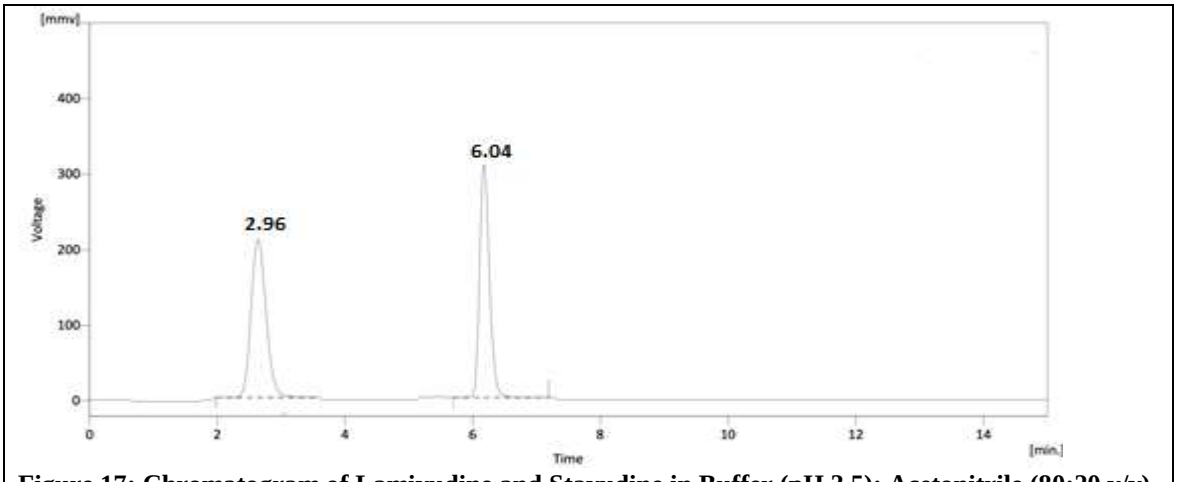

Figure 17: Chromatogram of Lamivudine and Stavudine in Buffer (pH 3.5): Acetonitrile (80:20 v/v).

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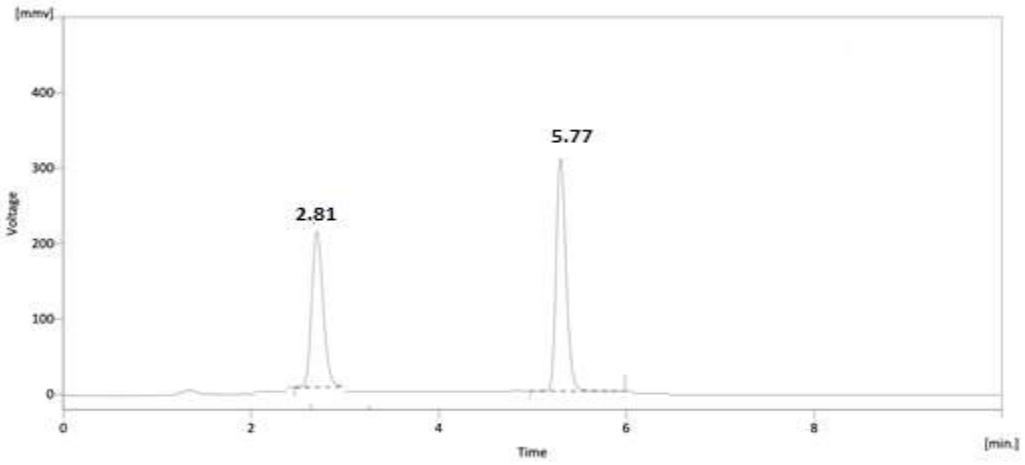

Figure 19: Chromatogram of Lamivudine and Stavudine in Buffer (pH 3.5): Acetonitrile (85:15 v/v).

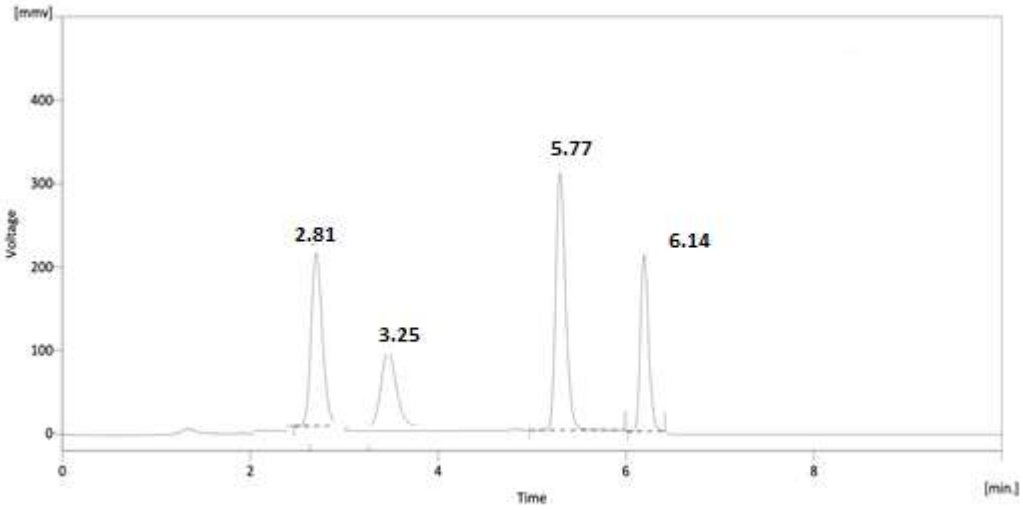

Figure 20: Chromatogram of Lamivudine and Stavudine along with its Related Impurities in Buffer (pH 3.5): Acetonitrile (85:15 v/v).Final

The mobile phase Buffer (pH 3.5): Acetonitrile (85:15 v/v). was selected because it was found to ideally resolve the peaks with retention time (RT) 5.77 and 2.81 min for Lamivudine and Stavudine and the retention time of Lamivudine impurity and Stavudine impurity were found to be 6.14 min and 3.25 min respectively respectively and the same is shown in fig 6.20.

Final Chromatographic Condition for Lamivudine and Stavudine

- **Stationary Phase :** BDS Hypersil C18 (250 mm×4.6 mm, 5 µm particle size)
- **Mobile Phase:** Buffer (pH 3.5): Acetonitrile (85:15 v/v).
- **Flow Rate :** 1 ml/min
- **Detection Wavelength :** 212 nm
- **Run Time :** 10 min
- **Injection Volume :** 20 µl

Observed values for System Suitability Test

1. Retention Time (Rt): Retention Time was observed depicted in Table 6.6.

2. Column efficiency (N): Number of plates observed for Lamivudine and Stavudine was observed depicted in Table 6.6.

3. Symmetry factor (S): Tailing factor observed for Lamivudine and Stavudine was observed depicted in Table 6.6.

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Parameters	Lamivudine	Stavudine	Lamivudine Impurity	Stavudine Impurity
Retention Time	7.460	5.280	8.440	6.577
Theoretical plates per column	7708	7180	7681	4160
Tailing factor	1.245	1.232	1.310	1.310

Table 6: Results for System Suitability Test.

METHOD VALIDATION

System Suitability Parameters

System suitability tests are used to verify that the resolution and repeatability of the system were adequate for the analysis intended. The parameters used in this test were the chromatographic peak, retention time, resolution, theoretical plate number and tailing factor.

Parameters	Lamivudine	Stavudine	Lamivudine Impurity	Stavudine Impurity
Retention Time	5.77	2.81	6.14	3.25
Theoretical plates per column	7516	8125	7934	8462
Tailing factor	1.32	1.51	1.45	1.54

Table 7: System Suitability Parameters

Specificity

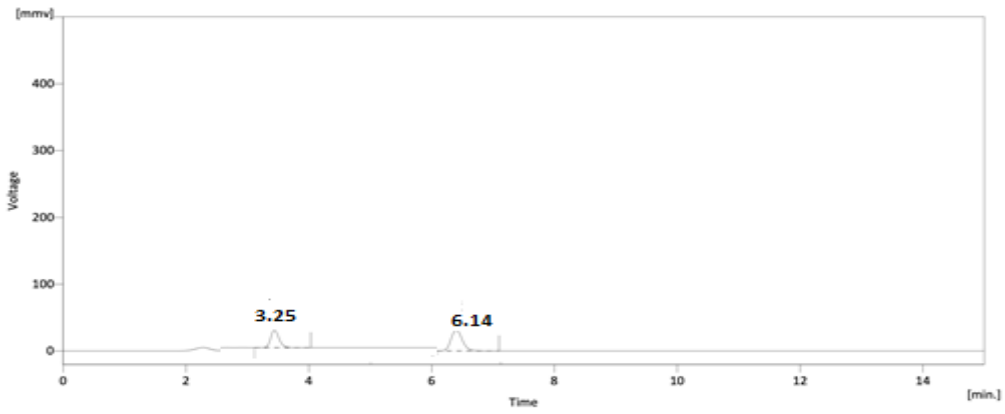

Figure 21: Chromatogram of Lamivudine Impurity and Stavudine Impurity Standard

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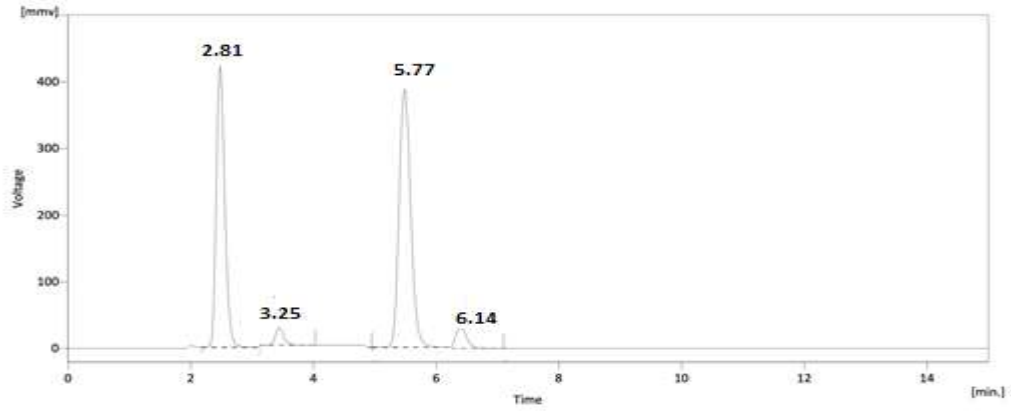

Figure 22: Chromatogram of Lamivudine and Stavudine Test

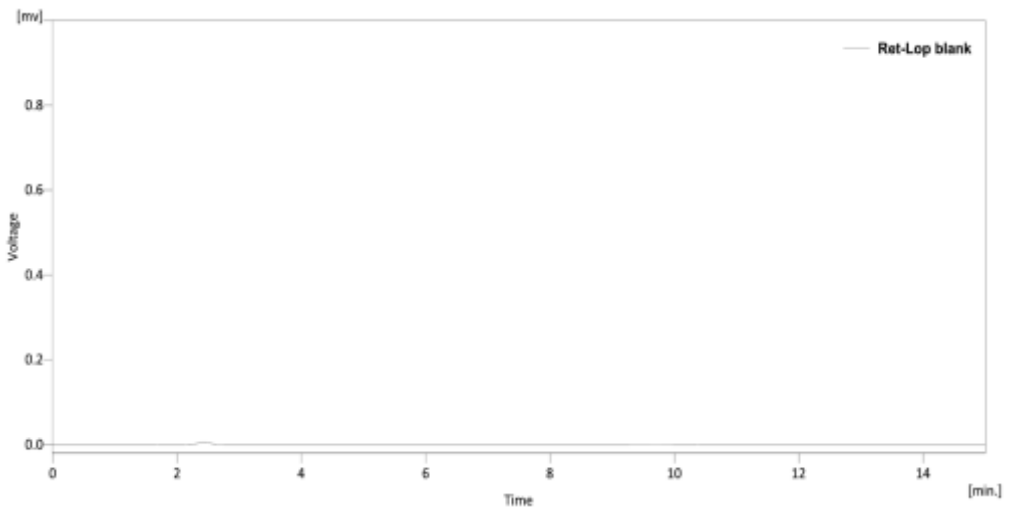

Figure 23: Chromatogram of Lamivudine and Stavudine Blank

The Chromatograms of Lamivudine and Stavudine Impurity standards and Lamivudine and Stavudine sample show no interference with the Chromatogram of Lamivudine and Stavudine Blank, so the Developed method is Specific.

Linearity and Range

The linearity for Lamivudine Impurity and Stavudine Impurity were assessed by analysis of combined standard solution in range of 2.5-7.5µg/ml and 10-30µg/ml respectively. Correlation co-efficient for calibration curve Lamivudine Impurity and Stavudine Impurity was found to be 0.9987 and 0.9991 respectively.

The regression line equation for Lamivudine and Stavudine are as following:

For Lamivudine Impurity: $y = 33.119x - 4.5574$ and

For Stavudine Impurity: $y = 11.144x - 17.248$

Sr. No	Concentration (µg/ml)	Area
1	2.5	75.664
2	3.75	121.361
3	5	164.248
4	6.25	201.146
5	7.5	242.764

Table 8: Linearity Data for Lamivudine Impurity.

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Sr. No	Concentration (µg/ml)	Area
1	10	96.475
2	15	149.487
3	20	201.214
4	25	262.351
5	30	318.647

Table 9: Linearity Data for Stavudine Impurity

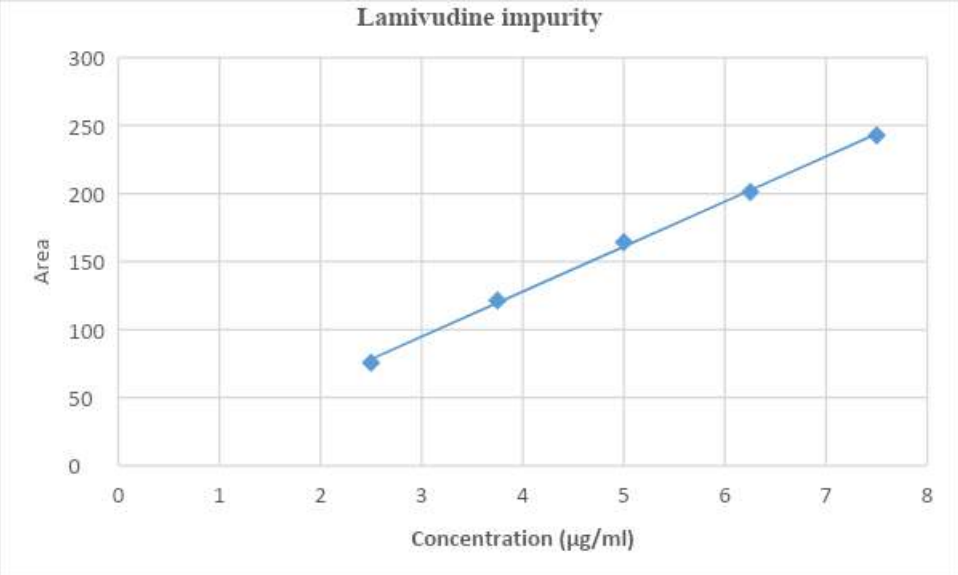

Figure 24: Calibration Curve of Lamivudine Impurity

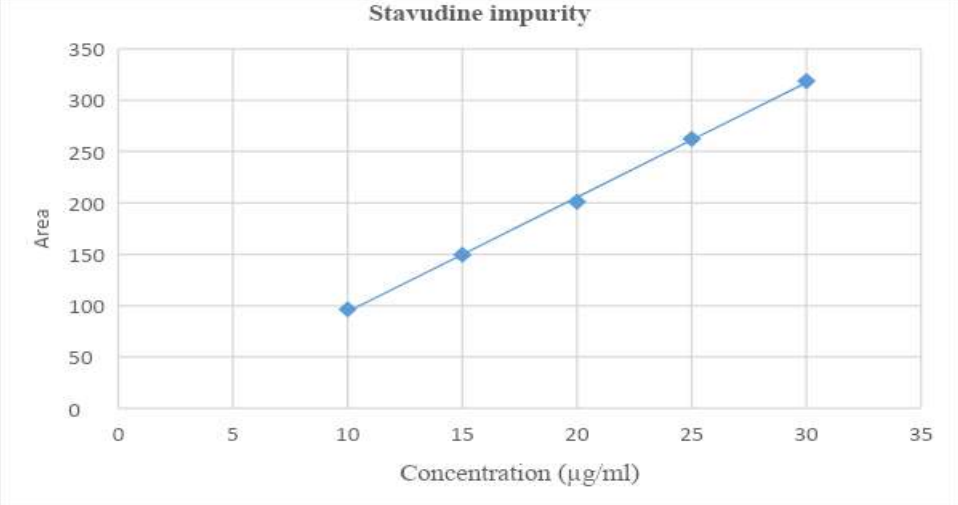

Figure 25: Calibration Curve of Stavudine Impurity

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Precision

Repeatability

The data for repeatability of peak area measurement for Lamivudine and Stavudine Impurity, based on six measurements of same solution of Lamivudine and Stavudine Impurity are depicted in table 6.14 and 6.15. The % RSD for Lamivudine Impurity and Stavudine Impurity was found to be 1.80 and 1.91 respectively.

Lamivudine Impurity				
Sr. No.	**Conc (µg/ml)**	**Area**	**Mean ± S.D (n=6)**	**% R.S.D**
1	5	164.321 168.147 170.345 165.754 164.865 171.645	167.512±3.026	1.806

Table 10: Repeatability Data for Lamivudine Impurity.

Stavudine Impurity				
Sr No.	**Conc (µg/ml)**	**Area**	**Mean ± S.D (n=6)**	**% R.S.D**
1	20	211.324 208.645 215.486 218.647 209.486 216.467	213.342±4.086	1.915

Table 11: Repeatability data for Stavudine Impurity

Intraday precision

The data for intraday precision for Lamivudine and Stavudine Impurity is shown in table 6.16. The % R.S.D. for Intraday precision was found to be 4.022-1.689 for Lamivudine Impurity and 4.217-1709 for Stavudine Impurity.

Sr. No.	**Lamivudine Impurity**			**Stavudine Impurity**		
	Conc. (µg/ml)	**Area Mean ± S.D. (n=3)**	**% R.S.D**	**Conc. (µg/ml)**	**Area Mean ± S.D. (n=3)**	**% R.S.D**
1	LOQ	25.235± 1.015	4.022	LOQ	41.589± 1.754	4.217
2	5	163.245± 2.758	1.689	20	214.849±4.345	2.022
3	7.5	248.678± 4.658	1.873	30	311.457±5.324	1.709

Table 12: Intraday precision data for Estimation of Lamivudine and Stavudine Impurity.

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Interday precision

The data for intraday precision for Lamivudine and Stavudine Impurity is shown in table 6.17. The % R.S.D. for interday precision was found to be 4.405-1.402 for Lamivudine Impurity and 2.290-0.990 for Stavudine Impurity.

Sr. No.	Lamivudine Impurity			Stavudine Impurity		
	Conc. (µg/ml)	Area Mean ± S.D. (n=3)	% R.S.D	Conc. (µg/ml)	Area Mean ± S.D. (n=3)	% R.S.D
1	LOQ	28.356 ± 1.249	4.405	LOQ	45.625 ± 1.045	2.290
2	5	168.346± 2.361	1.402	20	221.345± 2.541	1.148
3	10	241.365± 4.321	1.790	30	324.654± 3.214	0.990

Table 13: Interday Precision data for Estimation of Lamivudine and Stavudine Impurity.

Accuracy

Accuracy of the method was confirmed by recovery study from marketed formulation at three level of standard addition. The results are shown in table 6.18 and 6.19. Percentage recovery for Lamivudine Impurity was 100.30-106.0%, while for Stavudine Impurity, it was found to be in range of 99.94-104.04 %.

Sr. No.	Conc. Level (%)	Amount Added (µg/ml)	Amount recovered (µg/ml)	% Recovery	% R.S.D
1	LOQ	1.25	1.281	102.48	2.785
2		1.25	1.254	100.32	
3		1.25	1.325	106.00	
4	80%	4	4.012	100.30	1.395
5		4	4.124	103.10	
6		4	4.085	102.13	
7	100%	5	5.214	104.28	1.897
8		5	5.024	100.48	
9		5	5.156	103.12	
10	120%	6	6.275	104.58	2.169
11		6	6.034	100.57	
12		6	6.257	104.28	

Table 14: Recovery Data for Lamivudine Impurity.

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Sr. No.	Conc. Level (%)	Amount Added (µg/ml)	Amount recovered (µg/ml)	% Recovery	% R.S.D
1	LOQ	2.5	2.542	101.68	2.048
2		2.5	2.601	104.04	
3		2.5	2.497	99.88	
4	80%	16	15.986	99.91	1.215
5		16	16.375	102.34	
6		16	16.234	101.46	
7	100%	20	20.378	101.89	1.184
8		20	20.058	100.29	
9		20	19.912	99.56	
10	120%	24	24.254	101.06	1.069
11		24	24.075	100.31	
12		24	24.587	102.45	

Table 15: Recovery Data for Stavudine Impurity.

LOD and LOQ

Calibration curve was repeated for five times and the standard deviation (SD) of the intercepts was calculated. Then LOD and LOQ were calculated as follows:

LOD = 3.3 * SD/slope of calibration curve

LOQ = 10 * SD/slope of calibration curve

Limit of Detection

Lamivudine Impurity	Stavudine Impurity.
LOD = 3.3 x (SD / Slope) = 3.3 x (0.524/24.354) = 0.071 µg/ml	LOD = 3.3 x (SD / Slope) = 3.3 x (0.234/21.621) = 0.035 µg/ml

Table 16: Limit of Detection Data for Lamivudine Impurity and Stavudine Impurity.

Limit of Quantitation

Lamivudine Impurity	Stavudine Impurity
LOQ = 10 x (SD / Slope) = 10 x (0.524/24.354) = 0.215 µg/ml	LOQ = 10 x (SD / Slope) = 10 x (0.234/21.621) = 0.108 µg/ml

Table 17: Limit of Quantitation Data for Lamivudine Impurity and Stavudine Impurity.

Robustness

The effect of changes was found to be within the acceptance criteria as shown in table 6.22 and table 6.23**.** The % RSD should be less than 5%.

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Sr No.	Area at Flow rate (+ 0.2 ml/min)	Area at Flow rate (- 0.2 ml/min)	Area at pH (-0.2)	Area at pH (+0.2)	Area at Mobile phase(-2)	Area at Mobile phase(+2)
1	152.361	171.345	184.623	156.482	191.356	163.457
2	156.457	175.642	178.647	151.875	195.485	168.782
3	151.145	177.245	180.321	148.486	186.546	170.986
% R.S.D	1.815	1.746	1.701	2.636	2.341	2.308

Table 18: Robustness data for Lamivudine Impurity.

Sr No.	Area at Flow rate (- 0.2 ml/min)	Area at Flow rate (+ 0.2 ml/min)	Area at pH (- 0.2)	Area at pH (+ 0.2)	Area at Mobile phase(-2)	Area at Mobile phase(+2)
1	221.356	208.654	229.345	205.364	256.356	228.647
2	215.632	198.658	225.645	211.645	261.864	225.486
3	211.561	201.356	231.654	215.845	251.478	231.654
% R.S.D	2.276	2.549	1.324	2.500	2.025	1.349

Table 19: Robustness data for Stavudine Impurity

Calculation of Known Impurities of Lamivudine and Stavudine

Applicability of the proposed method was tested by analyzing the commercially available Tablet formulation Emletra. The results are shown in table 6.24.

Impurity	Conc (µg/ml)	Area	% Impurity	% R.S.D
Lamivudine	5	121.365	0.254	2.262
		118.694	0.216	
		128.645	0.271	
Stavudine	20	104.321	0.124	4.066
		98.645	0.289	
		102.754	0.215	

Table 20: Calculation of Known Impurities of Lamivudine and Stavudine

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The results indicate that the developed method is accurate, precise, simple and rapid. It can be used in the routine quality control of dosage form in industries.

METHOD VALIDATION SUMMARY

Sr. No.	Parameter		Lamivudine	Stavudine
1	Specificity		Specific	
2	Linearity & Range		2.5-7.5	10.0-30.0
3	Regression equation		y = 33.119x - 4.5574	y=11.144x -17.248
4	Correlation co-efficient (r^2)		0.9987	0.9991
5	Precision (% RSD)	Repeatability	1.806	1.915
		Interday	4.022-1.689	4.217-1709
		Intraday	4.405-1.402	2.290-0.990
6	Accuracy (% recovery)		100.30-106.0	99.88-104.04
7	Limit of Detection(LOD)		0.071 µg/ml	0.035 µg/ml
8	Limit of Quantification(LOQ)		0.215 µg/ml	0.108 µg/ml
9	Robustness (% RSD)		The system suitability parameters were found well within the acceptance criteria as per system suitability	

Table 21: Summary of Validation Parameters for Lamivudine and Stavudine Related Impurities

DISCUSSION

A new Related Impurities RP-HPLC method has been developed for estimation of Lamivudine and Stavudine Impurity in tablet dosage form was rapid, accurate, precise, economic and easy to perform. The linearity was investigated in the range of 2.5-7.5 µg/mL (r^2 = 0.9987) for Lamivudine Impurity and 10-30 µg/ml (r^2 = 0.9991) for Stavudine Impurity. The LOD were 0.071µg/ml and 0.035µg/ml for Lamivudine and Stavudine Related Impurities, respectively. The LOQ were 0.215µg/mL and 0.108µg/mL for Lamivudine and Stavudine Related Impurities, respectively. This method was found to be simple, accurate, robust and reproducible.

IV. SUMMARY AND CONCLUSION

- There is no analytical work has been available regarding Related Impurities RP-HPLC method for Lamivudine and Stavudine in a literature. Data regarding behaviour of drug and its related impurities in chromatographic conditions and other relevant analytical properties are not available.
- A novel attempt in a field of research has been made to develop and validate Related Impurities method via RP- HPLC.
- Lamivudine and stavudine are often used in combination as part of antiretroviral therapy for the treatment of HIV. This combination, along with other antiretroviral medications, is used to suppress the replication of the HIV virus and manage the infection.
- RP-HPLC method was developed for simultaneous estimation Lamivudine and Stavudine. In RP-HPLC method, good resolution and separation of two drugs and its related impurities was achieved. 0.05 M Sodium Dihydrogen phosphate (pH 3.5):

acetonitrile (85:15 v/v) was used as mobile phase.

- Retention time of Lamivudine and Stavudine were found to be 5.77 and 2.81 min respectively with a flow rate of 1 ml/min. The proposed method was accurate and precise. Therefore proposed method can be used for routine analysis of Lamivudine and Stavudine in tablets.
- The suitability, performance and applicability of developed method has been validated as per ICH guideline by applying various validation parameters like specificity, linearity and range, accuracy precision and robustness.
- The RP-HPLC method developed for the determination of related impurities of Lamivudine and Stavudine is found to be specific, linear, sensitive, precise, accurate and robust in nature.
- The method was successfully validated in terms of specificity, precision, linearity, accuracy and robustness as per ICH guidelines.
- It can be concluded that the proposed method can be used for routine analysis for estimation of related impurities of Lamivudine and Stavudine in combined dosage form by RP-HPLC.

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